Molecular Analysis of Reproductive Neuroendocrine Function in Broiler Breeders

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Declaration

The experiments described within this Thesis were the sole work of the author, where this is not so credit has been duly given. No part of the work has been previously submitted in support of any other degree.

N A Ciccone
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This Thesis is dedicated to Nerine Joseph for "the course of true love never did run smooth" and in memory of Rosie (2001-2004).

Nick Ciccone, Edinburgh, 2005
Quotations

The chicken is the egg's way of ensuring the production of another egg

by Samuel Butler (1835-1902)

A woman possessed a hen that gave her an egg everyday. She often pondered how she might obtain two eggs daily instead of one, and at last, to gain her purpose, determined to give the hen a double allowance of barley. From that day the hen became fat and sleek, and never once laid another egg.

The Woman and Her Hen

by Aesop (620-560 B.C.)

Translated into English by the Reverend George Fyler Townsend (1814-1900).

Old Woman with a Hen by
Artolomé Esteban Murillo (1618-1682)
Alte Pinakothek,
Munich, Germany.
Publications Arising

Refereed Papers


Review Chapters


Others


Publications Arising

Others


The aim of this Thesis was to increase understanding of the regulation of gonadotrophin (LH and FSH) secretion in poultry with particular reference to ageing broiler breeder hens (*Gallus domesticus*). Measurements were made of plasma LH and FSH, and seven mRNAs encoding gonadotrophin subunits (common α-subunit, FSH β, LH β), activin βB, follistatin and gonadotrophin releasing hormone-I (GnRH-I) and gonadotrophin inhibitory hormone (GnIH). Observations were made *in vitro* on the gonadotrophic responses of cockerel pituitary fragments to GnRH-I and GnIH.

In broiler breeder hens, plasma FSH and LH were higher in peak-of-lay than in end-of-lay birds and this was correlated with higher numbers of yellow yolky ovarian follicles and pituitary common α-subunit mRNA, but not with the other mRNAs measured. Old out-of-lay hens had higher pituitary FSH β mRNA and circulating FSH plasma levels than laying hens. This higher plasma FSH in out-of-lay hens was suggested to be due to a lifting of the inhibitory feedback effects of ovarian factors.

Lifting food restriction in laying broiler breeder hens, which are normally food restricted to prevent obesity, increased the number of yellow yolky ovarian follicles. This was associated with an increase in hypothalamic GnRH-I and pituitary common α-subunit and follistatin mRNAs and plasma LH, while plasma FSH was depressed. There was no change in other mRNAs measured, including GnIH mRNA. The expression of incubation behaviour was associated with depressed plasma LH, decreased hypothalamic GnRH-I, common α and LH β subunit mRNAs and increased GnIH mRNA. In photosensitive short day female quail, exposure to 3 long
days increased ovarian weight in association with increases in common $\alpha$ and FSH $\beta$ subunit mRNAs with no change in LH $\beta$ mRNA.

Incubation of cockerel pituitary fragments with pulses of GnRH-I increased pituitary LH release and increased common $\alpha$, but not LH $\beta$ subunit mRNAs. Follistatin mRNA was also increased with no associated change in FSH $\beta$ mRNA. Incubation of cockerel pituitary fragments with GnIH for 120 minutes suppressed LH and FSH release and common $\alpha$ and FSH$\beta$ mRNAs, but not LH$\beta$ subunit mRNAs.

It is concluded that the decrease in egg laying in broiler breeders is a consequence of decreased LH and FSH secretion. The decrease in LH secretion in older hens is associated with a reduction in common $\alpha$-subunit mRNA with no change in LH$\beta$ subunit mRNA. In contrast to mammals, common $\alpha$ subunit mRNA synthesis may be a rate-limiting step in LH release in birds. The decrease in FSH secretion in older laying hens is not a consequence of reduced FSH$\beta$ mRNA but could involve interactions of an intrapituitary activin/follistatin loop and ovarian oestrogen and inhibin.
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CHAPTER 1. INTRODUCTION

1.1 General introduction

The chicken (*Gallus domesticus*) is of great economic importance to man for meat and egg production. Approximately one quarter of the world’s meat consumption is derived from poultry, and table egg production is around $280 \times 10^9$/year (Sharp, 1998). The chicken belongs to the order Galliformes, that also includes other commercially important domestic birds such as the turkey (*Meleagris gallopavo*), quail (*Coturnix coturnix*) and guinea fowl (*Acryllium vulturinum*). Intensive selection has produced two distinct lines of commercial chicken with contrasting phenotypes (Etches, 1996). Egg laying chickens have been genetically selected for high egg productivity, but usually have small bodies that make them undesirable as meat producers. In contrast, meat type (broiler) chickens are selected for fast growth rate and larger body size with emphasis on meat yield and more efficient feed conversion. Selection for growth rate in broiler chickens has impaired the reproductive performance of meat-type parent (broiler breeder) stocks. Similar effects of selection for growth rate on reproduction also occur in Japanese quail (Marks, 1985) and turkeys (Nestor, *et al.*, 1980). Fertile egg production in obese broiler breeders is low due to poor egg shell quality as a result of a lack of coordination of the eggshell calcification process, to a high incidence of multiple-yolked eggs (such eggs are rejected by hatcheries because chicks rarely hatch out of them), and to a reduction in thePersistency of egg laying (Sharp, 1998). The number
of eggs produced during a laying year by a broiler breeder flock is ~170 compared to ~280 by a layer flock (Robinson et al., 1993).

Major objectives of commercial poultry breeders are to improve desirable traits in poultry in order to maximise efficiency and profit as well as to provide nutritious, high quality and appealing food at affordable prices. Our understanding of the reproductive physiology of birds can be applied to broiler breeders to understand the causes of their poor reproductive performance.

1.1.1 The origin of domestic fowl

Drawing on his own observations, Charles Darwin suggested that domestic chickens are descendants of wild junglefowl (Darwin, 1875). Archaeological evidence provides support for Darwin’s hypothesis showing that chickens were first domesticated from red junglefowl (Gallus gallus) in South-east Asia before the sixth millennium BC. From there they spread north to become established in China by 6000 BC. It has also been proposed that the main dispersion of chickens throughout Europe was during the Iron Age and with the aid of molecular tools, this hypothesis has been shown to be correct (West and Zhou, 1989). By screening domestic and jungle fowl population genomes, with microsatellite molecular markers, it has been possible to construct phylogenetic trees which demonstrate evolutionary relationships (Romanov and Weigend, 2001). In this way, it has been shown that
jungle fowl populations “form a separate, ancestral cluster” which have subsequently given rise to modern domestic fowl.

**1.1.2 Commercial Egg Layers and Broilers**

During 8,000 years of domestication the appearance and reproductive physiology of the chicken considerably changed. The ancestor of the domestic fowl, the Red Junglefowl, lays 10 to 15 eggs per year in the wild, whereas commercial laying hens are capable of producing 300 eggs per year. Current breeding strategies for commercial laying type poultry have generated specialised grandparent and parent lines derived by intense selection, of which by cross breeding produce populations with hybrid vigour for key production traits (Preisinger and Flock, 2000). Studies on the genetics of the growth rate of poultry began to appear in the 1930s (Asmudson and Lerner, 1934) when the economic importance of genetic purity and ornamental breeds was shifting to the development of multipurpose breeds with both meat and egg production potential. By the 1950s two distinct lines of commercial chicken were emerging for egg or meat production, egg layers and broilers, with opposing extremes in body weight and reproductive efficiency (Fig 1.1). Small bodied egg laying type lines direct more nutrients into egg production than do large bodied broilers. Selection of broilers for rapid growth and meat production has resulted in a strong negative relationship between body weight and reproductive efficiency (Robinson et al., 1993).
Figure 1.1 Comparison of laying (left) and broiler (right) strains of commercial chicken at 9 weeks old illustrating the differences in body size.

1.1.3 Broiler Breeders: Problems of Reproductive Efficiency

Broiler breeders must not only have the genetic potential for fast and efficient growth, but also for efficient reproduction. Continuous improvements in growth performance of meat-type chickens have made the challenge of maintaining a good rate of egg production in the parent stocks a difficult task. The poor reproductive efficiency of broiler breeder hens is due to problems with the development of an orderly ovarian follicular hierarchy and, later in the laying year, with poor persistency and erratic egg laying (Hocking et al., 1987, Robinson et al., 1993, Sharp et al., 1992) and is a consequence of selection for genes controlling growth rate, and genes which are closely linked to them. Both broilers and broiler breeders eat at or near the capacity of their gastrointestinal tract when fed ad libitum and appear to have lost the mechanism which regulates appetite (Bokkers and Koene, 2003). This is in contrast to egg laying stocks that consume only enough food to meet their
growth, metabolic and reproductive requirements without excessive food deposition (Etches, 1996). Thus, unless the food intake of broiler breeders is limited, the resulting obesity has detrimental effects on reproduction, vigour, and viability. For this reason, it is commercial practice to restrict the food intake by about 50% of ad libitum broilers at 2 weeks of age.

The chicken ovary is composed of follicles that are divided into hierarchical and non-hierarchical subclasses. The hierarchical subclass is composed of large yellow yolky follicles (YYFs) (Fig 1.2a). These follicles are the largest in the ovary, ranging from ~8 and 35mm in diameter. The largest of these is designated F1 and is be the next follicle to be ovulated, the smaller follicles in the hierarchy are sequentially numbered F2-F6 (Fig 1.2a). This hierarchy tends to be disrupted in the broiler hen, particularly at the onset of lay with an over development of YYFs (Hocking, 1993; Yu et al., 1992) (Fig 1.2a,b). The effect of this over development is a high incident of soft, thin or poorly shelled, double yolked eggs and a high incidence of internal ovulation which is a consequence of the simultaneous ovulation of multiple follicles (Hocking, 1987).

In order to support good hatchable egg production, broiler breeder chickens require a sophisticated programme of food restriction to prevent obesity and decrease the number of YYFs within the ovary (Fig 1.2a-b; Hocking, 1993; Yu et al., 1992). The reasons for the recruitment of excessive YYFs into the ovarian hierarchy of broiler
breeder hens at the onset of lay are not known, but are associated with ovulation of two or more ova on one day.

Figure 1.2 Problems affecting the reproductive efficiency of broiler breeders. 

A high rate of egg production in parents of meat type chickens (broiler breeders) is restricted to a relatively brief period of 5-7 months after the onset of egg laying.

(Robinson et al., 1993, Fig 1.2c) Egg-laying subsequently falls progressively in
association with a reduction in the recruitment of ovarian follicles into the yellow-yolky follicular hierarchy (Palmer and Bahr, 1992; Waddington et al., 1985), a reduction in the rate of follicular maturation (Johnson et al., 1986), alterations in the sensitivity of the hypothalamus to steroid feedback (Williams and Sharp, 1978) and an increased incidence of hens that are completely out of lay with no yellow-yolky follicles (YYFs) (Fig 1.2c: Sharp et al., 1992). Poor persistency of egg production later in the laying year has a genetic basis, but the genes involved are not known. In contrast layer type chickens produce eggs at a high rate for over a year (Etches, 1996). Identification of genes controlling persistency of egg laying will aid future selection programmes aiming to negate unfavourable reproductive traits within broiler breeder flocks (see section 1.2).

1.2 The hypothalamic-pituitary-gonadal (HPG) axis

Gonadal function in vertebrates is regulated by the secretion of the gonadotrophins, lutenising hormone (LH) and follicle stimulating hormone (FSH), which are secreted by the adenohypophysis of the pituitary gland. The control of synthesis and secretion of LH and FSH involves an interplay between hormones secreted by gonads, hypothalamus and anterior pituitary gland which form the hypothalamic-pituitary-gonadal (HPG) axis and are maintained in homeostatic relationships (Fig 1.3). The biosynthesis and secretion of the gonadotrophins (see section 1.2.1)
are regulated by hypothalamic neuropeptides, particularly gonadotrophin releasing hormone (GnRH) (see section 1.2.2) and gonadal hormones both steroids (oestrogen and progesterone) and proteins (e.g. inhibin) (see section 1.4). Gonadal hormones regulate gonadotrophin secretion directly at the level of the pituitary, or indirectly by modulating the action of hypothalamic GnRH neurones (see section 1.2.2.3) by inhibitory feedback mechanisms that maintain tonic gonadotrophin secretion and by a stimulatory feedback mechanism to generate the pre-ovulatory LH surge. Pituitary activin and follistatin control FSH secretion in the rat and probably other mammals (see section 1.2.4) and, are in turn, controlled by GnRH, but there is no information available for birds.

Figure 1.3 The hypothalamic-pituitary-gonadal (HPG) axis of the hen.
1.2.1 The gonadotrophins: structure, function and constitutive release

The discovery of the gonadotrophins can be traced back to the 1920s when surgical hypophysectomy (removal of the pituitary gland) in animals was discovered to result in gonadal regression and loss of reproductive function. Philip Smith pioneered parapharyngeal (through the roof-of-the-mouth) hypophysectomy in the rat, and by 1927, Smith demonstrated that pituitary hormones controlled gonad development (Smith, 1932; Sawyer, 1991). During the subsequent 50 years two gonadotrophin molecules were identified with different properties, referred to as luteinising hormone (LH) and follicle stimulating hormone (FSH). The discovery of their subunit structure and subsequent elucidation of the complete amino acid sequences came in the 1960s and 1970s (Li, 1973). The 1980s saw the characterisation of gonadotrophin subunit gene sequences in mammals, and the profiling of their expression [common α-subunit (Fiddes and Goodman, 1981; Chin et al. 1981) LHβ subunit (Jameson et al., 1984) FSHβ subunit (Maurer et al., 1987; Esch et al., 1986)].

LH and FSH are members of the family of pituitary glycoprotein hormones that include thyroid stimulating hormone (TSH). All members of this family are heterodimers formed by non-covalent association of a distinct β-subunit that confers biological specificity (Pierce and Parsons, 1981) and an α-subunit that is common to all the members of the family, except for certain fish species where there are two α subunits (Van Der Kraak et al., 1992).
In mammals, pituitary gonadotrophin α- and β-subunits are co-expressed in a specific cell type in the anterior pituitary gland, the gonadotroph (Childs et al., 1983), but in the chicken LH and FSH are synthesised in different cell types (Proudman et al., 1999; Puebla-Osorio et al., 2002). The LHβ gene has evolved in primates and equine species to produce a fourth glycoprotein hormone subunit: chorionic gonadotrophin β(CGβ) (Li and Ford, 1998), which is synthesised predominantly in the placenta. It forms a heterodimer with the α-subunit to make chorionic gonadotrophin. The gonadotrophin subunits are encoded for by different genes (Maurer, 1987; Jameson et al., 1984; Fiddes and Goodman, 1981; Godine et al., 1980) and consequently the synthesis of a mature, biologically competent gonadotrophin is dependent not only on β-subunit biosynthesis but also on α-subunit biosynthesis. In mammals α-subunit mRNA is produced in excess of β-subunit mRNAs (Shupnik, 1996; Nilson et al., 1983) implying that gonadotrophin β-subunit mRNAs production could be the rate limiting step for gonadotrophin synthesis. It is unknown whether this is the case in birds. The cloning of gonadotrophin subunit genes in birds [α-subunit: turkey (Foster and Foster, 1991), quail (Ando and Ishii, 1994); LHβ subunit: chicken (Noce et al., 1989), turkey (You et al., 1995), quail (Ando and Ishii, 1994); FSHβ subunit: chicken (Shen and You, 2002)] makes it possible to investigate the mechanisms controlling their expression.

LH and FSH act on the gonads of both sexes through their respective receptors to regulate reproductive activity. In the hen, FSH receptor protein, FSH receptor mRNA and FSH stimulated adenyly cyclase activity have been observed to be
prominent in the granulosa layer of prehierarchical follicles up to the third (F3) largest YYF (You et al., 1996). Moreover, FSH receptor mRNA is lower in atretic than in morphologically normal 3- to 5-mm follicles (You et al., 1996). These observations indicate that FSH is important in maintaining the viability of prehierarchical follicles and in initiating granulosa cell differentiation at the time follicles are selected to enter the preovulatory hierarchy. The role of chicken FSH is to stimulate granulosa cell proliferation, induce steroid synthesis from F6-F3 YYFs, and to increase numbers and growth of small white and yellow follicles (Palmer and Bahr, 1992). Responsiveness of ovarian follicles to FSH decreases as they mature with a decrease in FSH-stimulated steroidogenesis and adenylyl cyclase activity between F3 and F1 YYFs. This is associated with a decline in granulosa cell FSH receptor numbers (Ritzhaupt and Bahr, 1987). This decline in FSH receptor numbers with ovarian follicular maturation is inversely related to an increased responsiveness to LH which is correlated with progressive increases in LH receptor mRNA as follicles approach ovulation (Zhang et al., 1997). LH stimulates progesterone production in the preovulatory follicles and ovulation of the F1 follicle (Etches and Cunningham, 1976).

Biological activity is only exhibited by gonadotrophin β-subunits when they are bound to common α-subunit and are appropriately glycosylated (Willey, 1999). The observation that glycoprotein hormone α-subunit combines non-covalently with both the gonadotrophin β and TSHβ -subunits to confer biologically specificity
suggests that regions within the three dimensional protein structure of FSHβ, LHβ and TSHβ subunits are very similar, which makes it difficult to develop specific antibodies for glycoprotein hormone quantification assays. In addition, the circulating gonadotrophins are secondarily modified through saccharide moieties which makes them highly heterogeneous, and creates further specificity problems in the immunoassays used to measure them. The saccharide component of glycoprotein hormones play a role in protein folding, determining the circulating half-life, binding to receptors and signal transduction (Ulloa-Aguirre et al., 2001). Human FSH is heavily secondarily modified with sialic acid-enriched oligosaccharides while LH is sialylated to a lesser extent (Green and Baenziger, 1988).

There is evidence that FSH release is predominately constitutive in mammals and birds. Using pulse-chase labelling Muyan and colleges (1994) showed that one third of newly synthesised FSH enters a storage pathway, while most is released immediately after biosynthesis. Further, in rats, GnRH antagonists (Grady et al., 1985; Kartun and Schwartz, 1987) or GnRH immunoneutralization (Culler and Negro-Vilar, 1986) show that while LH secretion is suppressed, FSH release remains relatively unchanged. In cultured Japanese quail pituitary glands the amount of FSH released into media was found to be three times more than the original pituitary content indicating that constitutive FSH secretion also occurs in birds (Hattori et al., 1986). Taking into account that most FSH secretion is constitutive, it can be argued that measuring FSHβ mRNA provides an alternative to plasma FSH measurements in evaluating FSH secretory dynamics.
In mammals, LHβ and α-subunit mRNAs are poorly correlated with plasma LH (Winters, 1996) and, after synthesis, LH is predominantly packaged and stored in secretory granules prior to GnRH-stimulated release (McNeilly et al., 2003; see section 1.2.2). However, there is evidence that LH release, at least in part, is also constitutive in mammals. For example, after the preovulatory LH release in sheep gonadotrophs contain few LH positive granules. This reduction in stored LH is associated with an absence of GnRH-stimulated, pulsatile LH release (see section 1.2.2.3), and the maintenance of tonic LH secretion and LHβ subunit protein in the rough endoplasmic reticulum (Crawford et al., 2000). This observation suggests that tonic LH secretion after preovulatory release may be partly constitutive.

1.3 Hypothalamic control of gonadotrophin subunit gene expression and release

In birds, as in other higher vertebrates, reproductive activity is controlled by developmental and environmental cues [photoperiod (Dunn and Sharp, 1999; Dunn et al., 2003; Thiery et al., 2002); social (Moffart, 2003); developmental (Wilson et al., 1989; Dunn and Sharp, 1999; Dunn et al., 2003), stress (Cameron, 1997; Smith et al., 2003)]. The information derived from these stimuli is integrated in the brain to regulate concentrations of plasma gonadotrophins and gonadal hormones. The brain area known as the hypothalamus is the link between the higher sensory central nervous system (CNS) and the pituitary-gonadal axis (Fig 1.3 and 1.4). The
hypothalamic control of LH and FSH is mediated by regulatory hormonal factors neuropeptides secreted by parvocellular (small-cell) neurosecretory cells. The axons of the parvocellular neurosecretory cells project terminate on, and secrete neuropeptides into a special set of capillaries, the hypophysial portal vasculature, covering the base of the hypothalamus over a structure called the median eminence, which drain into the anterior pituitary gland. The median eminence is of key importance within the hypothalamic-pituitary-gonadal axis being the interface of communication between hypothalamus and pituitary and hence an integrative area of the nervous and endocrine system. The portal vasculature carries hypothalamic neuropeptides to the anterior pituitary where they bind to respective receptors to mediate cellular changes that control the synthesis and secretion of gonadotrophins (Figure 1.4).

Figure 1.4 Sagittal section of Japanese quail (Coturnix japonica) brain illustrating the anatomical relationship between hypothalamus, median eminence and anterior pituitary gland. C= Cerebellum HB= Hind Brain H= Hypothalamus ME= Median Eminence A= Anterior Pituitary OC= Optic Chiasma CH= Cerebral Hemisphere. (Courtesy of P. J. Sharp).
1.3.1 Neurosecretion and discovery of gonadotrophin releasing hormone (GnRH) and its isoforms

It was first demonstrated in the 1930s that electrical stimulation of the hypothalamus induces ovulation in the rabbit (Harris, 1937). Initially the link between hypothalamus and pituitary was assumed to be neural. Subsequent studies established that “releasing factors” produced in neurones of the hypothalamus were released into hypophysial portal vasculature through terminals in the median eminence and are transported to the anterior pituitary to stimulate gonadotrophin secretion (see section 1.2.2.2).

This conclusion was drawn from the observation that transplantation of the anterior pituitary to the kidney capsule in female rats abolished reproductive function, but ovulatory cycles resumed if the pituitary was returned to its normal location close to the median eminence (Nikitovitch-Winer and Everett, 1958). Direct inference with hypothalamic activity by means of surgical or electrolytic lesions or by sterotaxically positioned knife cuts showed that this part of the brain plays a central role in the regulation of gonadotrophin secretion in birds including the Japanese quail (Sharp and Follett, 1969), chicken (Ravona et al., 1973), and turkey (Opel, 1979). The fact that surgical interference with the hypothalamus inhibits reproductive function implied the existence of an avian gonadotrophin releasing factor. The first direct evidence for such a factor came from the demonstration that infusion of chicken hypothalamic extract into the pituitary gland induced ovulation in the hen (Opel and
Lepore, 1972), and that incubation of chicken pituitaries with quail hypothalamic
extract stimulated gonadotrophin release (Follett, 1970).

### 1.3.2 Structure and function of GnRH and GnRH receptor isoforms

Research to establish the identity of hypothalamic gonadotrophin releasing factors started in the 1960s and by the early 1970s the amino acid sequence of both porcine and ovine GnRH was shown to be a decapeptide (Table 1.1; Matsuo et al., 1971; Burgus et al., 1972). This decapeptide sequence was found to be the same in human, mouse and rat (Seeburg and Adelman, 1984; Mason et al., 1986; Adelman et al., 1986). The amino acid sequence of a non-mammalian form of GnRH was first described in birds when chicken (ch)GnRH-I was purified and the structure (Table 1.1) was found to differ from the mammalian sequence at position 8 where arginine was substituted for glutamine (King and Millar, 1982; Miyamoto et al., 1982). The amino acid sequence of a second form of chicken GnRH, GnRH-II, was described by Miyamoto and colleagues (1984). This second form of GnRH was also a decapeptide, with three different amino acid residuals compared with both mammalian (m)GnRH-I and chGnRH-I. The three differences are histidine at position 5, tryptophan at position 7, and tryrosine at position 8. A third GnRH, lamprey (l) GnRH-III (Sower et al., 1993), has been also been reported in the avian brain.
(Bentley et al., 2004; Berghman et al., 2000) and differs from chGnRH-I in positions 3, 5, 6 and 8 (Table 1.1). The conserved carboxyl and amino terminal amino acid sequences and peptide length within the three GnRH isoforms indicates that these features are critically important for receptor binding and activation (Miller et al., 2004).

Table 1.1 The amino acid structure of chicken GnRH-I, GnRH-II and lamprey GnRH-III. Bold letters indicate the differences in the amino acid sequence of these decapeptides. Data from King and Millar (1982), Miyamoto et al., (1982, 1984), Sower et al., 1993

<table>
<thead>
<tr>
<th>Neuropeptide</th>
<th>amino acid structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken (ch)GnRH-I</td>
<td>Glu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Gln⁸-Pro⁹-Glu¹⁰NH₂</td>
</tr>
<tr>
<td>Mammalian (m)GnRH-I</td>
<td>Glu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Glu¹⁰NH₂</td>
</tr>
<tr>
<td>Chicken GnRH-II</td>
<td>Glu¹-His²-Trp³-Ser⁴-His⁵-Gly⁶-Trp⁷-Tyr⁸-Pro⁹-Glu¹⁰NH₂</td>
</tr>
<tr>
<td>Lamprey (l)GnRH-III</td>
<td>Glu¹-His²-Tyr³-Ser⁴-Leu⁵-Glu⁶-Tyr⁷-Lys⁸-Pro⁹-Glu¹⁰NH₂</td>
</tr>
</tbody>
</table>

GnRH-II, which occurs in all non-agnathan vertebrates, is considered to be ancestral for most of the 15 isoforms of GnRH known to date (Dubois et al., 2002), since it is evolutionarily conserved (Millar, 2003; Neill et al., 2004). Molecular phylogenetic analysis shows that the gene encoding for GnRH-II is likely to be the result of gene duplication before the evolution of vertebrates (White et al., 1998). It is uncertain whether GnRH-II plays a physiological role in the regulation of gonadotrophin secretion at the level of the pituitary gland in birds and mammals but there is evidence in fish that GnRH-II controls gonadotrophin function (Khakoo et al., 1994; Kim et al., 1995).
GnRH mediates its action on the pituitary gonadotrophs by activating second messenger systems after binding to its receptor. For example, an important mediator of cellular GnRH signaling is intracellular Ca\(^{2+}\) - a net influx of calcium ions causes LH secretion from the gonadotrophs of both mammals (Anderson, 1996) and birds (Liu et al., 1995) and also results in increased gonadotrophin subunit gene expression in rats (Haisenleder et al., 2001). The amino acid sequence of the GnRH receptor was first deduced in the mouse (Tsutsumi et al., 1992) and provided the basis for the cloning of GnRH pituitary receptors from the rat (Kasier et al., 1992), human (Chi et al., 1993), sheep (Brooks et al., 1993), cow (Kakar et al., 1993) and pig (Weesner and Matteri, 1994). Homologues of the mammalian GnRH receptor have been cloned from marsupials (King et al., 2000), chickens (Sun et al., 2001), amphibians (Wang et al., 2001) and fish (Tensen et al., 1997). The non-mammalian GnRH receptors with the greatest homology to the mammalian pituitary receptors have ~45% amino acid identity but show 58-67% identity among themselves (Millar et al., 2004). Although there is some debate on nomenclature, in this Thesis these GnRH receptors are designated type I. This poor conservation of the sequence of the mammalian type I GnRH receptors with the non-mammalian GnRH receptors maybe explained by a sudden acceleration in evolutionary change between non-mammals and mammals that has resulted in the loss of the carboxyl terminal tail in the mammalian type I receptor, a unique feature among G protein-coupled receptors. The change in the structure of the mammalian type I GnRH receptor has altered its
function (see section 1.2.2.3). The presence of three GnRH isoforms in most vertebrates including birds could be associated with three specific GnRH receptor subtypes. In addition to the type-I GnRH receptor, a type II GnRH receptor has been cloned in new and old world monkeys, pigs, amphibians and reptiles, although premature stop codons or deletions occur in the type-II GnRH receptor mRNA transcripts observed in the human, chimpanzee, cow and sheep (Millar, 2003). The transcripts in these species are therefore incapable of being translated to a full length protein and presumably are non-functional as a receptor.

With respect to the control of gonadotrophin secretion, by far the most intensively studied GnRH isoform is mGnRH-I. The pivotal role played by mGnRH-I in reproduction was illustrated by observations on the hypogonadal (hpg) mouse (Cattanach et al., 1977). In this animal, an autosomal recessive mutation truncates the mGnRH-I gene resulting in undetectable gonadotrophin secretion and hypogonadism. Transplantation of hypothalamic tissue from wild type mice (Krieger et al., 1982) or introduction of a complete mGnRH-I gene into the genome of the hpg mouse (Mason et al., 1986) corrects HPG axis activity and supports full reproductive function. Research on the hpg mouse has demonstrated that full gonadal activity is primarily dependent on a functional mGnRH-I neuronal system (Seeburg et al., 1989).

Most of GnRH-I is synthesised and processed in the preoptic area (POA) of the hypothalamus and packaged into storage granules that are transported down axons to the external zone of the median eminence (Seeburg et al., 1987; Fink, 1988).
The secretion of GnRH-I is controlled first at the level of biosynthesis, which may involve changes in gene transcription, mRNA stability and in enzymatic processing of the GnRH-I pre-propeptide (Srinivasan et al., 2004; Son et al., 2003; Dunn and Millam, 1998) and secondly at the level of storage and release from the median eminence (Terasawa, 2001; Bakker and Baum, 2000; Ebling and Cronin, 2000) (Fig 1.5).

![Figure 1.5 The GnRH-I neurone.](image)

**Figure 1.5 The GnRH-I neurone.** GnRH-I neuronal function is controlled at the level of synthesis and processing in the cell body and at the level of GnRH-I release within the terminals of the median eminence.

Investigations into the functional significance of GnRH-I in birds were initiated using mGnRH-I in the chicken, where it was found to induce ovulation and elicit an increase in plasma LH (Bonney et al., 1974, Johnson et al., 1984; Van Tienhoven and Schally, 1972) and to stimulate LH secretion from *in vitro* pituitary preparations (Tanaka et al., 1974). Following the identification of the amino acid sequence of chGnRH-I, King and colleges (1986) showed chGnRH-I also stimulates LH release from chicken pituitary cells *in vitro*. 

20
In mammals, mGnRH-I plays an important role in stimulating the transcription of the gonadotrophin α- and LHβ and FSHβ subunits (see section 1.2.2.3) but there is limited information on the effect of GnRH-I on the synthesis of gonadotrophin subunits in birds. In a study using cultured turkey pituitary cells, chGnRH-I stimulated LHβ subunit mRNA and LH secretion (You et al., 1995) while a similar experiment in the chicken failed to demonstrate an effect of chGnRH-I on LHβ subunit mRNA production (Marsden et al., 1994). There is also evidence in the turkey that α-subunit mRNA (Foster et al., 1992) and in the chicken, that FSHβ subunit mRNA (Shen and Yu, 2002) is stimulated by chGnRH-I.

In the chicken, the secretion and biosynthesis of FSH may be autonomous or at least markedly less dependent on chGnRH stimulation than LH. Using the Krishnan chicken FSH RIA (Krishan et al., 1993) to measure FSH, chGnRH-I injections do not increase plasma FSH in juvenile hens (Dunn et al., 2003), cockerels (Krishnan et al., 1993) nor in laying hens (Buggerman et al., 1998b). These results contrast with data obtained using a chicken FSH immunoassay developed by Sakai and Ishii (1980). Using this assay, plasma FSH increased in quail and chicken after the administration of chGnRH-I although to a lesser extent than an increase in plasma LH (Hattori et al., 1985). Further, using a heterologous mammalian FSH radioimmunoassay (Follett, 1976), chGnRH-I was observed to stimulate chicken FSH release in vitro (Millar et al., 1986). These discrepancies in the effect of chGnRH-I on chicken FSH release may by explained by the relative lack of
specificities of the Sakai and Ishii and Follett assays compared with the more recently developed Krishnan et al. assay.

The likely absence of an acute stimulatory effect of chGnRH-I in FSH secretion suggests that LH and FSH release in the chicken are differentially controlled. This view is supported by the observation that, in broiler breeder cockerels, the pattern of pulsatile FSH secretion is different from that of LH (Vizcarra et al., 2004), and in the hen, the preovulatory surge of plasma LH is not accompanied by a change in the concentration of plasma FSH (Lovell et al., 2000; Vanmontfort et al., 1994; Krishnan et al., 1993). Early studies in mammals addressed the possibility that FSH is controlled by a FSH-releasing factor (RF), which might be an analogue of mGnRH-I (Dhariwal et al., 1965). In support of this view, lesions in the median eminence of castrated rats suppressed LH pulses, but not FSH pulses, whereas animals with posterior to mid-median eminence lesions had no FSH pulses but maintained LH pulses (Marubayashi et al., 1999). Similarly, ablation of the dorsal anterior hypothalamus of ovariectomised female and castrated male rats suppressed FSH, but not LH secretion (Mc Cann et al., 1983). Collectively these results raise the possibility that there is another hypothalamic factor that preferentially releases FSH.

A possible candidate is GnRH-II, the neuropeptide preferentially stimulates both mammalian (Millar et al., 2001; Padmanabhan et al., 2003) and avian (Millar et al., 1986) FSH secretion. In fact, GnRH-II is reported to be more than ten fold more potent than chGnRH-I in stimulating chicken FSH release in vitro (Millar et al.,...
1986). In this study, avian FSH was measured using a heterologous mammalian FSH radioimmunoassay (Follett, 1976), and consequently these data must be accepted with caution (see above). Further, in sheep, a bolus injection of GnRH-II produces a higher ratio of FSH to LH secretion than observed with GnRH-I (Millar et al., 2001). The possibility that GnRH-II is a selective FSH-RF is further supported by the presence of type II GnRH receptors in some mammalian pituitary gonadotrophs (Millar, 2003). In the chicken functional evidence points to one GnRH receptor being present in the pituitary gland (type I GnRH receptor) that mediates the action of both chGnRH-I and GnRH-II (King et al., 1988). Moreover, GnRH-II stimulation of FSH and LH in mammals is completely blocked after administration of specific type I GnRH receptor antagonists (Gault et al., 2003; Densmore et al., 2003; Okada et al., 2003) suggesting that the action of GnRH-II on the gonadotrophs is mediated by the type I GnRH receptor as may be the case in birds (Millar et al., 1986; King et al., 1988). However, a chicken type II GnRH receptor has recently been cloned (accession number: NM 0010126009), but it remains to be established whether this preferentially mediates the gonadotrophic action of either chGnRH-I or GnRH-II.

The physiological relevance of GnRH-II in controlling gonadotrophin secretion is unclear in mammals and birds. In the rat, mGnRH-I fibers are abundant in the median eminence (Jennes and Conn, 1994) while, in contrast, GnRH-II fibers are rarely seen in the musk shrew median eminence (Rissman et al., 1995). Furthermore, hpg mice that lack a functional mGnRH-I peptide and do not ovulate or secrete LH, have GnRH-II immunoreactive cells and fibers within the hypothalamus.
(Chen et al., 1998). In the chicken GnRH-II is absent or not abundant in the median eminence (Sharp et al., 1990; Mikami et al., 1988), but in the Japanese quail, a combined immunocytochemical and high pressure liquid chromatography study shows the median eminence contains abundant GnRH-II terminals (Van Gils et al., 1993). Critically, no detectable release of GnRH-II has been reported from the avian median eminence (Millam et al., 1998) and active immunization against chGnRH-I but not GnRH-II induces ovarian regression in the laying hen (Sharp et al., 1990). Although the functional significance of GnRH-II is not clear, exogenous administration of GnRH-II is more potent in releasing LH than chGnRH-I in the laying hen (Sharp et al., 1990; Wilson et al., 1989) and in the turkey hen (Guemene and Williams, 1999) which may be due to the higher affinity of GnRH-II for the chicken pituitary type I GnRH receptor (Hasegawa et al., 1984).

A second possible candidate FSH-RF is GnRH-III. Yu et al. (1997) reported that, in the rat, GnRH-III has selective FSH releasing activity, however, more recent studies on the effects of GnRH-III on mammalian FSH release do not confirm this finding (Kovacs et al., 2002, Amstalden, et al., 2004). Further, GnRH-III terminals are abundant in the chicken (Berghman et al., 2000) and sparrow (Bentley et al., 2004) median eminence.
1.3.3 GnRH pulsatility

As demonstrated in the horse (Irvine and Alexander, 1993), sheep (Clarke, 2002; Padmanabhan et al., 1997), cow (Rodriguez and Wise, 1989), and rat (Levine and Remirez, 1982) mGnRH-I is secreted from the hypothalamus in pulses that vary in their frequency and amplitude to change the information content of the message to the anterior pituitary gland. The pulsatile release of GnRH-I results in the pulsatile release of LH (e.g. humans: Reame et al., 1984, rats: Fox and Smith, 1982, sheep: Moenter et al., 1991) Due to the technical difficulty of measuring the concentration of the chGnRH-I in the hypophysial portal vasculature of birds, the pattern of chGnRH-I release has been inferred from measurements of the concentration of LH in blood samples taken at frequent intervals in chicken (Wilson and Sharp, 1975a; Vizcarra et al., 2004), quail (Urbanski, 1984), and turkey (Bacon et al., 1991) or from in vitro observations of GnRH-I secretion from the quail hypothalamus (Ottinger et al., 2004; Millam et al., 1998; Li et al., 1994).

In adult male chickens, pulses of LH release typically recur at intervals of 1-3 hours with the increase in plasma concentrations of LH being as high as 300% of baseline values (Wilson and Sharp, 1975a; Vizcarra et al., 2004). In the laying hen (Wilson and Sharp, 1975a) and turkey (Bacon and Long, 1995) episodic release of LH is undetectable. This does not necessarily mean that in vivo chGnRH-I is not episodically released and evidence for episodic LH discharges has been obtained from ovariectomised hens (Wilson and Sharp, 1975a) while in vitro release of
GnRH-I from female quail hypothalamic slices is pulsatile (Ottinger et al., 2004). The absence of a detectable episodic LH release in the laying hen may be a consequence of the inhibitory effects of high concentrations of plasma oestrogen on the pituitary gland (see section 1.4).

In mammals an episodic pattern of mGnRH-I release is required for normal pituitary function. The secretion of LH and FSH can only be maintained with pulsatile mGnRH-I stimulation: continuous exposure causes a desensitisation of pituitary gonadotrophs and a reduction or complete loss of LH and FSH secretion (Belchetz et al., 1978; Weiss et al., 1990a; Weiss et al., 1990b). Differences in mGnRH-I pulse frequency and amplitude provide a mechanism for regulating LH and FSH secretion differentially, with high frequency GnRH pulses favouring LH secretion and low frequency pulses favouring FSH release (Wildt et al., 1981; Jayes et al., 1997). In the rat, these frequency-dependant effects have been shown to occur in vivo and are mediated by regulating concentrations of gonadotrophin subunit mRNAs (Haisenleder et al., 1991; Dalkin et al., 1989). Similarly, exposure of cultured rat pituitary cells to varying mGnRH-I pulse frequencies result in differential changes LHβ and FSHβ gene expression, and in LH and FSH release (Burger et al., 2002; Kasier et al., 1997) that are similar to observation made in vivo. Fast pulses of mGnRH-I up-regulate LHβ subunit mRNA and LH release as well as type I GnRH receptor mRNA, while slow mGnRH-I pulses up-regulate FSHβ subunit mRNA and FSH secretion (Kasier et al., 1997). This up-regulation of gonadotrophin β-subunit mRNAs in response to differential mGnRH-I pulse
frequency is due, at least in part, to an increase in gene transcription (Bedecarrats and Kaiser, 2003). In contrast, there are no differences in the degree of stimulation to α-subunit mRNA in response to exposure to different frequencies of mGnRH-I pulses and there is evidence that α-subunit mRNA increases in response to continuous exposure to mGnRH-I (Weiss et al., 1990b). The number of GnRH receptors present on the cell surface of a gonadotroph is important in mediating pulse frequency dependent changes in FSHβ gene expression and release in the rat. Over expression of GnRH receptor in the gonadotroph cell line, LβT2 causes a reduction in mGnRH-I stimulated FSHβ promotor activity (Bedecarrats and Kaiser, 2003). It has not been established if differential GnRH pulse frequency affects concentrations of chicken gonadotrophin subunit mRNAs, and LH and FSH secretion.

Although direct evidence for pulsatile secretion of chGnRH-I is lacking in birds, cultured chicken pituitary cells, like mammalian cells, become desensitised in response to continuous stimulation with chGnRH-I (King et al., 1986) and this has been attributed to internalisation of the type I GnRH receptor (Pawson et al., 1998). Such desensitisation is consistent with the requirement for a pulsatile manner of chGnRH-I presentation to support gonadotrophin secretion. This view is further supported by the observation that prolonged treatment of laying hens with long acting GnRH agonists results in an inhibition of ovulation, cessation of egg laying and ovarian regression (Dickerman and Bahr, 1989). Similarly treatment of cockatiels with GnRH agonist blocks photoinduced egg laying (Millam and Finney, 1994). However, chicken pituitary cells differ from mammalian cells in that
desensitisation of the type I GnRH receptor is more rapid (King et al., 1986). This has been ascribed to the cytoplasmic C-terminal tail of chicken type I GnRH receptor (Pawson et al., 1998), which is lacking in the mammalian type I GnRH receptor (Davidson et al., 1994).

1.3.4 Gonadotrophin Inhibitory hormone (GnIH)

In 2000, a novel 12 amino acid neuropeptide (Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH₂) was isolated from the Japanese quail hypothalamus which was found to inhibit gonadotrophin secretion in vitro and was accordingly named gonadotrophin inhibitory hormone (GnIH) (Tsutsui et al., 2000). GnIH is a member of a family of neuropeptides classified as Rfamides, which are characterised by a C-terminal Arg-Phe-NH₂ motif and are accordingly referred to as RFamides. Immunocytochemical studies using RFamide antiserum show that these RFamide-peptides are localised in the nervous system suggesting that they act as neurotransmitters or neuromodulators.

The first RFamide peptide, Phe-Met-Arg-Phe-NH₂ (FMRFamide) to be described was isolated from the nervous system of the bivalve mollusc Macrocallista nimbosa (Price and Greenberg, 1977), and RFamides were found to occur in all classes of cnidarians, suggesting that these peptides may have been among the first neurotransmitters to evolve (Grimmelikhuijzen et al., 1996). A large family of RFamide peptides were subsequently identified in the nervous system of mammals
(Yang et al., 1985; Hinuma et al., 1998), fish (Fujimoto et al., 1998), amphibians (Koda et al., 2002), reptiles (Vallarino et al., 1994), and birds (Dockray et al., 1983) (Table 1.2). One of the functions of RFamide peptides may be a role in the neuroendocrine control of the pituitary gland. This hypothesis is derived from the discovery of hypothalamic RFamide peptides with prolactin releasing activity in the rat (Hinuma et al., 1998) and growth hormone releasing activity in amphibians (Koda et al., 2001). It may be relevant to note that another RFamide, kisspeptin (KiSS 14, Table 1.2) has been found to stimulate gonadotrophin secretion in the rat by acting on the mGnRH-I neuronal system (Messager et al., 2005; Seminara and Kaiser, 2005).

Table 1.2. Alignment of hypothalamic RFamide peptides found in various species with bovine NPAF

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Species</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPAF</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>bovine</td>
<td>Pain modulation</td>
</tr>
<tr>
<td>NPSF</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>human</td>
<td></td>
</tr>
<tr>
<td>NPFF</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>bovine, human</td>
<td></td>
</tr>
<tr>
<td>GnIH-RP-2</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>quail</td>
<td></td>
</tr>
<tr>
<td>FGRP</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>frog</td>
<td></td>
</tr>
<tr>
<td>RFRP-2</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>bovine</td>
<td></td>
</tr>
<tr>
<td>RFRP-3</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>bovine</td>
<td></td>
</tr>
<tr>
<td>GnIH</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>quail</td>
<td></td>
</tr>
<tr>
<td>LPLRFamide</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
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<td></td>
</tr>
<tr>
<td>PrRP20</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>rat</td>
<td></td>
</tr>
<tr>
<td>PrRP31</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>bovine</td>
<td></td>
</tr>
<tr>
<td>C-RFa</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>carp</td>
<td></td>
</tr>
<tr>
<td>KISS-14</td>
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<td>P518</td>
<td>ABCDEFGHIJKlMNOPQRST</td>
<td>human</td>
<td></td>
</tr>
</tbody>
</table>

Avian GnIH cDNA was first cloned in the Japanese quail (Satake et al., 2001) and antiserum against the Japanese quail GnIH peptide sequence has been produced (Tsutsui et al., 2000). In the chicken, GnIH mRNA is found only in the hypothalamus and not in other brain areas or body tissues (Fig 1.6).

Immunocytochemistry shows that the highest numbers of GnIH-immunoreactive cell bodies are in the paraventricular nucleus with abundant immunoreactive nerve terminals in the median eminence (Song Sparrow: Bentley et al., 2003, Quail: Ukena et al., 2003, chicken: Fig 1.7). A quail gene encoding GnIH receptor has been cloned, and shown to be expressed in the quail pituitary gland (Yin et al., 2005; Ikemoto and Parks, 2005).

![Figure 1.6. Tissue distribution of GnIH mRNA in the chicken. a). RT-PCR amplification of mRNA from chicken tissues using oligos specific to the GnIH cDNA sequence (see section chapter 2, section 2.5.5). b). Test of cDNA integrity by amplification of β-actin. 1=Basal Hypothalamus 2=Anterior Hypothalamus 3=Pituitary 4=Olfactory bulb 5=Hippocampus 6=Striatum 7=Cerebellum 8=Optic lobe 9=Hindbrain 10=Fat 11=Liver 12=Proventriculus 13=Gizzard 14=Small intestine 15=Large intestine 16=Adrenal gland 17=Kidney 18=Lung 19=Spleen 20=Heart 21=Skeletal muscle 22=Ovary (stroma) 23=Oviduct B=negative control (H2O blank). (RT-PCR performed by N. A. Ciccone with cDNA provided by T. Boswell).](image-url)
Figure 1.7 Immunostaining of GnIH within the chicken hypothalamus using a GnIH antibody raised against the quail GnIH (Tsutsui, et al., 2002) a), GnIH cell bodies and fibres within the paraventricular nucleus (PVN) and b), GnIH nerve fibres within the median eminence. (Courtesy of S. Lumineau and P. J. Sharp).

It is therefore concluded that GnIH and the GnIH receptor are located appropriately to be involved in the neuroendocrine control of pituitary gland function. The observation that some GnRH-I neurones appear to make synaptic contact with GnIH fibres in sparrows suggests that GnIH may also control gonadotrophin release at the level of the hypothalamus by regulating GnRH-I neuronal activity (Bentley et al., 2003) as well as at the level of the pituitary gland.

The GnIH precursor polypeptide in the quail, White-crowned sparrow and chicken includes at least three RFamide peptide sequences (Table 1.3), comprising a single copy of GnIH, SIRPSAYLPLRF flanked by a glycine C-terminal amidation signal and a single basic amino acid on each end as an endoproteolytic site and GnIH gene-related peptide 1 (GnIH-RP-1), and GnIH-RP-2 (Table 1.3, Satake et al., 2004).
Both of these related peptides are flanked by amidation signals. Their peptide sequences contain a C-terminal LPXRF sequence (X= L in GnIH-RP-1 or Q in GnIH-RP-2), which is identical with or similar to the sequence LPLRF at the C-terminus of GnIH. The high conservation of LPXRF sequence in the chicken, quail and White-crowned sparrow (Table 1.3) suggests that all three RFamides in the GnIH precursor polypeptide may be of functional significance. It is of interest to note that the mammalian orthologues of the avian GnIH precursor polypeptide indicate that they can not be further processed to form GnIH, but could be processed to form mammalian orthologues of avian GnIH-PP-1. Consequently GnIH-RP-1 may have a similar function in birds and mammals, while GnIH and GnIH-RP-2 may be unique to birds.

Both GnJH and GnIH-RP-2 peptides has been demonstrated in the quail brain (Satake et al., 2001) but only the functional significance of GnIH has been investigated. This peptide inhibits LH but not prolactin secretion from cultured adult male quail pituitary glands, but an inhibitory effect on FSH release has not been demonstrated conclusively (Tsutsui et al., 2000). In vivo, GnIH has a transiently depressive effect on plasma LH in the white crowned sparrow (Zonotrichia leucophrys gambelii), and co-injection of a GnIH rapidly attenuates GnRH-I stimulation of LH release in song sparrows (Melospiza melodia) (Osugi et al., 2004). The effect of GnIH on LH and FSH secretion in the chicken has not been reported.
Table 1.3 Amino acid sequence of avian GnIH precursor polypeptides and mammalian orthologues

<table>
<thead>
<tr>
<th>Species</th>
<th>Signal peptide sequence</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>HEIIISSLFIILTLATTSSTLTSNIFSDELYVSMNSHSEK-MYQCVSEPQVYP---QGERSL</td>
<td>AF330057</td>
</tr>
<tr>
<td>Bovine</td>
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<td>AB040291</td>
</tr>
<tr>
<td>Mouse</td>
<td>HEIIISSLFIILTLATTSSTLTSNIFSDELYVSMNSHSEK-MYQCVSEPQVYP---QGERSL</td>
<td>AF330058</td>
</tr>
<tr>
<td>Rat</td>
<td>HEIIISSLFIILTLATTSSTLTSNIFSDELYVSMNSHSEK-MYQCVSEPQVYP---QGERSL</td>
<td>AF330059</td>
</tr>
<tr>
<td>Chicken</td>
<td>HEIIISSLFIILTLATTSSTLTSNIFSDELYVSMNSHSEK-MYQCVSEPQVYP---QGERSL</td>
<td>AB120325</td>
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<tr>
<td>Quail</td>
<td>HEIIISSLFIILTLATTSSTLTSNIFSDELYVSMNSHSEK-MYQCVSEPQVYP---QGERSL</td>
<td>AB039815</td>
</tr>
<tr>
<td>Sparrow</td>
<td>HEIIISSLFIILTLATTSSTLTSNIFSDELYVSMNSHSEK-MYQCVSEPQVYP---QGERSL</td>
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</tbody>
</table>

Signal peptide sequence predicted by Yang et al. (1985). GenBank accession numbers: Human AF330057; Bovine AB040291; Mouse AF330058; Rat AF330059; Chicken AB120325; Quail AB039815; White-crowned sparrow AB128164.

1.4 Modulation of the hypothalamic-gonadotroph axis by feedback of gonadal hormones

The classical method to investigate the feedback effects of gonadal hormones on gonadotrophin release is to measure gonadotrophin function in gonadectomised animals with or without gonadal hormone replacement. The loss of feedback by gonadal factors at the hypothalamic level, results in altered GnRH-I pulse amplitude and frequency (see section 1.3.3), and at the level of the anterior pituitary gland, in
changes in responsiveness to GnRH-I as well as direct effects on gonadotrophin biosynthesis and secretion (Gharid et al., 1990). In the chicken, the effects of gonadectomy on chGnRH-I (Sharp et al., 1994) and gonadotroph (Sharp and Gow, 1983; Terada et al., 1997) function is in part reversible by treatment with gonadal steroids (e.g. oestrogen or testosterone).

In addition to gonadal steroids, the ovary secretes protein hormone, inhibin, which acts as an inhibitory feedback signal to control FSH secretion. Inhibin was first identified in the testes (McCullagh, 1932; De Jong, 1979) and subsequently isolated from ovarian follicular fluid (De Jong, 1988). Ovarian-derived inhibin, has been demonstrated or proposed to be of importance in suppressing plasma FSH, with little or no effect on circulating LH, in mammals and birds by a classical endocrine feedback mechanism (see section 1.6). The ovary also produces two other proteins, activin (Ling et al., 1986; Vale et al., 1986), and follistatin (Robertson et al., 1987; Ueno et al., 1987), which have paracrine functions (Lin et al., 2003; Lovell et al., 2003). Activin and follistatin also occur in the mammalian pituitary where they are involved in the paracrine control of FSH synthesis and release (see section 1.5).

There is no information available on the occurrence of activin or follistatin in the avian anterior pituitary gland.

Inhibins belong to the transforming growth factor β (TGFβ) superfamily of extracellular signaling molecules that are structurally conserved but functionally diverse. Inhibins are dimeric glycoproteins consisting of an α subunit disulphide-linked to one of two distinct β subunits (βA or βB) to generate inhibin-A or inhibin-B
respectively (Fig 1.8). Other members of TGFβ family, the activins, are disulphide linked homo- or heterodimers of βA and/or βB subunits producing activin-A (βA-βA) and activin-B (βB-βB) or activin-AB (βA-βB) (Fig 1.8). The TGFβ family also includes the bone morphogenic proteins (BMPs, Faure et al., 2005; Otuska and Shimaski, 2002; Onagbesan et al., 2003) and Mullerian inhibiting substance (MIS, Bedecarrats et al., 2003) that are also involved in the development and function of the reproductive axis.

Follistatin is a single chain polypeptide with a structure unrelated to inhibin and activin, that is co-expressed with activin in all tissues but not necessarily in the same cell type (Phillips and De Krester, 1998; Kawakami et al., 2001). Follistatin is a high affinity activin-binding protein that when bound the biological activity of activin is abolished (Fig 1.8).
1.4.1 Inhibitory and stimulatory effects of gonadal steroids on the hypothalamic-gonadotroph axis

The frequency and amplitude of LH pulses change during the menstrual cycle of women and these are correlated with plasma oestrogen concentrations (Reame et al., 1984) indicating that oestrogen influences the LH pulse generator. Major steps in understanding the inhibitory effect of plasma gonadal steroids on mammalian
pituitary gonadotroph function began in the early 1980’s when Boime and co-workers showed that RNA isolated from castrated cattle pituitary tissue directed the synthesis of greater amounts of LHβ and α-subunit synthesis than RNA from the pituitaries of intact cows (Keller et al., 1980; Fetherston and Boime, 1982).

Subsequently, Counis and colleagues showed that oestrogen inhibits the translation of mRNAs encoding all three gonadotrophin subunits in the rat (Counis et al., 1983) with similar observations by others in the sheep (Alexander and Miller, 1981; Landefeld et al., 1983; Landefeld et al., 1984). In male rats, castration results in an increase in plasma LH and all three gonadotrophin subunit mRNAs (Papavasiliou, et al., 1986; Dalkin et al., 2001; Burger et al., 2004). Testosterone suppresses this post-gonadectomy increase in pituitary LHβ and common α-subunit mRNAs, but has stimulatory effects on FSHβ mRNA (Paul et al., 1990). In female rats, after ovariectomy, plasma LH and pituitary LHβ and common α-subunit mRNAs increase. Treatment of ovariectomised rats for 2 days with oestrogen prevents the increase in LHβ and α-subunit mRNAs, while FSHβ mRNA unlike LHβ or α-subunit gene expression is only partially suppressed (Dalkin et al., 1990). These findings suggest that FSHβ subunit gene expression may be controlled by other ovarian hormones such as inhibin (see section 1.4), or indirectly by oestrogen through a suppression of mGnRH-I pulse frequency that, in turn, favours FSHβ mRNA and constitutive release.
Gonadectomy results in increased hypothalamic GnRH-I mRNA in male and female rats (Toranzo et al., 1989) and male chickens (Sun et al., 2001) and long term treatment with oestrogen or testosterone, completely prevents the post-gonadectomy mGnRH-I mRNA rise. Long term exposure of ovariectomised female rats to oestrogen results in a decrease in the transcription factor cFos within neurones that co-express mGnRH-I indicating a decrease in mGnRH-I neuronal activity (Tsai and Legan, 2002). *In vitro* studies show that oestrogen reduces mGnRH-I promoter activity and provides evidence that oestrogen suppresses mGnRH-I at the transcriptional level (Wierman et al., 1992; Roy et al., 1999).

Other studies, in the rat, show that the pituitary is also a site of the inhibitory action of oestrogen on gonadotrophin secretion. For example, the rat pituitary gland grafted to the kidney capsule responds to mGRH-I treatment by secreting substantial amounts of LH, but in the presence of oestrogen, this mGnRH-I-stimulated LH secretion is attenuated (Strobl and Levine, 1989). Further, LH pulses induced in ovariectomized rhesus monkeys bearing hypothalamic lesions with intermittent mGnRH-I infusion, are abolished after oestrogen administration (Plant et al., 1978). *In vitro*, in the rat, oestrogen decreases pituitary gland LH responsiveness to mGnRH-I (Frawley and Neill, 1984; Miller and Wu, 1981), while, in sheep, oestrogen suppresses FSHβ (Alexander and Miller, 1981; Alexander and Miller, 1982) and α-subunit (Hall and Miller, 1986) mRNAs. Therefore, it is concluded that oestrogen exerts inhibitory effects on LH release at both hypothalamic and pituitary levels. At the hypothalamic level, the inhibitory effects of oestrogen result in
reduced mGnRH-I pulse frequency and/or amplitude. At the pituitary level, oestrogen may finely tune this effect by altering the response of pituitary gland to mGnRH-I by decreasing GnRH receptor numbers on the surface of the gonadotrophs. In support of this view, oestrogen treatment of gonadectomised female rats decreases pituitary GnRH receptor mRNA (Kasier et al., 1993).

In the chicken, castration induces an increase in hypothalamic chGnRH-I mRNA (Sun et al., 2001) and this is associated with an increase in chGnRH-I peptide (Knight et al., 1983; Sharp, 1990; Wilson et al., 1990b) and chGnRH receptor (Sun et al., 2001) in the anterior pituitary gland. Castration also increases the number of visible chGnRH-I neurones observed using immunocytochemistry (Sharp et al., 1994) and increases both basal and K+ stimulated release of chGnRH-I from perifused hypothalami (Lal et al., 1990). Further, long term treatment of cultured male quail hypothalami with oestrogen decreases baseline GnRH-I release (Li et al., 1994) which is likely to be functionally significant as testosterone is converted to oestrogen by aromatisation within the hypothalamus (Wilson et al., 1983). In support of this view, testosterone has been shown to specifically stimulate hypothalamic aromatase in the quail (Schumacher and Hutchison, 1986). The increase in hypothalamic chGnRH-I mRNA, chGnRH receptor mRNA, chGnRH-I peptide content and chGnRH-I cell numbers in the cockerel induced by castration is reversed after oestrogen treatment (Sun et al., 2001; Sharp et al., 1994). The increase in chGnRH-I neuronal function after castration is associated with an increase in LH secretion in the cockerel (Knight et al., 1983; Lal et al., 1990; Wilson
et al., 1990a) and increased plasma FSH in Great Tits (Parus major) (Silverin et al., 1999).

In the laying hen administration of the oestrogen synthesis blocker, tamoxifen increases hypothalamic chGnRH-I peptide consistent with a suppressive effect of oestrogen on chGnRH-I neuronal function (Wilson et al., 1990b). Oestrogen depresses both FSH pituitary content and plasma FSH in intact hens and this effect does not appear to be secondarily to a depression in chGnRH-I release since chGnRH-I does not directly stimulate FSH secretion in the hen (Krishnan et al., 1998; Dunn et al., 2003; see section 1.3.2). This suggests that oestrogen directly suppresses FSH biosynthesis at the level of the pituitary. Ovariectomy increases circulating LH concentrations and mRNAs encoding LHβ and α subunits in the pituitary gland of chickens (Terada et al., 1997) and turkeys (Petrowski et al., 1993). The ovariectomy-induced increase in plasma LH and pituitary LHβ and α subunit mRNAs in the chicken is prevented by oestrogen treatment (Terada et al., 1997) although it is unclear whether the inhibitory effect of oestrogen is mediated at the levels of the hypothalamus or pituitary gland.

The inhibitory effects of oestrogen on the chGnRH-I neurone or gonadotroph in laying hens (Cockrem and Rounce, 1994) may account for the sex differences in both plasma FSH (Krishnan et al., 1993) and LH (Sharp et al., 1987), which are lower in laying hens than in adult cockerels. The LH response to chGnRH-I in vivo is lower in laying hens than in adult cockerels which is thought to reflect the greater
inhibitory effect of oestrogen in the hen, since plasma oestrogen is much higher in the hen than in the cockerel (Sharp et al., 1987). This view is strengthened by the finding that juvenile chickens of both sexes, which have low circulating oestrogen, show similar sensitivity and responsiveness to chGnRH-I (Wilson et al., 1989; Sharp et al., 1987). Additionally, pituitary responsiveness to chGnRH-I decreases in female chickens (Knight et al., 1985) but not in male chickens during sexual maturation (Wilson et al., 1989; Sharp et al., 1987), correlating with increased oestrogen secretion in the female but not the male. Oestrogen may also exert an inhibitory effect on chGnRH-I release as treatment of intact cockerels with oestrogen increases chGnRH-I in the basal hypothalamus which might indicate an inhibition of chGnRH-I release (Wilson et al., 1990). Further, chGnRH-I peptide is higher in laying hens (high oestrogen) than in adult cockerels (low oestrogen) (Liu, 1993) again indicating a reduced release of chGnRH-I in the hen.

There is also evidence that oestrogen acts at the level of the anterior pituitary gland in birds to reduce gonadotrophin synthesis and release. Observations in vitro using cultured chicken pituitary cells show that chGnRH-I stimulated LH release is attenuated by oestrogen (King et al., 1989). Further, anterior pituitary glands from laying hens contain 10 times less LH than adult cockerels (Liu, 1993). In addition, K+ induced release of pituitary LH in vitro is lower in laying hens than in adult cockerels (Liu, 1993). In contrast, there is no sex difference in pituitary content or K+ induced in vitro release of LH in juvenile male and female chickens (Liu, 1993).
In addition to their inhibitory effects on tonic gonadotrophin biosynthesis and secretion, gonadal steroids are also involved in generating the pre-ovulatory LH surge in females in both mammals and birds. When rat pituitary fragments are exposed to oestrogen in short term culture (2 or 6 hours), no effects are seen on α or FSHβ mRNAs while LHβ mRNA increases (Shupnik et al., 1986), highlighting a mechanism that might facilitate the preovulatory LH release. In female sheep oestrogen exerts a positive feedback effect at the hypothalamic level that culminates in mGnRH-I release and a preovulatory surge in LH (Karsch et al., 1997; Clarke, 1987) and increases pituitary GnRH receptor mRNA and protein (Brooks and McNeilly, 1994; Turzillo et al., 1998). In the female rat, the preovulatory LH surge also seems to be triggered by the positive feedback action of oestrogen (Ferin et al., 1969) acting on the mGnRH-I neurone and is enhanced by progesterone which increases the amplitude of the LH surge (Freeman et al., 1967). In the chicken, in contrast, progesterone rather than oestrogen, elicits the preovulatory LH surge, although oestrogen primes the hypothalamic-gonadotroph axis to make it responsive to the stimulatory effects of progesterone on LH secretion (Wilson and Sharp, 1976). It appears that chGnRH-I also mediates the increase LH release during a preovulatory surge in the hen since increase in plasma LH which is induced in a laying hen after an injection of progesterone is associated with a decrease in hypothalamic GnRH-I peptide content indicating substantial chGnRH-I release (Wilson et al., 1990b).
1.5 Intra-pituitary control of gonadotrophin gene expression and release

In mammals, gonadotroph function is also regulated by autocrine factors produced by gonadotrophs themselves, and by paracrine factors from surrounding pituitary cells. Such factors include pituitary adenylate cyclase-activating polypeptide (PACAP), adenosine triphosphate (ATP), nitric oxide (NO) as well as activin and follistatin (Winters and Moore, 2004). While some studies have sought to identify a unique hypothalamic FSH releasing factor to explain instances in which FSH and LH secretion diverge (Dhariwal et al., 1965; Yu et al., 1997; Koppan et al., 1998) there is evidence that FSH secretion is selectively regulated by autocrine/paracrine interactions in the pituitary. The best known of these are an interaction between activin and follistatin which respectively increase and decrease constitutive FSH release (Burger et al., 2002; Besecke et al., 1996). Activin stimulates FSH release by increasing FSHβ mRNA stability (Carrol et al., 1991; Attardi and Winters, 1993) and rate of FSHβ gene transcription (Huang et al., 2001) through activin receptor subtypes present in the pituitary (Dalkin et al., 1996). The importance of activin in controlling FSH release is demonstrated by depriving pituitary gonadotrophs of exposure to pulsatile GnRH-I (Koppan et al., 1998; Farnworth et al., 1995; Kartun and Schwartz, 1987; Culler and Negro-Vilar, 1986; Sheridan et al., 1979; Hattori et al., 1986). Under these circumstances for a short time, in both mammals and birds, the gonadotrophs do not secrete much LH, but continue to secrete substantial
amounts of FSH. Additionally, activin treatment is essential for the expression of FSHβ mRNA in the LβT2 gonadotroph cell line (Alarid et al., 1996; Nicol et al., 2004; Graham et al., 1999).

Follistatin and inhibin/activin α and β subunit mRNAs are present in the anterior pituitary gland of sheep (Baratta et al., 2001), cow (Gospodarowicz and Lau, 1989), rat (Besecke et al., 1996; Meunier et al., 1988) and monkey (Kawakami et al., 2002). Anterior pituitary cell preparations from rats secrete both activin A and activin B (Bilezikjian et al., 1993a) as well as follistatin (Bilezikjian et al., 1993b). Immunoreactive inhibin α-subunit also occur in gonadotroph secretory granules (Roberts et al., 1992), which raises the possibility that inhibin is present in gonadotrophs. However, inhibin α-subunit within these secretory vesicles are monomeric and in precursor or partly processed forms. There is therefore doubt whether the mammalian pituitary synthesises biologically active inhibin (Farnworth, 1995). It is therefore concluded that gonads are the principal source of inhibin which regulates FSH release at the level of the pituitary gland (Welt et al., 2002).

While inhibin is readily measurable in peripheral circulation (Welt et al., 2002) there is very little free circulating follistatin or free activins in different physiological states in mammals (Padmanabhan and Sharma, 2001). Immunoneutralisation of activin B produced by cultured rat pituitary cells demonstrates that locally secreted activin B provides a positive signal that drives FSH secretion by an intrapituitary mechanism (Corrigan et al., 1991). Addition of inhibin to rat pituitary cell cultures to block activin receptors (see section 1.6) or addition of follistatin to bind activin
reduces FSHβ mRNA and FSH secretion. This demonstrates that inhibin and follistatin suppress FSH release by inhibiting activin signaling (Ying et al., 1988). Furthermore, activin stimulated FSH release can be enhanced by treating rat pituitary cultures with a follistatin-specific antibody, providing evidence that follistatin plays a significant intra-pituitary role in inhibiting FSH secretion (Bilezikjian et al., 1993b).

Experimental work in the rat focusing on the role of intra-pituitary regulation of FSH production indicate that mGnRH-I and gonadal hormones exert their effects on FSH production, at least in part, by modulating pituitary follistatin and activin βB subunit expression and thus altering the local availability and ratio of activin B and follistatin (Burger et al., 2004). After gonadectomy pituitary follistatin, activin βB and activin receptor type-II mRNAs (see section 1.6) increase in parallel with FSHβ mRNA (Burger et al., 2004) and different patterns of mGnRH-I pulse frequency produce different effects on activin βB and follistatin mRNAs (Kirk et al., 1994; Besecke et al., 1996; Burger et al., 2002). In the rat, continuous exposure to mGnRH-I (Bauer-Dantoin et al., 1996) or exposure to high mGnRH-I pulse frequency (Besecke et al., 1996) increases follistatin mRNA while exposure to low mGnRH-I pulse frequency increases activin βB mRNA (Fig 1.10). These observations suggest that FSH release is preferentially induced by exposure of the pituitary gland to low mGnRH-I pulse frequency.
In contrast, gonadectomy in the monkey does not stimulate pituitary follistatin mRNA (Kawakami et al., 2002) and mGnRH-I does not stimulate follistatin mRNA in cultured monkey pituitary cells (Winters and Moore, 2004). This suggests that there are species differences in the role of follistatin and activin in the control of FSH secretion in mammals. The role activin and follistatin might play in controlling intra-pituitary FSH secretion in poultry is unknown.

### 1.6 Effects of inhibin on FSH secretion

The finding that oestrogen treatment of gonadectomised rats inhibits FSHβ mRNA substantially less than LHβ or α-subunit mRNAs suggests that a non-steroidal gonadal hormone regulates FSHβ mRNA and subsequent constitutive FSH release (Dalkin et al., 1990). Administration of inhibin A to adult rats suppresses FSHβ
mRNA and FSH secretion but not LHβ and α-subunit mRNAs (Carroll et al., 1991a). Furthermore, inhibin A injection inhibits the increase of FSH after gonadectomy in male and female rats (Carroll et al., 1991a). Inhibin-based antigens have been used to immunoneutralise endogenous inhibin in cattle (Scanlon et al., 1993; Martin et al., 1991) sheep (Wrathall et al., 1990) pigs (King et al., 1990), rats (Rivier and Vale, 1989; Rivier et al., 1986), quail (Moreau et al., 1998), turkey (Ahn et al., 2001) and broiler hens (Lovell et al., 2001; Satterlee et al., 2002). In general, these studies show that immunisation against inhibin enhances ovarian follicular development or ovulation rate, but this is not always associated with an increase in plasma FSH. Although there is circumstantial evidence in the chicken that inhibin controls FSH secretion in vivo (Lovell et al., 2000), there is no increase in plasma FSH in broiler hens (Lovell et al., 2001) or turkey hens (Ahn et al., 2001) after inhibin immunisation, although inhibin immunisation does increase FSHβ but not LHβ subunit mRNAs in the turkey pituitary gland (Ahn et al., 2002).

The relationship between follicular growth, plasma inhibin and FSH has been investigated in domestic fowl. Both Vanmontfort et al. (1992) and Lovell et al. (2000) showed that levels of plasma inhibin fall and plasma FSH increase coincident with ovarian regression. The granulosa layer of the largest hierarchical follicles (F1 to F4) contains the most inhibin A protein (Lovell et al., 2001) and surgical removal of the hierarchical follicles (F1 to F4) is associated with a reduction in plasma inhibin (Johnson et al., 1993a).
The cellular mechanism by which inhibin suppresses pituitary FSH release remains unclear but probably involves antagonism of activin signaling (see section 1.5) in mammals (Phillips and Wooruff, 2004), and this may also be the case in birds (Sweeney and Johnson, 2005). Although conclusive evidence for the existence of a cognate inhibin-specific signaling receptor has not emerged (Phillips and Wooruff, 2004) other ligands of the TGFβ family, such as activin, bind to, and form hetero-oligomeric complexes with two types of cell surface receptors, designated type-I and type-II. Several type-I and type-II activin receptors have been cloned and characterised in mammals (Abe et al., 2004) and three activin receptor-subtypes have been reported in birds (Lai et al., 2000; Nohno et al., 1993). The association of activin with type-I receptor requires the presence of type-II receptors and both receptor types are required to mediate ligand signaling (Abe et al., 2004). Inhibin also binds to activin type-II receptors, although with an affinity ~20 times lower than activin (Zimmerman and Mathews, 1996). The binding of inhibin to type-II receptors prevents the recruitment of type-I receptors to the complex, and appears to be the mechanism through which inhibin antagonises the biological actions of activin (Gray et al., 2002; Phillips and Wooruff, 2004). Inhibin binds to betaglycans, also known as type-III TGFβ receptor (Lewis et al., 2000), which enhances inhibin binding to activin type-II receptors and so renders inhibin a more effective activin antagonist. Inhibin A has been shown to bind betaglycan with high affinity and, in the presence of an activin type II receptor (ActRII), forms a highly stable complex (Lewis et al., 2000). In the chicken, betaglycan mRNA and protein are abundantly
expressed within the anterior pituitary (Sweeney and Johnson, 2005). Moreover, betaglycan protein colocalises to FSH-expressing pituitary gonadotrophs with a greater abundance than in other pituitary cell types (Sweeney and Johnson, 2005). The avian pituitary gland therefore has receptors required for inhibin to suppress FSH release by potentially antagonising activin signalling. However, it has not been established whether activin occurs in the avian pituitary gland.

1.7 Reproductive senescence in mammals and birds

Reproductive ageing in female mammals and birds is characterised by reduced numbers of ovarian follicles and fertility in rats (Anzalone et al., 2001), mice (Gosden et al., 1983) and chickens (Sharp et al., 1992). The onset of reproductive senescence is characterised by increased incidence of attenuated and delayed preovulatory LH surges (Wise, 1984; Wise, 1982; Cooper et al., 1980) and increased plasma FSH in sheep (De Souza et al., 1998), rats (Meredith and Butcher, 1985) and women (Klein et al., 1996). This age-related rise in plasma FSH is associated with a reduction in plasma inhibin in sheep (De Souza et al., 1998) and rats (De Paolo, 1987).

Diminished preovulatory LH surges in aging rodents are associated with an attenuation of pituitary LHβ subunit gene expression and reduced LH protein synthesis (Krieg et al., 1995). Attenuated LH preovulatory surges in ageing rats are
also caused in part, by reduced gonadotroph sensitivity to mGnRH-I associated with decreased GnRH receptor mRNA (Rubin and Jimenez-Linan, 1999) and mGnRH-I binding capacity (Marchetti and Cioni, 1988). Ageing female rats also have longer intervals between LH pulses and decreased LH pulse amplitude relative to young rats (Scarbrough and Wise, 1990), which may be due to decreased mGnRH-I pulse frequency with advancing age (see section 1.3.3).

Other observations in rats, also points to a decline in mGnRH-I neuronal function in old age. For example, mGnRH-I peptide content in the mid-hypothalamic area is higher in old than in young rats (Takahashi et al., 1990; Rubin et al, 1985) while secreted mGnRH-I is lower in old compared to young rats (Rubin and Bridges, 1989). These data point to an age-related accumulation of mGnRH-I peptide which may be a consequence of impaired in release.

A similar mechanism might account for the reduced persistency of egg laying in ageing hens. A reduction in pituitary and plasma LH is related to a decline in ovarian function in old laying broiler breeders (Sharp et al., 1992) and turkeys (Guemene and Williams, 1999) and, although, this does not appear to be associated with reduced hypothalamic chGnRH-I peptide (Sharp et al., 1992), it may be due to a reduction in chGnRH-I release, reflecting a decrease in chGnRH-I pulse frequency or amplitude.
In addition to a reduction in chGnRH-I release during ageing, an increase in GnIH synthesis (see section 1.3.4) may also contribute to the age-related reduction in egg production in the broiler breeder hen. To date, this possibility has not been investigated.

1.8 Effects of nutrition on reproduction in mammals and birds

Broiler breeders are fed a restricted diet to maximise egg production (see section 1.1.3), but the neuroendocrine mechanisms affected are not understood. The link between food intake and reproductive function are well established in mammals (e.g. Hoffer et al., 1986, Campbell et al., 1977) and birds (Tanabe et al., 1981, Vanmontfort et al., 1994) where periods of under nutrition result in low circulating concentrations of LH and gonadal steroids. Re-feeding of food-restricted rats restores circulating levels of LH while FSH levels tend to be less sensitive to changes in nutritional status (Nakanishi, 1976, Howland, 1975). The inhibitory effects of reduced food intake on gonadotrophin secretion is associated with decreased hypothalamic GnRH-I release (rats: Berghandal et al., 1991) rather than to decreased responsiveness of anterior pituitary gland to GnRH-I (rats: Foster et al., 1989; chicken: Bruggerman et al., 1998). This view is supported by the observation in mammals that starvation-induced impairment of gonadotrophin and sex steroid secretion is reversed by pulsatile administration of mGnRH-I (Bergendahl, 1991). Further, food restricted broiler breeder hens (Bruggerman et al., 1998) and fasted men and women (Rojdmarl, 1987) have higher plasma LH than control groups after
administration of GnRH-I. Taken together these findings provide evidence that the pituitary LH response to GnRH-I administration during a period of reduced food intake is increased. The underlying mechanism responsible for the reduction in GnRH-I secretion in fasted animals could be the result of decreased GnRH-I gene expression or GnRH-I mRNA stability or a reduction in stimulatory inputs or increase in inhibitory inputs to GnRH-I terminals in the median eminence to reduce GnRH-I release. Alternatively, at least in birds, GnIH neuronal function (see section 1.3.4) may be stimulated during periods of reduced food intake which would also account for reduced plasma LH.

Hypothalamic mGnRH-I mRNA (McShane et al., 1993) and peptide content (I'Anson et al., 2000) are not affected by food restriction in sheep, indicating an inhibition of mGnRH-I release rather than biosynthesis. On the other hand, in the rat, numbers of visible mGnRH-I mRNA-positive neurones are reduced in the preoptic area (POA) after 60 hours food restriction (Gruenewald and Matsumoto, 1993), while there is a reduction in mGnRH-I primary transcripts in the POA if oestrous cycles are disrupted as a consequence of 48 hour food deprivation (Nappi and Rivest, 1997). In chronically food restricted broiler hens chGnRH-I peptide stored within the median eminence is decreased (Bruggerman et al., 1998) and, in chronically food-restricted cockerels, in vitro baseline and K+ induced release of hypothalamic chGnRH-I is reduced (Lal et al., 1990). This suggests that in chickens food-restriction reduces chGnRH-I release. In contrast, basal mGnRH-I secretion from median eminence of acutely fasted rats was not reduced while stimulation with
K+ resulted in a higher release of mGnRH-I than in the control group (Warnhoff et al., 1983). The mechanisms underlying the alterations of GnRH-I release and biosynthesis by fasting or food restriction are therefore unclear. It could involve either, or both, an inhibitory effect at the level of the GnRH-I terminal or at the level of the GnRH-I cell to reduce GnRH-I mRNA production or stability.

The view that metabolic signals generated by changes in food intake may directly affect hypothalamic GnRH-I mRNA is supported by the observation that mGnRH-I mRNA increases in cultures of mouse hypothalamic neuronal cells after incubation with insulin and leptin, which are key hormones involved in metabolic signaling (Burcelin et al., 2002). Another candidate providing a link between metabolic signals and the reproductive system is the neurotransmitter neuropeptide Y (NPY). The release of NPY secretion increases in response to food restriction in both mammals (Kalra et al., 1991) and birds (Boswell et al., 1999) and could therefore decrease in response to increased food intake. Changes in GnRH-I mRNA and LH secretion associated with a change in food intake may be mediated by NPY neurones (McShane et al., 1993). In support of this view, NPY peptide has been implicated in the control of pre-ovulatory release of LH (Contijoch et al., 1993) and of the onset of puberty in the chicken (Kuenzel and Fraley, 1995). RFamides have also been implicated in the control of feeding behaviour (Dockray, 2004), this points to the possibility of an involvement of GnIH as a mediator between food intake and reproductive function.
1.9 Effects of incubation on reproductive function in birds

The survival of a species depends on the ability of parents to produce viable young and consequently, the quality of parental care has been selected for and maintained by natural selection (Rosenblatt, 2003). Incubation behaviour in poultry is associated with strong inhibition of the reproductive axis resulting in the cessation of egg production (Sharp et al., 1998). In the chicken the onset of incubation behaviour is characterised by an increasing attachment to a nest site and a growing clutch of eggs (Sharp et al., 1998). Incubation can be terminated, and a return to egg laying can be initiated, by nest deprivation (Richard-Yris et al., 1998; Dunn et al., 1996).

Hypothalamic chGnRH-I peptide in chickens (Burke et al., 1990) and turkeys (El Halawani et al., 1993; Rozenboim et al., 1993) has been reported to decrease during incubation, although other studies fail to demonstrate this (Dunn et al., 1996; Millam et al., 1989). Hypothalamic chGnRH-I mRNA is very low during incubation and rapidly increases when incubation behaviour is terminated by nest deprivation (Dunn et al., 1996). This provides strong evidence that chGnRH-I neurones are inhibited in incubating hens.

The consequence of reduced GnRH-I neuronal activity on gonadotrophin function in incubating turkey hens is reflected in a reduction in plasma LH and LHβ subunit mRNA (Wong et al., 1992). However, there is a robust LH response to chGnRH-I injection in incubating hens (Sharp and Lea, 1981) and turkeys (Guemene and Williams, 1999), which supports the hypothesis that chGnRH-I secretion is reduced.
in incubating hens. However, it is relevant to note that incubation behaviour is characterised by hyperprolactinemia, and that nest deprivation causes plasma prolactin to decrease (Ramesh et al., 2001) while plasma LH and oestrrogen increases (Richard-Yris et al., 1998). It is therefore possible in incubating birds, that high plasma prolactin inhibits reproductive function at all levels of the HPG axis. In support of this view, in the turkey, systemic administration of ovine prolactin decreases hypothalamic chGnRH-I and GnRH-II peptide and plasma LH (Rozenboim et al., 1993), while incubation of anterior pituitary cells with prolactin inhibits in vitro LHβ subunit gene expression and blocks the observed in vivo GnRH-I stimulated rise in LHβ subunit mRNA and secreted LH (You et al., 1995). There is also evidence in the turkey that prolactin inhibits the steroidogenic activity of LH in the ovary (Camper and Burke, 1977), although there is doubt whether this is physiologically relevant (Porter et al., 1991).

It is also possible that the decrease in plasma LH in incubating hens is also due, in part, to an increase in the activity of the GnIH neuronal system (see section 1.3.4). This possibility has not been investigated.

1.10 Avian reproductive photoperiodism

Natural selection favours genotypes whose young are reared at the most favourable time of year for survival. In temperate and high latitudes the most common time to breed is during spring when daylength increases immediately before an increase in
food resources required to feed young. The ancestral progenitor of the chicken, the red junglefowl, is photoperiodic and in spite of thousands of years of domestication the commercial chicken reproductive system remains responsive to changes in daylength (Sharp, 1993).

Egg laying in domestic hens is stimulated by increasing daylengths and inhibited by decreasing daylength. Early photoperiodic studies on commercial hens (reviewed: Sharp et al., 1983; 1993), designed to discover the optimal lighting pattern for around the year egg production led to the discovery that prolonged exposure of adult or juvenile birds to long days reduces reproductive function through a phenomenon that has been termed photorefractoriness (Nicholls et al., 1988).

The onset of sexual maturity and the production of a first egg in domestic chickens reared on short days occurs around 26 weeks of age and is advanced by photostimulation at around 17-18 weeks of age. Direct evidence for the early maturation of a stimulatory photoperiodic response comes from a study in which hens were reared from hatch on 8 h light/day and transferred to 20h light/day at 3, 7, 11, 15 and 19 weeks of age (Dunn and Sharp, 1990). A photoinduced release of LH was observed at all these ages although this was not reflected in an accelerated rate of ovarian growth until the birds were more than 7 weeks of age. Plasma FSH concentration increases in response to photostimulation by 8 weeks but not before 5 weeks of age and is therefore a better predictor of gonadal development than LH (Lewis et al., 1999). From 15 weeks of age, the increase in plasma LH in
response to photostimulation is reduced probably because of the inhibitory feedback actions of oestrogen and other steroids from the maturing ovary (Dunn and Sharp, 1990; see section 1.4).

The stimulatory effect of long day lengths is associated with increased hypothalamic chGnRH-I content and increased chGnRH-I immunoreactivity in the hypothalamus and median eminence in all avian species studied: European starling (Foster et al., 1987; Goldsmith et al., 1989; Dawson et al., 1984), Garden warbler (Bluhm et al., 1991), dark-eyed junco (Saldanha et al., 1994), house sparrow (Hahn and Ball, 1995) and Japanese quail (Foster et al., 1988; Perera and Follett, 1992). In the domestic chicken photostimulation is associated with increased hypothalamic chGnRH-I mRNA (Dunn and Sharp, 1999).

The response of chGnRH-I neurones to photostimulation is developmentally regulated, as hypothalamic GnRH-I mRNA content increases after photostimulation in peripubertal and somatically mature chickens, but not in juvenile cockerels before 8 weeks of age (Dunn and Sharp, 1999). However, if juvenile cockerels are treated with oestrogen, hypothalamic GnRH-I mRNA content increases after photostimulation (Dunn and Sharp, 1999). It seems that oestrogen initially stimulates the maturation and response of the reproductive neuroendocrine system to photostimulation in juveniles, but after puberty the action of sex steroids shifts to an overall inhibitory effect. In support of this view, treatment with oestrogen increases plasma LH and FSH in photostimulated juvenile but not adult hens (Dunn et al., 2003).
Measurements of hypothalamic chGnRH-I mRNA in peri-pubertal cockerels and adult hens, indicates that chGnRH-I neuronal activity is increased within a week of an increase in photoperiod from 8 to 14 or 16 hours of light (Gladwell et al., 1996; Dunn et al., 1996; Dunn et al., 1997). The increase in chGnRH-I mRNA is associated with an increase in LH secretion demonstrating that the photoperiodic control of LH release is mediated by a change in chGnRH-I gene expression or chGnRH-I mRNA stability.

The stimulatory effect of increased photoperiod on reproduction is eventually attenuated or terminated by development of absolute or relative photorefractoriness (see below). In Galliforms such as the domestic chicken and Japanese quail the gonads remain mature indefinitely under constant long day lengths, but regress if the photoperiod drops to a photoperiod which is longer than that required to stimulate gonadal growth in a fully photosensitive short day bird. As a consequence in chickens or quail exposed to natural lighting, the annual cycle of gonadal activity is asymmetrical with respect to the seasonal changes in day length with gonadal regression occurring in the autumn when daylength are still longer than that which stimulated gonadal activity in the spring (Sharp, 1984, 1993). To explain this phenomenon Robinson and Follett (1982) suggested that quail become “relatively” photorefractory and the same mechanism has been proposed for the chicken (Sharp et al., 1992; Lewis et al., 2003). These investigators proposed a mechanism whereby the critical photoperiod required to maintain full gonadal function increases with
time of exposure to long photoperiods. A reduction in gonadal function in chickens that are suggested to be relatively photorefractory is not associated with a reduction in hypothalamic chGnRH-I peptide content (Sharp et al., 1992). Therefore, it is likely that the development of relative photorefractoriness in quail and chickens involves a mechanism that inhibits GnRH-I release rather than GnRH-I biosynthesis.

The development of photorefractoriness is more pronounced in passerine species such as the European starling (Sturnus vulgaris) in that they become “absolutely” refractory to stimulation by light. In the starling, and in contrast to quail and chickens, photorefractoriness is characterised by a decrease in concentration of chGnRH-I peptide in the hypothalamus and a decrease in the number and size of chGnRH-I immunoreactive cell bodies, and chGnRH-I neurone immunoreactive terminals in the median eminence disappear (Goldsmith et al., 1989).

1.11 Aims of Thesis

The overall aim of this Thesis was to gain further understanding of the control of reproduction in domestic poultry with particular reference to the age-related decline in ovarian function in broiler breeder hens. The studies presented in this Thesis focus on measuring reproductive neuroendocrine mRNAs and LH and FSH secretion in relation to ovarian function in chicken and quail. After profiling changes in reproductive neuroendocrine mRNAs and LH and FSH in ageing broiler breeders, experiments were conducted in vivo to change ovarian function in laying hens.
supplemented with *in vitro* observations, in order to further understand neuroendocrine mechanisms responsible for age-related loss of persistency of lay. Observations were made on changes in reproductive neuroendocrine gene expression associated with reduced ovarian function in incubating hens, in food restricted broiler breeder hens, and in quail after transfer from long to short days. Reproductive neuroendocrine mechanisms which may be responsible for an age-related loss of persistency of lay is hypothesised to involve chGnRH-I and GnRH-II neural systems and gonadotrophin subunits, and additionally the less well investigated GnIH neuronal system, and intra-pituitary activin and follistatin.

The specific aims of this Thesis were:

1. To characterise changes in the HPG axis of ageing broiler breeder hens with reduced egg production including the relationship between plasma LH, FSH and hypothalamic chGnRH-I and GnIH mRNAs and pituitary gonadotrophin subunit and follistatin and activin βB mRNAs. It is hypothesised that a decrease in chGnRH-I neuronal function and an increase in GnIH neuronal function is responsible for the age-related loss of reproduction in broiler breeder hens. The expression of follistatin and activin βB mRNAs in the pituitary is predicated to be altered as a consequence of diminished hypothalamic chGnRH-I release and reduced gonadal hormones during reproductive ageing and results in decreased plasma FSH. This research is presented in chapter 3.
2. To test the hypothesis that, the food-restricted laying broiler breeder hen, *ad libitum* feeding stimulates ovarian development by increasing chGnRH-I gene transcription or chGnRH-I mRNA stability. The possible increase in chGnRH-I neuronal activity could be mediated by altering hypothalamic NPY gene expression, resulting in altered pituitary gonadotrophin subunit, activin $\beta_{B}$ and follistatin mRNAs and gonadotrophin secretion. In addition, it was hypothesised that a decrease in GnIH mRNA is associated with increased ovarian growth after *ad libitum* feeding. This research is presented in chapter 4.

3. To determine the effects of ovarian regression induced by the development of incubation behaviour (Chapter 5) and transfer from long to short days (Chapter 6) on gonadotrophin subunit mRNAs and LH and FSH release. It is hypothesised that expression of incubation behaviour depresses both FSH and LH secretion by inhibiting chGnRH-I gene transcription or chGnRH-I mRNA stability, which, in turn, inhibits gonadotrophin subunit mRNAs. Additionally it is hypothesised that GnIH mRNA increases in incubating hens to result in an additional suppression of plasma gonadotrophins. It is hypothesised that inhibition of ovarian function by reducing daylength results in decreased plasma LH associated with decreased gonadotrophin subunit mRNAs.

4. To determine *in vitro* the effects of pulsatile chGnRH-I, GnRH-II and GnIH on pituitary gonadotrophin function. It was hypothesised that chGnRH-I and II would
stimulate gonadotrophin gene expression while GnIH would inhibit it. Further, it was hypothesised that pulsatile chGnRH-I is required to regulate follistatin and activin $\beta_B$ mRNAs which in turn would regulate FSH$\beta$ mRNA. This research is presented in chapter 7.
CHAPTER 2. Materials and Methods

2.1 Animals

Broiler breeder hens (Cobb Vantress) were either obtained from a commercial pedigree flock (chapter 3) or purchased at one day old (chapter 4 and 8) from a local hatchery (Whitburn hatchery, Grampian Country Foods, Whitburn, Scotland). White Leghorn and Silkie hybrid hens (chapter 5), adult Japanese quail, *Coturnix japonica* (chapters 6) were taken from the Roslin Institute’s breeding flocks. Pituitary glands for studies *in vitro* (chapters 7) were from adult ISA Brown cockerels (ISA Poultry Services Ltd, Peterborough, UK) or broiler breeder hens (Cobb Vantress) purchased at one day old.

![Figure 2.1. Broiler breeder hens reared at the Roslin Institute](image)

2.1.1 Husbandry and welfare

All experimental procedures were carried out under United Kingdom Home Office Project Licence number PPL 60/3178 and Personal Licence number SCT-E/03/211.
Broiler breeder hens from the commercial breeding flock were killed on site and biological samples were processed in the laboratory. All birds obtained from Roslin Institute were held on a 18L:6D lighting pattern with free access to food and water. Birds were housed in individual cages apart from broiler breeder and incubating hybrid hens. Broiler breeder hens purchased from the local hatchery were reared in floor pens at the Roslin Institute (Fig. 2.1). Chicks were fed a commercial restricted feeding programme from 2 weeks old as recommended by Cobb Vantress (Cobb management guide 2001) to maximize egg laying and to prevent obesity. Water was freely available during rearing and throughout the experimental period. White Leghorn and Silkie hybrid birds were housed in floor pens. Incubation was encouraged by adding nest boxes with 4-6 boiled eggs to the pens just before the onset of lay at 16 weeks of age. A poultry attendant maintained a daily record of the absence or expression of incubating behaviour of each hen. Incubating hens selected for the experiments (Chapter 5) had been incubating for 8-21 days before sample collection. All incubating hens had fully regressed ovaries with no yellow yolky follicular hierarchy and laying hens had fully developed ovaries with fully developed yellow yolky follicular hierarchies.

2.2 Administration of test substances and blood sampling

Intravenous injections were administered into a brachial wing vein. Blood samples (~1 ml) were taken from a brachial vein using a heparinised (PUMP-HEP®, Leo laboratories Ltd, Princes Risborough, Bucks, UK) 2ml syringe (Terumo Europe,
Leuven, Belgium) with a 25 G, 0.5mm needle (Teruno Europe). Plasma fractions obtained by centrifugation at 250 x g, 4°C for 15 minutes were stored at -20°C.

2.3 Collection of neuroendocrine tissues

All birds were killed by cervical dislocation (Home Office Schedule 1 - killing). After killing the bird was decapitated and the lower mandible, excess bone and tissue removed with a sharp pair of dissecting scissors to expose the skull.

2.3.1 Collection of pituitaries for in vitro cell culture

For studies of anterior pituitary function in vitro the gland was removed as rapidly as possible using a dorsal approach. A transverse cut was made across the rear of the skull and two additional lateral cuts were made to connect the transverse cut with the rear of the eye sockets. The base of the skull was broken transversely behind the eye sockets by pulling up the skull to the front of the transverse cut and pressing down on the rear portion of the base of the skull. The brain stalk was cut and the whole brain was reflected forward with the base uppermost. This procedure allowed rapid access to the anterior pituitary gland which remained in or was pulled out of the sella turcica, the cavity that surrounds the gland. A pair of fine forceps was used to remove the anterior pituitary. Freshly dissected pituitaries were pooled into a sterile
15 ml tube (Greiner) containing chilled phosphate buffered saline (PBS). The time elapsed from killing to removing the anterior pituitary gland was less than 3 minutes.

2.3.2 Collection of neuroendocrine tissues for studies in vivo

For studies involving the measurements of pituitary and hypothalamic mRNAs the tissues were dissected using a ventral approach. Tissue and bone from the base of the skull was removed using fine scissors and a rongeur. Once the posterior of the optic nerve and sella turcica were located the bone overlaying the base of the anterior pituitary was removed using fine scissors cutting rostrally and bilaterally from the optic chiasma. The anterior pituitary was removed with a pair of fine forceps and placed into a sterile 2 ml screw cap tube (Sarstedt) weighed on a balance (Sartorius model BP210s, Epsom Surrey) and quickly snap frozen in liquid N₂ and stored at -80°C. All bone underlaying the basal hypothalamus was removed to reveal the median eminence. Additional bone was removed from the base of the brain to reveal all of the optic chiasma and the base of the pre optic hypothalamus and mid-fore brain. The optic chiasma was removed and bilateral cuts 2 mm to each side of the midline were made with a sterile scalpel blade from rostral preoptic area (POA) to the roots of the occulomotor nerves. Transverse cuts were then made rostral to the POA and caudal to the roots of the occulomotor nerves and the whole hypothalamus was lifted out of the brain using a small pair of curves scissors. This tissue block contained all GnRH-I (Dunn et al., 1996) and GnIH (Ukena et al., 2003) neurones.
The hypothalamic block was placed into a sterile 2 ml screw cap tube and quickly snap frozen in liquid N$_2$ and stored at -80°C.

### 2.4 Chicken gonadotrophin radioimmunoassays

Gonadotrophin quantification in blood plasma or culture medium was achieved by radioimmunoassay (RIA) which involves measuring the ability of an unlabelled gonadotrophin to compete with $^{125}$I labelled gonadotrophin for binding sites on an antibody. If the quantity of antibody and radioactive hormone are held constant, and the amount of unlabelled hormone within the reaction mixture increased, the amount of labelled hormone bound to the antibody is reduced. This relationship underlies the principle of the RIA standard curve (Figure 2.2).

![Principles underlying the standard curve for a radioimmunoassay](Image)
Evaluation of the amount of radioactive hormone bound to the antibody in the presence of varying amounts of non-radioactive hormone is used to generate the standard curve. By comparing the inhibition of binding of radioactive hormone in an unknown sample by a standard of known concentration, it is possible to determine the quantity of hormone present in the test sample (Figure 2.2).

### 2.4.1 Specificity

An important parameter for determining reliability of a RIA is to demonstrate it quantifies only the hormone that it is intended to measure. This characteristic is known as specificity. Specificity is defined as the lack of interference from substances other than the one to be measured. Similarities in the structures of the anterior pituitary glycoprotein hormones FSH, LH and TSH have made production of specific antisera difficult, because these hormones share a common $\alpha$ subunit and have homologies in the amino acid sequences of the hormone specific $\beta$ subunits. Proteins with high homology would share numerous common antigenic determinants. Further compounding this problem is that a purified preparation of chicken TSH is currently unavailable to test for cross reactivity in chicken FSH and LH assays. This problem has been overcome by screening a large number of antibodies for high specificity and ensuring that biological samples dilute out parallel to the standard curve.
2.4.2 Iodination of radiolabelled hormone

The radioisotope most commonly used to label proteins is iodine$^{125}$ (I$^{125}$) that is purchased as a solution of sodium salt NaI$^{125}$. The release of nascent iodine from NaI$^{125}$ is brought about by the use of an oxidising agent like chloramine-T (CL-T) which catalyses the iodination reaction. The oxidant used in the reaction has potential to denature the protein into which the radioiodinate is being incorporated. To minimise this, exposure of the protein to the oxidizing agent is kept to a minimum. To be certain that a molecule of radioiodine is incorporated into each molecule of protein excess NaI$^{125}$ is included into the reaction mixture. This necessitates separation of I$^{125}$ bound to unbound I$^{125}$. Separation is accomplished by column chromatography. Contents of the radioiodination mixture are applied to a column containing a resin that separates compounds based on size. Since the molecular size of the radiolabelled protein is much larger than NaI$^{125}$, the protein passes through the column much more rapidly (Figure 2.3). The radioiodinated label is saved and free I$^{125}$ discarded.
2.4.3 RIA procedure

The first step in a representative RIA procedure involves dispensing standards and samples (1) accompanied by an antibody (2) (Fig 2.4). This mix is typically incubated for 24 hours. The next step involves the addition of a fixed amount of radiolabelled hormone (3) and allowed to incubate for a further 24 hours. On the third day of a typical RIA secondary (NARS) and tertiary (DARS) antibodies (4-5) are added to assay tubes to produce an antibody-antigen matrix, which facilitates precipitation. Following a final incubation the tubes are spun down and excess radiolabelled hormone is removed. The remaining radioactivity is measured and the data is processed (6).
Chapter 2

Material and Methods

Figure 2.4 Typical RIA procedure

2.4.4 Chicken LH RIA

The double antibody method for radioimmunoassay of chicken LH developed by Sharp and colleagues (1987) was used in the studies presented. All reagents were available “in house”. The intra-assay coefficient of variation was 5.4%. Medium samples were diluted serially in LH radioimmunoassay diluent.

An adapted version of Greenwood’s (1963) chloramine T procedure for iodination was followed to radiolabel purified LH (LH-RI-1) preparations. All reagents used in the preparation of the radiolabelled LH were dissolved in 50mM sodium phosphate buffer, pH 7.5. The iodination of LH-RI-1 was carried out in a specified radioactive hot lab.
Before the start of the iodination procedure a PD-10 Sephadex G-25 column (LKB-Pharmacia) was equilibrated with column buffer (50 mM phosphate buffer + 0.2% gelatin).

**Preparation of 50mM phosphate buffer**

0.25 M Na$_2$HPO$_4$ (17.75 g Na$_2$HPO$_4$ in 500 ml dH$_2$O)

0.25 M NaH$_2$PO$_4$·H$_2$O (17.2 g NaH$_2$PO$_4$·H$_2$O in 500 ml dH$_2$O)

A 1000 ml beaker containing 500 ml 0.25 M Na$_2$HPO$_4$ (base) and a stirring bar was placed on a magnetic stirrer, the solution was mixed and the probe of a pH120 Microprocessor pH meter (Hanna instruments) was placed in the solution. 0.25 M NaH$_2$PO$_4$·H$_2$O (acid) was added to the solution using a Pasteur pipette until the solution had a pH 7.5. This solution was 0.25 M phosphate buffer and was kept as a stock. 100 ml 0.25 M phosphate buffer in 400 ml of dH$_2$O gave a working concentration of 50 mM phosphate buffer.

6.25 µl of 1.25µg LH-R1-1 in a 1.5ml microcentrifuge tube (Sarstedt) was clamped over a magnetic stirrer and surrounded by a lead shield. A small stirring bar (~2mm piece of paper clip) was added to the reaction vessel. The iodination procedure was performed with constant mixing of the magnetic stirrer. 19 µl of 50 mM phosphate buffer and a 37 Mbq unit of [125I]-NaI (IMS-30, APBiotech) in 10 µl was
introduced into the microcentrifuge tube and the reaction started with 10μl of freshly prepared 1 mg.ml⁻¹ chloroamine T sodium salt (Fison). The reaction proceeded at room temperature (17-19°C) for precisely 45 seconds. The reaction was terminated by the addition of 100μl of 1mg.ml⁻¹ sodium metabisulphate, Na₂S₂O₅ (Fisons) and 100μl of 0.1g.ml⁻¹ potassium iodide, KI (Fisons). The content of the reaction tube was then transferred to the equilibrated PD-10 column. The reaction tube was rinsed with 250μl of column buffer and also transferred to the PD-10 column. Column buffer was continuously supplied to the column reservoir until approximately 20 fractions, each containing 10 drops, were eluted into LP4 plastic tubes (Denley-Luckham).

The amount of gamma radioactivity in each tube was then counted using a Mini-Assay type 6-20 counter (Mini-instruments). Intact radiolabelled LH-RI-1 was typically located between fractions 5 – 8 (Figure 2.3), this was preceded by a smaller peak which contained denatured, radiolabelled LH-RI-1. The free iodine eluted between fractions 16 – 20 (see figure 2.4). Tubes containing the intact LH label were pooled and diluted in radioimmunoassay diluent at approximately 500 000 counts per minute (cpm) per 10μl and stored for up to 4 weeks at 4°C.

Stock LH-RI-1 was stored lyophilised in 192μg aliquots and was reconstituted into 192μg.ml⁻¹ solution with 50 mM phosphate buffer (1ml) for use in the assay. Further dilutions of this stock were made using radioimmunoassay diluent as shown in table 2.1.
Table 2.1 Serial dilution of LH-RI-1 stock for standard curve

<table>
<thead>
<tr>
<th>Standard No.</th>
<th>LH-RI-1</th>
<th>Diluent</th>
<th>pg / tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26 μl of stock</td>
<td>974 μl</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>100 μl of 1</td>
<td>900 μl</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>100 μl of 2</td>
<td>900 μl</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>400 μl of 3</td>
<td>3.6 ml</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td>1.6 ml of 4</td>
<td>400 μl</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>1.0 ml of 4</td>
<td>1.0 ml</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>1.0 ml of 5</td>
<td>1.0 ml</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>1.0 ml of 7</td>
<td>1.0 ml</td>
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</tr>
<tr>
<td>9</td>
<td>1.0 ml of 8</td>
<td>1.0 ml</td>
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<tr>
<td>10</td>
<td>1.0 ml of 9</td>
<td>1.0 ml</td>
<td>6.25</td>
</tr>
<tr>
<td>11</td>
<td>1.0 ml of 10</td>
<td>1.0 ml</td>
<td>3.125</td>
</tr>
<tr>
<td>12</td>
<td>1.0 ml of 11</td>
<td>1.0 ml</td>
<td>1.5625</td>
</tr>
<tr>
<td>13</td>
<td>1.0 ml of 12</td>
<td>1.0 ml</td>
<td>0.7812</td>
</tr>
<tr>
<td>14</td>
<td>1.0 ml of 13</td>
<td>1.0 ml</td>
<td>0.3906</td>
</tr>
<tr>
<td>15</td>
<td>1.0 ml of 14</td>
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<tr>
<td>16</td>
<td>1.0 ml of 15</td>
<td>1.0 ml</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.0 ml of 16</td>
<td>1.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

LH Radioimmunoassay diluent

9.875 g Na₂H₂PO₄
1.62 g NaH₂PO₄
17.5 g NaCl
5.84 g EDTA (disodium salt)
2 g sodium azide (BDH)
40 ml horse serum (GIBCO)
dH₂O Milli-Q to make a final volume of 2 litres

pH adjusted to 7.5 using 1 M NaOH using pH120 Microprocessor pH meter (Hanna instruments).
The assay was set up in LP2/2 plastic tubes using a Hamilton Microlab-M 500 series auto-dispense (Howe and Co ltd) as illustrated in table 2.2. Total counts (Tc), non-specific binding (NSB) and standard tubes were dispensed in triplicate and maximum binding (B₀) into six tubes. Quality controls were pooled cockerel plasma and were dispensed into four tubes. After each addition of a reagent the assay tubes were vortexed on a multi-tube vortexer (Alpha Laboratories) and returned to 4°C overnight incubation.

Stock solutions of rabbit anti-LH-R1-1, designated anti-LH 3/3 (Sharp et al., 1987), were stored at -20°C at 1 in 190 dilution in 300μl LH diluent. A working concentration of 1:19 000 was prepared in diluent and added to each assay tube (except Tc and NSB). On the second assay day approximately 12 000 cpm per 50μl of 125I-LH-R1-1 in LH diluent was added to all assay tubes. Normal rabbit serum (NRS) (Scottish Antibody Production Unit, Edinburgh, UK) and donkey anti-rabbit serum (DARS) (Scottish Antibody Production Unit) at 1:200 and 1:20 respectively were each added to the assay tubes in 50μl volumes (except Tc). On the final assay day all tubes were centrifuged (Sovrval RC-3B) at 1500 x g for 30 minutes at 4°C. The integrity of the pellet was preserved by adding 50μl of 6% starch solution (BDH) to each tube (except Tc) using a 125μl multi-dispensing pipette (Eppendorf, Cambridge, UK). Assay tubes were re-centrifuged for an additional 15 minutes. The supernatant fractions were aspirated to waste (apart from Tc) and pellets counted for 60 seconds on a gamma-counter (GammaMaster, LKB Pharmacia).
Table 2.2 Chicken LH RIA protocol

<table>
<thead>
<tr>
<th>TUBE</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD/Sample</td>
<td>Diluent</td>
<td>Anti-3/3</td>
<td>Label</td>
<td>NRS</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>NSB</td>
<td>0</td>
<td>250</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Bo</td>
<td>0</td>
<td>200</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>STD1 to</td>
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<td>0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>STD14</td>
<td>QC1</td>
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<td>50</td>
<td>50</td>
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<tr>
<td>QC2</td>
<td>20</td>
<td>180</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Sample 1</td>
<td>dilution in 200</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Sample 2</td>
<td>etc</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

2.4.5 Chicken FSH RIA

The double antibody method for radioimmunoassay of chicken FSH developed by Krishnan et al (1998) was used in the studies presented. A chicken FSH RIA kit was obtained from Dr. John Proudman of the U.S. Department of Agriculture (Beltsville, MD, USA). The intrassay coefficient of variation was 8.5%. Medium samples were diluted serially in FSH assay buffer. Chicken FSH, named USDA-cFSH-I-1, was radiiodinated using the chloramine T procedure. The iodination of USDA-cFSH-I-1 was carried out in a specified radioactive hot lab.

Before the start of the iodination procedure a PD-10 Sephadex G-25 column (LKB-Pharmacia) was equilibrated with column buffer (50mM sodium phosphate buffer + 0.2% gelatin).
Preparation of 50mM phosphate buffer

0.25 M Na₂HPO₄ (17.75 g Na₂HPO₄ in 500 ml dH₂O)

0.25 M NaH₂PO₄·H₂O (17.2 g NaH₂PO₄·H₂O in 500 ml dH₂O)

A 1000 ml beaker containing 500 ml 0.25 M Na₂HPO₄ (base) and a stirring bar was placed on a magnetic stirrer, the solution was mixed and the probe of a pH120 Microprocessor pH meter (Hanna instruments) was placed in the solution. 0.25 M NaH₂PO₄·H₂O (acid) was added to the solution using a Pasteur pipette until the solution had a pH 7.5. This solution was 0.25 M phosphate buffer and was kept as a stock. 100 ml 0.25 M phosphate buffer in 400 ml of dH₂O gave a working concentration of 50 mM phosphate buffer.

Five µg of cFSH was placed in a 0.5 ml micro-centrifuge tube and dissolved in 10 µl of distilled water. The micro-centrifuge was clamped over a magnetic stirrer and surrounded by a lead shield. A small stirring bar (~2mm piece of paper clip) was added to the reaction vessel. The iodination procedure was performed with the magnetic stirrer constantly rotating. 20 µl of 0.25 M sodium phosphate buffer, pH 7.4 and 10 µl Na¹²⁵I were then added to the iodination vial. The iodination reaction was initiated by addition of 55 µg.ml⁻¹ of freshly prepared chloramine T in 10 µl in 0.25 M sodium phosphate buffer, pH 7.4. The reaction proceeded for 5 minutes at room temperature and terminated with 100 µl transfer solution (100 mg potassium iodide, 1.6 g sucrose, 1 mg bromophenol blue diluted in 50 mM sodium phosphate
buffer) and 200 μl 25% BSA. The reaction mixture was transferred to the equilibrated PD-10 column. Twenty fractions, each containing 8 drops, were eluted into LP4 plastic tubes (Denley-Luckham) containing a few crystals of sucrose (BDH). The amount of gamma radioactivity in each tube was then counted using a Mini-Assay type 6-20 counter (Mini-instruments). Radiolabelled USDA-cFSH-I-1 was typically located between fractions 8-14. The peak iodinated protein fractions were pooled and further purified in an NR 200 column. The NR 200 column, connected to a pump (Gilson model 303), was pre-equilibrated before use and radiolabelled FSH was eluted at ~8 ml/hour with PBS + 0.01% thimerosal into tubes placed in a revolving carousel. Purified iodinated FSH typically fell within fraction numbers 30-40.

Stock reference standard, designated USDA-cFSH-K-I, was dissolved in FSH assay buffer (PBS + 0.1% BSA + 0.01% Thimerosal) at a concentration of 1000 ng.ml⁻¹. 100 μl of this stock was diluted in 900 μl assay buffer, this was used as the top standard.
Table 2.3 Serial dilutions of USDA-cFSH stock for standard curve

<table>
<thead>
<tr>
<th>Standard No.</th>
<th>USDA-cFSH-Kl (µl)</th>
<th>Assay Buffer (µl)</th>
<th>pg / tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 of stock</td>
<td>900</td>
<td>5000</td>
</tr>
<tr>
<td>2</td>
<td>560 of 1</td>
<td>140</td>
<td>4000</td>
</tr>
<tr>
<td>3</td>
<td>350 of 2</td>
<td>350</td>
<td>2000</td>
</tr>
<tr>
<td>4</td>
<td>350 of 3</td>
<td>350</td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>350 of 4</td>
<td>350</td>
<td>500</td>
</tr>
<tr>
<td>6</td>
<td>350 of 5</td>
<td>350</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>350 of 6</td>
<td>350</td>
<td>125</td>
</tr>
<tr>
<td>8</td>
<td>350 of 7</td>
<td>350</td>
<td>62.5</td>
</tr>
<tr>
<td>9</td>
<td>350 of 8</td>
<td>350</td>
<td>31.25</td>
</tr>
<tr>
<td>10</td>
<td>350 of 9</td>
<td>350</td>
<td>15.62</td>
</tr>
<tr>
<td>11</td>
<td>350 of 10</td>
<td>350</td>
<td>7.81</td>
</tr>
<tr>
<td>12</td>
<td>350 of 11</td>
<td>350</td>
<td>3.9</td>
</tr>
</tbody>
</table>

The assay was set up in LP2/2 plastic tubes using a Hamilton Microlab-M 500 series auto-dispenser (Howe and Co Ltd) as illustrated in Table 2.4. Total counts (Tc), non-specific binding (NSB) and standards and unknown samples were dispensed in triplicate and maximum binding (B₀) into six tubes. Quality controls were pooled cockerel and hen plasma that was dispensed into four tubes. After each addition of a reagent the assay tubes were vortexed on a multilube vortexer (Alpha Laboratories) and incubated at ambient temperature (~24°C).

Stock solutions of rabbit anti-cFSH anti-serum, designated USDA-AcFSH-16, was stored at -20°C in 1.0 ml of a 1:20 dilution. A working concentration of 1:200 was prepared in PBS-0.5M EDTA-1:20 NRS and dispensed in 20 µl volume into all tubes apart from Tc and NSBs. 20 µl of PBS-EDTA-NRS with no cFSH anti-serum was dispensed into NSB tubes. Assay tubes were then incubated at room temperature for
24 hours. On the second assay day approximately 12,000 cpm per 20 μl of USD-cFSH-I-1 in FSH assay buffer was added to all assay tubes and incubated for 48 hours. On the fourth assay day 20 μl DARS (Scottish Antibody Production Unit) at 1:20 dilution in PBS/EDTA (except Tc) was added to the assay tubes and incubated at room temperature for at least 16 hours.

On the final assay day all tubes were centrifuged (Sovrvall RC-3B) at 1500 x g for 30 minutes at 4°C. The integrity of the pellet was preserved by adding 50μl of 6% starch solution to each tube (except Tc) using a 125μl multi-dispensing pipette (Eppendorf). Assay tubes were re-centrifuged for an additional 15 minutes. The supernatant fractions were aspirated to waste (apart from Tc) and pellets counted for 60 seconds on a gamma-counter (GammaMaster, LKB Pharmacia).

Table 2.4 Chicken FSH RIA protocol

<table>
<thead>
<tr>
<th>TUBE</th>
<th>STD/Sample (μl)</th>
<th>Diluent (μl)</th>
<th>Anti-cFSH/PBS/EDTA/NRS (μl)</th>
<th>Label (μl)</th>
<th>DARS (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
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<tr>
<td>NSB</td>
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<td>50</td>
<td>0</td>
<td>20</td>
<td>20</td>
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<tr>
<td>Bo</td>
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<td>50</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
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<td>20</td>
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<td>QC2</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td>20</td>
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<tr>
<td>Sample 1</td>
<td>40</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Sample 2</td>
<td>40</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Day 5: SPIN TUBES and COUNT
2.5 Quantitative Competitive (QC) RT-PCR assays for neuroendocrine mRNAs

The quantitative competitive (QC) RT-PCR assay pioneered by Dunn and colleges (1996) to measure chicken (ch)GnRH-I mRNA was used in this Thesis to measure hypothalamic chGnRH-I gene expression and the methodology adapted to develop assays for six other neuroendocrine genes. These were pituitary gonadotrophin common α, FSHβ, LHβ subunits, follistatin and activin βB subunit and gonadotrophin inhibitory hormone (GnIH) mRNAs. The development of such assays draws upon a number of molecular biological techniques. The first steps in the development of a QC RT-PCR assay for the measurements of a mRNA transcript is the isolation of total RNA from neuroendocrine tissues for subsequent reverse transcription and designing of oligonucleotide primers for genes of interest for PCR amplification (figure 2.6).

PCR starts with double stranded DNA, and each cycle of the reaction begins with heat treatment to denaturise the two strands (step 1). After strand separation, cooling of the DNA with an excess of the two primer oligonucleotides allows these primers to hybridise to complementary sequences in the two DNA strands (Step 2). In the presence of DNA polymerase and the four deoxyribonucleoside triphosphates DNA is synthesised, starting from the two primers (step 3). The entire cycle starts again by a heat treatment to separate the newly synthesised DNA strands. Within a few cycles the predominant DNA is identical to the sequence encompassed by the two primers.
in the original template (see Figure 2.5). The principles of the QC RT-PCR assay is based on the application of this fundamental molecular biological technique.

**Figure 2.5** Amplification of DNA using the Polymerase Chain Reaction (PCR) technique.

**Figure 2.6** QC RT-PCR standard curve produced by a shortening of cloned standard to produce a smaller competitor while retaining 5' and 3' oligonucleotide recognition sites and b lengthening of cloned standard to produce a larger competitor. Amplified standard and competitor can be visualised by gel electrophoresis due to size differences.
The principle of QC RT-PCR assays to quantify mRNA transcripts in biological samples relies on co-amplification of a competitive template (competitor) that has the same oligonucleotide recognition sites as the target cDNA. A dilution series of the target cDNA of known concentrations (cloned cDNA standard) is added to a constant amount of competitor to create a standard curve, much like the RIA standard curve (see section 2.4.1). In the same QC RT-PCR assay an equal amount of competitor is added to each biological sample of unknown quantity. The ratio of unknown target to competitor can be compared with the standard curve to give a relative quantification.

The assays were carried out in tubes containing plasmid DNA standard and competitor for standard curves, or experimental sample cDNA and the competitor. Standard curves were made up of eight, two-fold dilutions of the respective standard plasmid. The concentration of standard plasmids ranged between $10^{-14}$ and $10^{-19}$ moles, depending on the assay, in a volume of 5μl. The competitor plasmids were diluted to a concentration that fell in the middle of the standard range. A fresh standard curve and competitor dilutions were made on the day of each assay. Each tube for all assays contained standard or cDNA neuroendocrine sample (5μl) and competitor (5μl) made up to a PCR mix of 20μl. The PCR assays were carried out in:

- PCR buffer (x10) with 1.5mM MgCl₂
- 2mM dNTP
- 0.025U/μl Taq DNA polymerase
- 0.5 μM of assay specific forward and reverse primers
The PCR conditions were optimised using a programmable gradient heating block (Hybaid MBS 0.2G) and the following conditions were found to be the most efficient at amplifying the respective neuroendocrine genes:

30 cycles (94°C, 20s; 62°C, 20s; 72°C, 20s) for FSHβ, LH β, common α subunits
30 cycles (94°C, 20s; 60°C, 20s; 72°C, 20s) for follistatin, GnRH-I and GnIH
40 cycles (94°C, 20s; 59°C, 20s; 72°C, 20s) for activin βB.

After the PCR amplification was completed, a final incubation step of 80°C for 20 minutes was added to ensure heterodimers of amplified competitor and standard or cDNA were eliminated (Figure 2.7).

**Figure 2.7** Heterodimers form due to the hybridization of amplified sets of competitor and standard/cDNA. A. illustrates the phenomenon of heterodimers visualized on an agarose gel. Heterodimer formations are undesirable when analysing results, although encouraging as this is evidence that the competitor and standard/cDNA are interacting and thus competing. An additional incubation step of 80°C for 20 minutes post PCR denatures these structures (B.) and allows for accurate analysis of the assay.
After agarose gel electrophoresis to separate bands (see ) the amplified cDNA in each lane was quantified using gel plotting macros in the Scion Image computer package (Scion Corporation, National Institutes of Health, USA). In order to obtain a linear standard curve, the ratio of the two bands, standard and competitor, was calculated, log transformed and plotted against the number of moles of standard plasmid present in each sample.

Figure 2.8 A-B. The intensity of standard/unknown and competitor bands are analysed in the Scion Image computer package C. linear regression of data using Minitab 14.
2.5.1 Validation of QC RT-PCR assays for neuroendocrine genes

To determine whether the developed QC RT-PCR assays for neuroendocrine genes are subject to non-specific interference experiments were carried out using dilutions of hypothalamic RNA for GnIH and pituitary RNA for common α, FSHβ, LHβ subunits. Samples of total RNA were prepared which contained decreasing amounts of hypothalamic or pituitary RNA with constant amounts of total RNA and constant amounts of hypothalamic or pituitary RNA with increasing amounts of total RNA. These samples were reverse transcribed (see 2.5.1.3) and assayed. Figure 2.9 illustrates the validation of LH β, the remaining QC RT-PCR assays gave similar results.

![Graph](image)

**Figure 2.9 Validation of LH β subunit QC RT-PCR assay.** a. Decreasing amounts of pituitary RNA with constant amounts of total RNA b. Constant amounts of pituitary RNA with increasing amounts of total RNA.
2.5.2 NPY QC RT-PCR assay

The QC RT-PCR assay to quantify chicken NPY mRNA was constructed by Dr. Tim Boswell. The NPY standard was constructed by cloning the product of RT-PCR amplification into the EcoRV multiple cloning site of a pBSK-II vector using chicken hypothalamic cDNA as a template and the oligonucleotides in Table 2.5. The cloned NPY plasmids were transformed into XL1 Blue competent E. coli cells (Stratagene Europe, Amsterdam, Netherlands). Plasmid DNA was purified using a Qiagen QIAprep Spin Midiprep kit (Qiagen Ltd., Crawley, West Sussex, UK) and sequenced to check gene identity.

The NPY competitor was constructed by a double enzyme digest of the cloned NPY standard. The restriction enzymes Bbs I and Bse RI cut within the cloned NPY cDNA sequence and produced a 59 bp digested fragment, the plasmid was re-ligated to produce the smaller competitor. The QC RT-PCR conditions for NPY quantification were 30 cycles (94°C, 20s; 62°C, 20s; 72°C, 20s) using specific oligonucleotide primers in Table 2.5.

2.5.3 Ribonucleic acid (RNA) extraction from neuroendocrine tissues

Total RNA was isolated from neuroendocrine tissues simultaneously and randomised according to experimental groups throughout the extraction process to avoid introducing unnecessary variation in quality between samples. A commercial
modification of the guanidinium thiocyanate phenol chloroform method proposed by Chomczynski and Sacchi (1987) was used for RNA tissue extraction. The amount of extraction solution, Trizol and chloroform that was added to samples was dependent on tissue type (see Figure 2.10).

![Pitutary RNA Extraction](image1)

![Hypothalamus RNA Extraction](image2)

**Figure 2.10** Volumes of Trizol and chloroform added to neuroendocrine tissue samples

Tissues were placed in matrix D tubes (Q-biogene-Alexis) containing the correct volume of the extraction solution Trizol (Invitrogen Life Technologies). Disruption of tissue samples was performed in a FastPrep FP120 homogeniser (Q-biogene-Alexis Ltd) using the parameters (time and speed) specified in Figure 2.10 for the respective tissue type. Chloroform (BDH) was then added (100 µl pituitary, 200 µl hypothalamus) and the tubes were vortexed and left on ice for 10 minutes. After incubation the tubes were centrifuged at 20 000 x g (14, 500 rpm) for 15 minutes. The upper aqueous phase was removed and transferred to a sterile 1.5 ml Eppendorf tube (Sarstedt) containing a 2 µl aliquot of molecular grade glycogen (Roche Diagnostics). Glycogen acts as a co-precipitate maximising RNA yield from small tissue samples. RNA was precipitated by the addition of an equal volume of
isopropanol followed by centrifugation for 15 min at 20 000 x g (14, 500 rpm). The RNA pellet was then washed twice with 1 ml of 70% ethanol. Residual ethanol was removed with a pipette and the RNA pellet was briefly dried under vacuum and dissolved in 100-150 μl dH₂O.

The yield of RNA was quantified by measuring the optical density of a 1/50 diluted sample of freshly extracted RNA at 260 nm and 280 nm on a spectrophotometer. The ratio between readings from the two wavelengths was determined and compared to pure RNA, which has a ratio of 2:1 (OD₂₆₀/OD₂₈₀). Ratios from extracted RNA ranged from 1:5 to 2:1. The quantity of total RNA in a sample was determined by multiplying the 260 nm reading by 40 because an optical density (OD) reading of 1 corresponds to approximately 40 μg.ml⁻¹ pure RNA and by the dilution factor.

\[
\frac{(\text{OD}_{260} \times 40 \times 50)}{1000} = \text{total RNA } \mu\text{g.μl}^{-1}
\]

An aliquot of the dissolved RNA from each sample was run on a formaldehyde gel in order to check RNA integrity. 10 μl of total RNA were loaded onto a 1.0% formaldehyde gel to verify the integrity of each sample. The visible bands correspond to the two ribosomal subunits (28s and 18s) within each total RNA sample (Figure 2.11).
A 10X formaldehyde (FA) buffer was prepared and kept as a stock. The composition of the stock solution (per liter) was:

10X formaldehyde buffer

41.9 g MOPS (BDH)

6.8 g sodium acetate.3H2O or 4.1 g sodium acetate

20 ml 0.5 M EDTA

pH adjusted to 7.0 using 1 M NaOH using pH120 Microprocessor pH meter (Hanna instruments).

Before running the samples the electrophoresis tank was cleaned with a commercially available detergent, RNaseZap® (Ambion), thoroughly rinsed with Milli-Q dH2O, and then rinsed with 70% ethanol and allowed to dry. 1% FA gel was prepared by adding 1.0 g of agarose (BDH) to 10 ml of 10X FA buffer and Milli-Q
d\textsubscript{2}H\textsubscript{2}O to 100 ml into an oven baked 500 ml flask. The flask was heated in a microwave with occasional swirling until the agarose was dissolved; to avoid build up of pressure during heating the lid of the flask was not tightened. If the volume of the liquid was significantly reduced due to heating additional Milli-Q d\textsubscript{2}H\textsubscript{2}O was added to ensure that the agarose concentration was correct. The dissolved agarose was cooled in a heated hot box set at 60°C. After cooling 20 ml of 12.3 M formaldehyde and 2 μl of 5 mg.ml\textsuperscript{-1} ethidium bromide was added in a fume cupboard while wearing nitrile gloves. The agarose solution was poured onto a gel tray in a fume hood. An appropriately sized comb for well formation was placed into gel tray before pouring. The gel was allowed to set for at least 30 minutes before use. Once the gel had set it was placed into the pre-washed electrophersis tank. The tank was filled with enough 1X FA buffer to cover the gel with at least 1 mm of liquid above the surface of the gel. The comb was carefully removed and the gel was allowed to equilibrate with the 1X FA buffer for at least 30 minutes. 10 μl of RNA of each sample was placed into individual wells of a 96 well plate (Abgene) containing 9 μl of 5X RNA loading dye and 1 μl ethidium bromide. The samples were then heated at 65°C for 10 minutes in a Hybaid MBS 0.2G programmable heating block and loaded into the gel immediately.
5X RNA loading dye
80 μl 500 mM EDTA, pH8.0
720 μl 37% formaldehyde
2 ml 100% glycerol
3084 μl formamide
4 ml 10X FA buffer
total to 10 ml with Milli-Q dH₂O
This loading dye remains stable for approximately 3 months if stored at 4°C.

2.5.4 Reverse transcription of total RNA to complementary deoxyribonucleic acid (cDNA)

A sample of total RNA was reverse transcribed using a First Strand synthesis kit (Amersham Pharmacia Biotech). Each RNA sample was heated at 65°C for 10 minutes to relax any secondary structures that may have been formed. 4μl of each sample (approximately 1μg) was added to a master mix containing 0.5μl of 200mM DTT solution, 0.5μl of 0.2μg/μl Not I-d(T)₁₈ primer solution and 2.5μl of 1st strand synthesis buffer for each sample to be reverse transcribed. The master mix was prepared in a sterile eppendorf tube in order to minimise the potential tube-to-tube variation due to pipette errors. To farther minimise this problem a multiple dispensing electronic pipette (Rainin EDP) was used to aliquot the master mix into
individual tubes of a 96 well plate. The plate was sealed with an Easy Peel heat sealing foil sheet (Abgene) and heated in a combi-thermo sealer (Abgene). Samples were pulse centrifuged (Jouan BR4i carrying a S20 rotor) and incubated on a Hybaid MBS 0.2G programmable heating block at 37°C for 1 hour and heated to 90°C for 5 minutes in order to denature the reverse transcriptase enzyme. After incubation samples were cooled on ice for 10 minutes and diluted into 40-80 µl dH2O before storing at -20°C.

2.5.5 Design of primers for use in polymerase chain reaction (PCR)

Oligonucleotide primers for the amplification of neuroendocrine genes were designed using the “primer” computer package version 0.5 (Whitehead Institute for biomedical research Cambridge, MA, USA) with sequences of published cDNA transcripts available on-line http://www.ncbi.nlm.nih.gov/entrez/. An effort was made to select primers with low GC content and sequences with significant secondary structures were avoided. A Tm of around 60°C was used to design primers. Oligonucleotide primers were synthesised by Sigma-Genosys. The sequences of all primers used in the cloning of neuroendocrine genes and subsequent QC RT-PCR assays are in table 2.5.
Table 2.5 Oligonucleotide primers used for QC RT-PCR assays with Genbank accession numbers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnRH-I</td>
<td>TGGGTGTTGTGATGGTGTGTTT</td>
<td>ATTTCCAGCGGGAAGAGTTT</td>
<td>X69491</td>
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<tr>
<td>GnIH</td>
<td>TGGAAAGCAGAGAGATGAGT</td>
<td>ACACAGTTTGGCAATTTCAGTT</td>
<td>AB039815</td>
</tr>
<tr>
<td>NPY</td>
<td>CACCATGAGGCTGTGGGTGTT</td>
<td>CAATGGCTGACTGACCTG</td>
<td>M87294</td>
</tr>
<tr>
<td>Alpha</td>
<td>ATGAAACAGAGGAGAAAGATCA</td>
<td>AACAGCCATCCATCTCATT</td>
<td>S70833</td>
</tr>
<tr>
<td>FSHβ</td>
<td>TGCTGGAAGCAATCTGGGTG</td>
<td>ACTGAGAGACGAGTGGATGG</td>
<td>bi392995</td>
</tr>
<tr>
<td>LHβ</td>
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<td>GGGCAGCGGCGCGCGGGCGCG</td>
<td>S70834</td>
</tr>
<tr>
<td>Activin β</td>
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<td>Z71594</td>
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<tr>
<td>Follistatin</td>
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<td>CTGCCAACCTAAAATACCA</td>
<td>X87609</td>
</tr>
</tbody>
</table>

2.5.6 Polymerase chain reaction (PCR)

All PCR amplification was carried out in a Thermo-Fast® low profile 96 well plate (Agene) on a Hybaid MBS 0.2G programmable heating block. PCR plates were sealed with Easy Peel heat sealing foil sheets (Agene) heated in a combi-thermo sealer (Agene). The PCR mix was pulse centrifuged in a Jouan BR4i with a S20 rotor arm before being placed in the heating block for PCR. Post PCR products were pulse centrifuged again before electrophoresis.

2.5.7 Agarose gel electrophoresis

Visualisation of DNA fragments for analytical or preparative purposes was by agarose gel electrophoresis in either standard (BDH) or ultra-pure agarose (NuSeive®, BMA) in 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA).

Agarose was melted in 1X TAE buffer in a microwave with occasional swirling until the agarose was dissolved; to avoid build up of pressure during heating the lid of the
flask was not tightened. If the volume of the liquid was significantly reduced due to heating H₂O was added to ensure that the agarose concentration was correct. After cooling ethidium bromide (0.5mg/ml) was added and the agarose was poured into a gel casting tray with an appropriate gel comb to form loading wells. After setting the comb was removed and the gel was submerged in 1X TAE buffer in an electrophoresis tank. DNA samples, typically 10μl, were loaded into the formed wells with 3μl of gel loading dye (30% glycerol, 70% 1x Tris-Acetate/EDTA (TAE), 0.4% Orange G) in parallel with a 1 Kb DNA ladder (New England Bio Labs) and electrophoresed at 6V/cm. DNA was visualised as a function of ethidium bromide fluorescence produced by UV illumination via a gel transilluminater at 312 nm (UVP), and captured using a video camera linked to a personal computer running the Multi-Analyst program (Bio-Rad Laboratories, Inc. USA).

2.6 Cloning of neuroendocrine cDNA sequences

Molecular DNA cloning is a process by which a specific DNA fragment from a genome is introduced into an autonomously replicating genetic element such as a plasmid, and then amplified by propagation in a rapidly growing host cell, often an appropriate strain of the bacterium, *Escherichia coli*.
A typical molecular cloning procedure requires: a DNA fragment of interest, often called foreign or passenger DNA, a cloning vector, restriction endonucleases, DNA ligase and a cell to serve as a biological host.

The molecular cloning vector plays an important role in recombinant DNA procedures. Since most DNA fragments are incapable of self-replication, particularly in a host cell from a different organism, an autonomously replicating segment of DNA must be used to allow replication of the desired DNA fragment. Most cloning vectors are derived from extrachromosomal replicons such as bactriophages, viruses and plasmids.

All neuroendocrine genes of interest were cloned into the pBSK-II vector and transformed into XL1 Blue *E. coli* competent cells. The purified plasmid carrying the clone DNA fragment was used as a standard in the QC RT-PCR assays and as a template for the construction of each respective competitor. Sequencing was carried out on positive clones to check the identity of the DNA fragment.

The plasmid Bluescript II SK (+/-) (pBSK-II) cloning vector is a derivative of plasmid BR322 and bactriophage M13 and is approximately 3000 bp that replicates to a very high copy number in *E. coli* host cells (figure 2.12). pBSK carries a gene that confers ampicillin (amp) resistance on its host, and also encodes for a gene that allows the selection of recombinant products by a colour reaction. The plasmid also contains a multiple cloning site (MCS) containing recognition sequences of 21 different endonucleases. The MCS is located within a mutated form of the LacZ gene encoding the enzyme, β-galactosidase, in which one end of the protein coding
region is missing. If the intact plasmid is transformed into a host cell that contains a β-galactosidase gene with a deletion at the other end such as XL1 Blue E. coli cells, then the partial proteins made from the partial genes fit together and make a partially active enzyme. This is a form of intragenic complementation. The enzyme cleaves the compound X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) creating a blue colour. The cells are plated on to selective media containing amp, IPTG (isopropyl-b-D-thiogalactopyranoside), which turns on the Lac operon, and X-gal, thus, colonies containing intact pBSK are amp resistant and are blue. If a piece of DNA is inserted into one of the restriction sites of the MCS the plasmid gene encoding the partial β-galactosidase will be inactivated, and no complementation between the plasmid-encoded and host-encoded enzyme will occur. Thus, a cell with a plasmid which has a DNA fragment cloned into the MCS site will be amp resistant, but will no longer make active β-galactosidase, and will form white colonies. PBSK-II exists in the form of a covalently closed circle of double stranded DNA. In order to clone a DNA fragment into the plasmid it must be linearised. The endonuclease Eco RV has only one recognition site located within the MCS of pBSK-II, which produces a single linear ds DNA.
fl (+) origin 135–441
β-galactosidase ω-fragment 460–816
multiple cloning site 653–760
lac promoter 817–928
pUC origin 1158–1825
ampicillin resistance (bla) ORF 1976–2833

Figure 2.12 The pBSK-II cloning vector showing the multiple cloning site (MCS)

Restriction enzymes were used to manipulate the pBSK-II cloning vector. A restriction enzyme or endonuclease functions by "scanning" the length of a dsDNA molecule. Once it encounters its particular specific recognition sequence, usually 4-6 nucleotides long, it will bond to the DNA molecule and make one cut in each of the two sugar-phosphate backbones of the double helix. Different endonucleases yield different sets of cuts, but one endonuclease will always cut a particular base sequence the same way, no matter what DNA molecule it is acting on. Once the cuts have been made, the DNA molecule will break into fragments often with 2 fold
symmetry. Restriction enzyme digests may result in both blunt and overhanging ends.

2.6.1 *Purification of RT-PCR products and blunt ending*

Purification of the amplified DNA fragment from contaminates such as unincorporated nucleotides, primers, salts, ethidium bromide and agarose is important for successful molecular cloning. All cloned DNA fragments were amplified from cDNA of pituitary or hypothalamic tissue depending on gene of interest. The RT-PCR product was blunt ended with 1 μl Klenow (Promega) to patch up overhanging DNA by incubating at 37°C for 15 minutes the reaction was terminated by a 75°C incubation for 5 minutes. Residual dNTPs within the post-PCR mix dictated that no additional nucleotides were required for the patch up. The product was then run on an ultra pure 1.2% nusieve agarose gel (BMA) in parallel with a 1Kb DNA ladder to determine product size. The band was then excised from the gel using a sterile size 11 scapel blade (Swann-Morton) under UV illumination using long wavelengths to minimise DNA damage and placed into a sterile 1.5mL Eppendorf tube. The commercially available QIAEX II gel extraction kit (Qiagen) was used to purify PCR products from agarose gels. The manufacturers protocol was followed. DNA yield from this kit was typically 0.4 μg.μl⁻¹.
2.6.2 Ligation

Eco RV (cleavage site boxed in red Fig 2.12) was routinely used to linearise the pBSK vector in preparation for ligation of a DNA fragment of interest.

**Reaction mix used to linearise pBSK-II**

0.2 μl 1μg.μl⁻¹ pBSK-II (Stratagene)
1.0 μl Buffer B (Roche Diagnostics)
0.1 μl Eco RV (Roche Diagnostics)
8.7 μl dH₂O

Total volume of 10 μl

The reaction mix was set up in a 0.5 mL microfuge tube (Sarstedt) and incubated at 37°C in an omni gene heated block (Hybaid) for 30 minutes. The reaction mix was then diluted to 40 μl with dH₂O to give a concentration of 5ng.μl⁻¹ of linearised plasmid.

An additional dephosphorylation step prior to ligation was performed on the linearised plasmid. This step reduces the chances of the plasmid re-circularising by removing 5'-terminal phosphates. The procedure makes use of shrimp alkaline phosphatase (SAP) that can be easily denatured after use by heat treatment at 65°C unlike other alternatives such as bacterial alkaline phosphatase (BAP) and Calf intestinal alkaline phosphatase (CIP), which are difficult to destroy by heating.
methods alone. A SAP kit (Roche Diagnostics) was used following the manufacture’s protocol.

The rapid DNA ligation kit (Roche Diagnostics) was used to clone DNA sequences. Reactions were set up in 1 ml Eppendorf tubes at room temperature.

7 µl Purified ~0.4 µg, µl⁻¹ RT-PCR product
1 µl Linearised 5ng, µl⁻¹ pBSK vector
1 µl 5x DNA dilution buffer
1 µl T4 DNA ligase
10 µl 2x T4 DNA ligation buffer
Total volume of 20 µl

The reaction mix was allowed to incubate at room temperature for 5 minutes. An additional Eco RV digest was performed after ligation to eliminate any plasmid that had recircularised without insert. The tubes were then chilled on ice prior to transformation.

2.6.3 Transformation of XL1 blue competent cells

The products of ligation reactions were transformed into XL1 Blue E. coli competent cells (Strategene). Stocks of competent cells were stored in liquid N₂ and defrosted on ice prior to use. 50 µl aliquots of thawed competent cells were then
added to each ligation reaction and left on ice for 30 minutes. The cells were heat shocked for 45 seconds at 45°C in a pre-heated water bath before cooling on ice for 1 minute. 700 μl of room temperature SOC was added to each sample and the cells were shaken at 250 rpm at 37°C for 45 minutes. The cells were then plated onto pre warmed agar plates with the aid of a sterilised glass spreader. After drying the plates were placed inverted into a 37°C oven overnight.

2.6.4 Microbiological sterile techniques and preparation of agar plates

All microbiological techniques were carried out in a Class I microbiological hood. Bacterial work surfaces were cleaned with 70% ethanol before and after use. Pipette tips, LB broth and LB agar were autoclaved and opened under flame. All plastic ware was sealed in sterile packaging. Glass spreaders were sterilised with burning ethanol before and after use. Waste was routinely decontaminated by autoclaving.

Autoclaved LB agar was melted in a microwave. The agar was allowed to cool before addition of 400 μl of 10 mg.ml⁻¹ ampicillin, 400 μl of 0.1 M IPTG and 800 μl of 20 mg.ml⁻¹ X-gal. The agar was set in 100 mm² plates and allowed to set. Plates were stored inverted in a sealed bag at 4°C. Before use plates were placed in an oven preheated to 55°C, to dry off condensation and make the LB agar warm to absorb the transformation mix.
2.6.5 Presequencing PCR

10 µl of LB broth was dispensed into either sterile 0.5 ml microfuge tubes or individual wells of a 96 well plate depending on number of positive colonies to be picked. White (positive) colonies from overnight growth, potentially carrying the desired insert, were picked using a sterile pipette tip. The tip carrying the colony to be amplified was placed into the LB broth and mixed vigorously. A pre-sequencing PCR mix containing the following PCR components per tube was used to amplify the insert:

**Pre-sequencing PCR mix per tube**

2 µl of PCR buffer (x10) with 1.5mM MgCl₂ (Roche Diagnostics)

2 µl of 2mM dNTP (Abgene)

0.1 µl of 0.025U/µl Taq DNA polymerase (Abgene)

4 µl of 5 M betaine (Sigma)

20 µM of assay specific forward and reverse pBSK-II primers (Sigma-Genosys)

19 µl of the mix was dispensed into each well, added to this was 1 µl of the LB broth containing the picked bacterial colony. The PCR conditions were: an initial melting temperature of 94°C for 5 minutes followed by 30 cycles (95°C 15s; 56°C 20s; 72°C 60s). Confirmation of positive clones were visualised under U.V illumination. False positives appeared as 250 bp bands.
The pBSK oligonucleotide primers were:

Forward CAATTTCACACAGGAAACAG

Reverse CGATTAAGTTGGTAACGC

The above primers were designed either side of the Eco RV multiple cloning site of the pBSK-II vector and were therefore universal for all inserts.

2.6.6 Plasmid purification

A bacterial colony carrying the desired DNA insert was used to inoculate 5 ml of LB broth containing 100 μg/ml ampicillin and was incubated at 37°C with shaking at 250 rpm, overnight. The culture was centrifuged at 6000 x g for 5 minutes at 4°C.

The supernatant was decanted and the tubes inverted to remove all medium. The remaining pellet was used to extract the plasmid carrying cloned DNA. Purification of plasmid DNA was prepared by a Qiagen midi kit according to the manufacturers protocol. Briefly, the bacterial pellet was resuspended in 4 ml buffer P1 and 4 ml of buffer P2 mixed gently by inverting, this lysis step was allowed to proceed for 5 minutes and terminated by addition of chilled buffer P3. The sample was mixed by inverting gently 4-6 times to avoid local precipitation and incubated on ice for 15 minutes. The tube was centrifuged at 20 000 x g for 30 minutes at 4°C and the supernatant containing the plasmid DNA was removed promptly. The supernatant
was re-centrifuged under the same parameters to avoid applying suspended material to the Qiagen column. During this additional centrifugation step a Qiagen column was equilibrated with 4 ml of buffer QBT, the supernatant was applied to the column. Two washes of 10 ml buffer QC was then applied to the column and allowed to rinse through by gravity flow. The DNA within the column was eluted by 5 ml of buffer QF. The DNA was precipitated by adding 3.5 ml of isopropanol and centrifuged at 15000 x g for 30 minutes at 4°C. The DNA pellet was washed with 2 ml of 70% ethanol and centrifuged at 5000 x g for 10 minutes. The supernatant was poured off and the pellet was air dried for 10 minutes before redissolving the DNA in 1 ml dH2O, typically resulting in a concentration of 400-500 ng.µl⁻¹.

2.6.7 Analysis of plasmid DNA quantity and quality

A double restriction enzyme digest using the endonucleases Hind III and Bam HI confirmed the presence of a cDNA insert in the plasmid. These restriction enzymes were chosen as they cut only once within the pBSK-II vector, either side of the Eco RV cloning site (see Fig. 2.11). The size of the cloned DNA fragment could be estimated by running the product of the enzyme digest on an agarose gel in parallel with a 3 Kb DNA ladder (discontinued product-Fermentas) (see Fig. 2.15). Comparisons between expected and actual size of the insert could be made at this point. Comparing with a size marker of known concentration can make an approximation of the concentration of the plasmid run on the gel.
The plasmid was diluted 1:50 prior to spectrophotometric analysis at 26 nm and 280 nm. The ratio between readings from the two wavelengths was determined and compared to pure ds DNA, which has a ratio of 1:5 (\( \text{OD}_{260}/\text{OD}_{280} \)). Ratios from extracted ds DNA ranged from 1:2 to 1:9. The quantity of total DNA in a sample was determined by multiplying the 260 nm reading by 50 because an optical density (OD) reading of 1 corresponds to approximately 50 \( \mu \text{g.mL}^{-1} \) pure DNA and by the dilution factor.

\[
\frac{\text{OD}_{260} \times 50 \times 50}{1000} = \text{total ds DNA} \ \mu \text{g.mL}^{-1}
\]
2.7 Sequencing of neuroendocrine cDNA sequences

To check that the cloned cDNAs were the desired neuroendocrine genes each respective standard and competitor plasmid was sequenced.

2.7.1 Sequencing reaction

The Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-Deaza-dGTP (Amersham Biosciences) was used for sequencing reactions. The components from the kit, ACGT reagents, were thawed on ice. A 96 well plate was marked in portrait orientation as illustrated in Figure 2.13. A master mix was made for each sequencing primer.

Volume for each reaction

1.5 μl of 100μM IRD-700 or 800 labelled primer
13.1 μl dH2O
1.4 μl DMSO

A 16 μl aliquot of the master mix was added to each tube in the row labelled R, followed by 5 μl of a positive pre-sequencing PCR reaction. 1.5 μl of A, C, G, and T kit reagents was placed into each tube of the accordingly marked row. The 96 well plate was sealed and placed on a heating block running the following programme:
M13 forward primer: TGTAAAACGACGACGGCCAGT
M13 reverse primer: CAGGAAACAGCTATGACC

**Sequencing reaction incubation**

Stage 1: (95°C 30 seconds) x 1 cycles

Stage 2: (95°C 10 seconds; 58.8°C 30 seconds; 65°C 30 seconds) x 20 cycles

Stage 3: (95°C 10 seconds; 95°C 10 seconds; 70°C 30 seconds) x 15 cycles

![Sequencing reaction layout on a 96 well plate](image)

*Figure 2.13 Sequencing reaction layout on a 96 well plate*
2.7.2 Li-cor automated DNA sequencer (MWG-Biotech)

The Li-Cor protocol was used for sequencing gel electrophoresis and data collection. 41 cm sequencing plates were routinely used to sequence cloned DNA fragments.

Composition of sequencing gel for 41 cm sequencing plates

7.5 ml Rapid Gel XL solution 40%
21 g UREA
5 ml 10X TBE long run buffer
28 ml dH₂O

TBE long run buffer
162 g tris base
27.5 g boric acid
9.3 g EDTA
made up to 1 litre with dH₂O

The solution was placed on a magnetic stirrer with a magnetic stirring bar placed inside the mixing vessel and mixed for 1-2 hours. During mixing the 41 cm plates were rinsed twice with water and 70% ethanol and dried with white paper tissues. If
the plates were used for the first time or were very dirty an additional rinsing step was carried out. This step involved one extensive rinse of the following: 1 M HCl acid, 1 M NaOH and again with 1 M HCl. The plates were placed together and held with appropriately sized spacers and rails. The plates were then placed in a horizontal position and raised at the end that the gel was to be applied. The following chemicals were then added to sequencing mix:

350 µl 0.1 M fresh ammonium persulphate
500 µl DMSO
50 µl TEMED

On addition of the final components to the mixture the gel was transferred to a syringe and poured immediately to avoid premature polymerisation. Gentle tapping facilitated the movement of the gel through the plates. The well former and casting plate was added and the gel was allowed to polymerise for at least 2 hours.

Prior to loading the sequencing reactions, the gel was transferred to the Li-cor DNA sequencer and incubated at 45°C for 30 minutes. Any urea that had accumulated in the wells was washed out with a Pasteur pipette. 4 µl of loading dye (formamide, EDTA and fuchsin), supplied with the sequencing kit, was added to each sample well i.e. A, C, G and T and heated at 65°C for 10 minutes. The samples were finally loaded onto the pre-heated gel using a multichannel gel loading syringe.
(Hamilton, Bonaduz, Switzerland) under illumination from a desk lamp. The gel was typically run overnight with the following conditions: 1500 V, 35mA, 40 W, 45°C.

2.7.3 Analysis of DNA sequences

The Li-cor software Baselmage 1 R 4.0v was used for analysing the digital image of the sequencing gel. The single lane automatic reader was used to delineate each lane and then double checked and corrected where appropriate. The sequence was then analysed using GCG, Wisconsin and the NCBI web site.

Figure 2.14 Analysis of DNA sequences. Sequences produced on the Li-cor are analysed on GCG. Sequences are Blasted on the NCBI database for a sequence match.
2.8 Sequences of cloned neuroendocrine genes used as standards for QC RT-PCR assays

2.8.1 Common $\alpha$-subunit

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCATA TAGGGCGAAT</td>
<td>Kpn I</td>
</tr>
<tr>
<td>TGGGTACCGG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGAT</td>
<td>Sal I</td>
</tr>
<tr>
<td>TGGGTACCGG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGAT</td>
<td>Hind III</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Bpf I</td>
</tr>
<tr>
<td>CACTTTGACC ATTTTGTCTG TGTTTCTGCA TCTTCTTCAT ACTTTCCCAG</td>
<td>Afl I</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Eco RI</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Sma I</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Not I</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Sac I</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Bpf I</td>
</tr>
<tr>
<td>ATGGAGAATT TCTCATGCAG GGTTGTCCAG ATGCAGCTGT</td>
<td>Eco RI</td>
</tr>
</tbody>
</table>

Figure 2.15 Sequence of cloned $\alpha$-subunit cDNA (5'-3') showing restriction site of endonuclease used to produce competitor plasmid and part of the MCS of pBSK-II (see section 2.9)
2.8.2 FSHβ subunit

TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCACTA TAGGGCGAAT
Kpn I Sal I Hind III
TGGGTACCGG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGAT

1 TGCTGGAAAG CAAATCTGTTG CTATAGCTGT GAACTCACCA ATATTACTAT

Pml I

51 AGCAGTGAAA AGAGGAAGAT GTGAACCTCTG CATTACAGTG AATGCCACGT
101 GGTCGCTCAGG ATACTGCTTTC ACAAGGGATC CAGTATACAA ATATCCACCA
151 GTCTCATCTG TTTAGCAAAAT ATGTACCTTTC AAGGAGGT TGATATGAAAC
201 AGTGAAGATC CCTGGCTGCC GTGACCATCC TGAATCTTTT TACTCATAAC

Bsg I

251 CAGTAGCTAC AGAGTGCCCAC TGTGAGACCT GTGACACTGA TAGCACTGAC
301 TGCACCTGTGA GAGGACTGGG GCCATCCTAC TGCTCCTTCA GT

Eco RI Sma I Not I Sac I
ATCGA ATTCCTGCAG CCCGGGGGAT CACTAGTTCT AGAGCGGCCG CCACCCGGGT
GGAGCTCCAG

Figure 2.16 Sequence of cloned FSHβ cDNA (5'–3') showing restriction site of endonucleases used to produce competitor plasmid and part of the MCS of pBSK-II (see section 2.9)
2.8.3 LHβ subunit

TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCCTAT TAGGGCGGAAT
Kpn I  Sal I  Hind III
TGGGTACCGG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGAT

Pflm I

1 ACGGTGGCGG TGGAGAAGGA CGGATGCCCC CAATGTATGG CTGTGACCAC
51 CACGGGCTGC GGGGGTACT GCAGGACCGG GGAGCCGGTG TATCGCAGCC
101 CTTTGGGCCG CCCCCCCCAG TCGGCGTGGCA TTTATGGGCC GCTGCGCTAC
151 GACCGTGGGG CGCTGTGGGG CTGCCCTATA GGGAGCGACC CCCCCGTCTC
201 CCTCCCCCCG GCTCTGAGCT GCCGTGCAC CCCTCCCTCC

EcoR I  Pst I  Sma I  Not I  Sac I
ATCGA ATTCCTGCAG CCCCCGGGAT CACTAGTTCT AGAGCGGCCG CCACCGCGGT
GGAGCTCCAG

Figure 2.17 Sequence of cloned LHβ cDNA (5'-3') showing restriction site of endonucl ease used to produce competitor plasmid and part of the MCS of pBSK-II (see section 2.9)
2.8.4 Activin $\beta_B$ subunit

TAAAACGACG GCCAGTGAGC CGCGTGAATA CGACTCACTA TAGGGCGAAT
  Kpn I  Sal I  Hind III
TGTTACGCC GCCCCCCCTC GAGGTAAGG GTATCGATAA GCTTGAT
  1  TCTGTTTGAG AGAGGAGAAA GGAGACTGAA CTTGAGTTT CAATGTGAGG
  51  GCTGTGAAGA GTATTCAGTG CTGCAATTT ATGTGGACC CGGGAGAA
  101  TCCCACCGGG CTTTTTTAGT GGTGAAGGC CGCCTGCTG ATACAAACA
       S\textit{tu} I
  151  CAGGATCCGG AAAAGAGGCC TGGAGTGCGA TGGCAGGACC AATCTATGTT
  201  GCAGGCAACA GTTTACATT GACTTTAGAC TCAATTGATG GAAATGACTGG
  251  ATCATAGCAC CATCAGGTTA CTATGGGAAT TACTGTGAAG GGAGCTGCCC
  301  GGCCTACCTG GTGGGCTCC CGGGGTCGGC TTCCTTCTTC CACACCCGGC
  351  TCGTGAATCA GTACCGAATG CCGGGCTGA ACCGGGACAC CGTGAACCTCC
  401  TGTTGCATT C CAAACAAACT TAGCACAATG
       Eco RI  Pst I  Sma I
       Not I  Sac I
ATCGA ATTCCTGCAG CCCGGGGGAT CACTGTTCT AGAGCGGCCG CCACCGCGGT
GGAGCTCCAG

Figure 2.18 Sequence of cloned activin $\beta_B$ cDNA (5'-3') showing restriction site of endonulease used to produce competitor plasmid and part of the MCS of pBSK-II (see section 2.9)
2.8.5 Follistatin

TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCAGTA TAGGGCGAAT
  Kpn I          Sal I          Hind III
TGGGTACC GG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTCTTGAT

1  CCGGATTGCT CTAATATCAC TTGGAAGGCG CCGGTGTGTG GCTTAGATGG
51  GAAAACCTAC AGGAACGAGT GTGCCCTTCT CAAAGCCAGA TGTAAAGAAC
101  AGCCCGAATCT TGAAGTCCAA TATCAGGGCA AGTGCAAAAA GACCTGTAGG

Xcm I

151  GATGTTTTAT GCCCAGGCAG CTCCACGTTG GTGGTTGATC AAAACTAATAA
201  CGCCTACTGT GTGACATGTA ATCGAATTGG CCGTCGAGCC ACCTCCCTTG
251  AGCAGTATCT CTGTCGGGAAT GATGGCATAA CCTAGCCAG AGTCTGACCC
301  CTCGAGAAAG CGACCTGCGT GCTGGGCGAG TCAATGGGTA TACGCTACGA
351  GGGAAAATGC ATCAAAGCGA AGTCCTGTGA AGATATTCAG TGGAGTGTGG
401  GGAAGAATTG CTTGTTGGAT TTCAGGTTG GCAG

Eco RI  Pst I  Sma I  Not I  Sac I
ATCGA ATTCTGCAG CCCGGGGAT CACTAGTTCT AGAGCCGCG AGGAGCTCCAG

GGAGCTCCAG

Figure 2.19 Sequence of cloned follistatin cDNA (5'-3') showing restriction site of endonuclease used to produce competitor plasmid and part of the MCS of pBSK-II (see section 2.9)
2.8.6 GnIH

TAAAACGACG GCCAGTGAGC GCCCGTAATA CGACTCACTA TAGGCGGAAT
Kpn I Sal I Hind III
TGGGTACCAG GCCCCCTC GAGCTCGACG GTATCGATAA GCTTGAT
1 TGGAAAG CAGAGAAGAT GATGATGATA AATATTATGA GACTAAAGAC
51 AGTATCTTG GAGAAGCGA GAGGATCTG AATTTTGAG AAATGAAAGA
101 CTGGGATCA AAAAAATTC TAAGGTGAA CACCCATCA GTAAACAAAG
151 TGCCAAATTC AGTTGCTAAT TTGGCTCCTA GATTTGGAA AAGCAATCCA
201 GAAGAAAGAA GCATTAAGCC AAGTGCTTAT TTGCCTCTGA GATTTGGAA
251 AGCTTTTTGA GAGAGCTCTC CTAGGCGTGC TCAAAATCTA TCAACAGGT
Bgl II

301 CTGGGAGATC TCCACCTGCT AGAAGTTCTA TTATCTCTA TCTAAATCTG
351 TCACAGAGAT TTGGGAGTGC AGTGCCCATC AGTCTATCTC AAGGTGTCCA
401 GGAATCTGAA CCAGGGATGT GAATTCTAAC AATTATGCTA GGTATATCA
451 GTTATGAGAA TGAAACCCGA AAGACCCGAG AAAGATGAC CGGTGATCCA
501 AAACTAAAGA TCTTGAAGG GGAATGTATA TGAGAACTGA AATGCAAACC
551 TGTGT

Eco RI Sma I Not I Sac I
ATCGA ATTCCTGCAG CCCGGGGGAT CACTAGTTCT AGAGCGGCCG CCACCGCGGT
GGAGCTCCAG

Figure 2.20 Sequence of cloned GnIH cDNA (5′-3′) showing restriction site of endonuclease used to produce competitor plasmid and part of the MCS of pBSK-II (see section 2.9)
2.9 Construction of competitor plasmids for QC RT-PCR assays

The specific cloned standard plasmid for each QC RT-PCR assay provides the bases for competitor production. Two strategies can be employed to develop a competitor plasmid for QC RT-PCR, the cloned standard gene sequence can either be shortened by sequence specific endonucleases or lengthened by insertion of a foreign fragment of DNA. Inserting a fragment of foreign DNA into the cloned standard cDNAs created competitor plasmids for LHβ, activin βb, follistatin and GnIH. The foreign DNA was a .174 bp fragment of a pBSK-II vector backbone produced by a Hae III digestion (Roche Diagnostics Ltd, East Sussex, UK) (Fig 2.21). The foreign DNA was subcloned into the standard cDNA sequences while retaining primer specific sequences at the 5’ and 3’ ends to allow PCR amplification. The LH β standard was cut by PflM I (New England Biolabs, Hitchin, Hertfordshire, UK), GnIH was cut using Bgl II (Roche Diagnostics), activin βb was cut by Stu I (Roche Diagnostics), and follistatin was cut with Xcm I (Roche Diagnostics). All restriction enzymes cut once within the cloned sequence and did not cut within the pBSK-II+ vector.

Figure 2.21 Sequence of 174 bp fragment of pBSK-II (5’-3’) cut by Hae III digest and inserted as foreign DNA into LHβ, activin βb, follistatin and GnIH standards to create competitors. GGCC = Hae III restriction site.
The common α subunit competitor was constructed using a Bpi I (New England Biolabs) and Afl II (New England Biolabs) double enzyme digest of the cloned alpha standard (Fig 2.22). Both endonucleases cut once within the standard sequence to produce a competitor fragment of 335 bp after blunt ending and re-ligation. The FSH β competitor was constructed by digestion with Pml I (Roche Diagnostics Ltd, East Sussex, UK) and Bsg I (Roche Diagnostics Ltd, East Sussex, UK) followed by blunt ending and religation.

Figure 2.22 Restriction enzyme digest of common alpha subunit standard plasmid for competitor construction run on a 1.0% ultra-pure agarose gel.

2.10 Studies in vitro

Freshly dissected pituitary glands were pooled into chilled phosphate buffered saline (PBS). The pituitaries were collectively diced into fragments and distributed randomly into the wells of a 12 or 24 well cell culture plates (Corning Costa). Each well contained 2-3 pituitary fragments, 1ml DMEM culture medium with phenol red
(Invitrogen Life Technologies) supplemented with 3.75% foetal calf serum (FCS), 6% horse serum (HS) and antibiotics: 100u.ml\(^{-1}\) streptomycin and 100μg.ml\(^{-1}\) penicillin. The cells were incubated at 37°C with gentle shaking (60 rpm/minute) on a revolving platform. Test substances were distributed in a latin square design.

Pituitary cell fragments were chosen over dispersed pituitary cells as the role of autocrine/paracrine factors controlling gonadotrophin function was of interest. It is likely that the dispersion process could alter the activity of such factors \textit{in vitro} (Halvorson \textit{et al.}, 1994). The use of pituitary fragments raises the need to quantify the amount of cells within each well in order to standardise between samples, the isometric density value of the 18s ribosomal RNA was chosen as a standardising factor. The 18s rRNA in each lane was quantified using gel plotting macros in the Scion Image computer package (Scion Corporation, National Institutes of Health, USA).

![Figure 2.23](image)

\textbf{Figure 2.23} Quantification of 18s rRNA from pituitary fragments.
2.11 Statistical analyses

All graphs were drawn in Sigma Plot 8.0 for windows (SPSS UK Ltd.). All analyses were carried out using Genstat 6 edition (VSN International Ltd, Oxford) or Minitab (Minitab® Release 14.13). Differences between experimental groups were considered significant at p< 0.05. Plasma LH and FSH and neuroendocrine mRNA data was routinely log transformed to normalise variance. Where possible data was analysed by two way ANOVA using treatment/group and time/plate position (for studies in vitro) as factors in the analysis and bird as a block in time course studies in vivo. One way ANOVA was used when two way ANOVA was not appropriate. Where ANOVA revealed statistical significance between groups post hoc analysis was by least significant difference or unpaired t test to reveal differences between means. Regression analyses were done using least-square regression analysis with the proportion of variance explained by the model and the significance values of the fit (r^2 and p values). Further information concerning specific statistical analyses of data is described in each chapter in the appropriate figure legend.
CHAPTER 3. Changes in the hypothalamic-pituitary-ovarian axis of Cobb broiler breeder hens during a laying year

3.1 Introduction

As the domestic hen becomes older, egg production progressively decreases and this loss of reproductive function is particularly pronounced in broiler breeder hens kept to produce chickens for meat consumption. Egg production is highest in broiler breeders at 5-7 months of age (Robinson et al., 1993; Sharp et al., 1992). The subsequent decrease in egg laying is associated with a reduced recruitment of ovarian follicles into the yellow-yolky follicular hierarchy (Palmer and Bahr, 1992; Waddington et al., 1985), decreased plasma and pituitary LH and decreased LH responsiveness to chGnRH-I (Sharp et al., 1992). Decreased plasma LH in old laying hens is not associated with reduced hypothalamic chGnRH-I peptide (Sharp et al., 1992). Similarly, decreased plasma LH in incubating hens with regressed ovaries is not associated with decreased chGnRH-I peptide but is associated with decreased hypothalamic chGnRH-I mRNA (Dunn et al., 1996), which suggests that the decrease in reproductive function in old laying hens could be mediated by a reduction in chGnRH-I mRNA transcription and/or stability. A change in chGnRH-I release during ageing is likely to affect LH and FSH secretion in different ways since, in rats, mGnRH-I directly stimulates LHβ mRNA synthesis and LH release (Kartun and Schwartz, 1987, Culler and Negro-Vilar, 1987, Shupnik, 1990) while it controls FSH secretion by regulation of follistatin and activin βB mRNAs to alter the ratios of the encoding proteins (Besecke et al., 1996, Dalkin et al., 1999; Burger et al., 2002).
The discovery of a putative avian gonadotrophin inhibitory hormone (GnIH, Tsutsui et al., 2000) presents a new possibility that reduced plasma LH in ageing laying hens could be a consequence of increased GnIH release.

Nothing is known in the domestic hen about the relationship between reproductive neuroendocrine gene expression and naturally reduced or terminated egg laying in the domestic hen. In Japanese quail, testicular regression induced by food withdrawal or transfer from long to short days is associated with decreased common α-subunit mRNA (Kobayashi et al., 2002, Kobayashi et al., 2004) and decreased plasma LH (Kobayashi et al., 2004). Food withdrawal also results in decreased pituitary LHβ and FSHβ subunit mRNAs (Kobayashi et al., 2002). Both the LHβ and common α-subunit mRNAs are likely to be regulated by an inhibitory action of oestrogen since in the chicken these mRNAs are increased after ovariectomy, while ovariectomy and oestrogen replacement prevents this increase in gonadotrophin mRNAs (Terada et al., 1997).

The objective of the research presented in this chapter was twofold, firstly to establish the percentage of non-laying hens in a commercial flock of old (60 weeks) broiler breeders and, secondly, to test the hypothesis that an age-related decline in ovarian function is due, at least in part, to a reduction in hypothalamic chGnRH-I mRNA and/or an increase in GnIH mRNA. To this end, in broiler breeder hens during reproductive ageing, the relationship between plasma LH, FSH and hypothalamic chGnRH-I and GnIH mRNAs and pituitary gonadotrophin subunits and follistatin and activin βB mRNAs were investigated.
3.2 Body, pituitary, oviduct weights and ovarian morphology

Hens were obtained from a flock of young pedigree broiler breeders (Cobb Vantress) at peak-of-lay at 30 weeks of age and from a flock of old hens of the same breed at 60 weeks of age. The birds were reared and maintained on a commercial restricted feeding programme, as recommended by Cobb Vantress, to maximize egg laying and were held on 16 hours light and 8 hours dark in floor pens. The hens in both flocks started laying at 22-24 weeks of age.

In order to determine the effect of reproductive ageing on the distribution of yellow yolky follicles (YYFs) greater than 8mm in the ovary and the proportion of hens out-of-lay, 30 hens were selected at random from the old flock and 15 hens from the young flock.

![Graph showing comparison of the numbers of yellow yolky ovarian follicles greater than 8mm in diameter in young (30 weeks) and old (60 weeks) broiler breeder hens sampled from a commercial flock.]

Figure 3.1 Comparison of the numbers of yellow yolky ovarian follicles greater than 8mm in diameter in a) young (30 weeks) and b) old (60 weeks) broiler breeder hens sampled from a commercial flock.
An analysis of the numbers of YYFs more than 8 mm diameter in 30 old hens selected at random within the sample flock showed that, compared with the young flock, there is a shift in the distribution of the numbers of YYFs greater than 8mm to a lower median, from 7 to 5 YYFs (Fig.3.1). Of the 30 hens selected at random from the old flock, 4 (13%) were out-of-lay as indicated by the absence of YYFs greater than 8mm and regressed oviducts. No young hens selected at random were out-of-lay (Fig 3.1).

Observations on the relationship between plasma gonadotrophins and reproductive neuroendocrine mRNAs were made on the 15 hens taken from the young flock for the analysis of the distribution of YYFs and on 12 old laying and 15 out-of-lay hens selected from the old flock by palpation of the pelvic bones which are softer and more widely spread in laying than in out-of-lay hens. Reproductive condition was assessed by dissection: out-of-lay hens were characterized by the absence of hierarchical YYFs larger than 8mm diameter and regressed oviducts in out-of-lay hens (n=15) while laying hens were characterized by the presence of 5-7 YYFs in the ovaries and fully developed oviducts (n=12) (Fig 3.2). All 15 young hens randomly sampled had 5-9 YYFs in their ovaries. Oviduct and residual ovarian weights were recorded after removal of follicles larger than 8mm. Pituitary glands and whole hypothalami were dissected into 1ml of “RNA later” (Ambion, Huntingdon, Cambridgeshire, UK) before transport to the laboratory where they were stored at -80°C until RNA extraction.
Body and pituitary weights were higher in old than in young laying hens, but were lower in old out-of-lay than in old laying hens selected for neuroendocrine mRNAs and plasma gonadotrophin measurements (Table 3.1). The mean number YYFs greater than 8 mm in diameter in the ovary of young laying hens was significantly higher than in old hens (Table 3.1).

Residual ovary weight was the same in young and old laying hens but was greater than out-of-lay hens, and the oviducts of young laying hens were heavier than old laying hens (Table 3.1).
Chapter 3  Reproductive Ageing

Table 3.1. Body, pituitary, ovary and oviduct weights and numbers of >8mm YYFs of young laying (30 weeks), old laying (60 weeks) and old out-of-lay (60 weeks) broiler breeder hens (means ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Young Laying (n=15)</th>
<th>Old Laying (n=12)</th>
<th>Old out-of-lay (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>3.57 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.11 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.35 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pituitary weight (mg)</td>
<td>9.70 ± 0.25&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.87 ± 0.61&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.67 ± 0.39&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>YYFs (&gt;8 mm)</td>
<td>6.76 ± 0.26&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.92 ± 0.23&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Residual ovary (g)</td>
<td>10.05 ± 0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.53 ± 0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.60 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oviduct (g)</td>
<td>65.40 ± 3.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.42 ± 1.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means within row followed by different superscripts are significantly different, <sup>a,b,c</sup> p< 0.001, <sup>d,e</sup> p<0.05, by analysis of variance and least-significant differences using body weight as a co-variant. <sup>f,g</sup> are significantly different, p< 0.05, using unpaired t-test. YYFs, number of yellow yolky follicles in the ovary.

3.3 Plasma LH and FSH

Plasma LH was higher in young than in old birds, while there was no difference in plasma LH between old laying and out-of-lay hens (Fig 3.3). Plasma FSH was higher in young than in old laying hens, while plasma FSH in old out-of-lay hens was higher than in young or old laying birds (Fig 3.3).
3.4 Gonadotrophin subunits, activin $\beta_B$ and follistatin mRNAs

Common $\alpha$-subunit mRNA pituitary content was higher in young than in old laying hens, and was higher in old laying than in old out-of-lay hens (Fig 3.4a). After correction for age-related differences in pituitary weight, the concentration of common $\alpha$-subunit mRNA was higher in young than in old laying hens but was not different between old laying and out-of-lay hens (Fig 3.4b). LH$\beta$ subunit mRNA pituitary content did not differ between young and old laying hens (Fig 3.4a) but after allowance was made for the age-related difference in pituitary weights the
concentration of pituitary LHβ mRNA was higher in young than in old laying hens (Fig 3.4b). Both pituitary content and concentration of LHβ mRNA were higher in old out-of-lay than in laying hens (Fig 3.4a, b). Pituitary FSHβ subunit mRNA concentration and content did not differ between young and old laying hens but they were higher in old out-of-lay hens compared to laying hens (Fig 3.4a, b).

**Figure 3.4** Comparison of a) pituitary content and b) pituitary concentrations of gonadotrophin subunit mRNAs in young laying (n=15), old laying (n=12) and old out-of-lay (n=15) hens. Means ± SEM. Different letters above bars, within each mRNA group, indicate a statistically significant difference between groups, p< 0.001, by least significant difference of one way ANOVA after logarithmic transformation of data.
Plasma FSH was correlated with pituitary FSHβ mRNA (Fig 3.5a) but not with α-subunit mRNA concentration (Fig 3.5b) while plasma LH was correlated with pituitary α-subunit mRNA (Fig 3.5d) but not with LHβ mRNA concentration (Fig 3.5c). Regression analysis showed that 52% of the variation in plasma LH was explained by the α-subunit mRNA concentration.

Figure 3.5 Linear regression analysis of the relationship between plasma gonadotrophins and pituitary gonadotrophin subunit mRNA concentrations for all hens in the study. a) plasma FSH versus FSHβ mRNA, b) plasma FSH versus a mRNA, c) plasma LH versus LHβ mRNA and d) plasma LH plasma versus a mRNA. Data were log transformed; values for the coefficient of determination (the proportion of the variation explained by the fit) $r^2$ and probability values associated with the $F$ test of the relationship between the fitted values for each regression analysis are shown.
Pituitary content and concentration of Activin $\beta_B$ mRNA did not change between young or old birds (Fig 3.6a, b) while pituitary follistatin mRNA content was higher in old out-of-lay than in young laying hens (Fig 3.6a). Correcting for differences in pituitary weight revealed follistatin mRNA was higher in old out-of-lay than old and young laying hens (Fig 3.6b). There was no difference between pituitary content or concentration of follistatin mRNA in old or young laying hens (Fig 3.6a, b).

**Figure 3.6** Comparison of a) pituitary content and b) pituitary concentrations of activin $\beta_B$ and follistatin mRNA in young laying (n=15), old laying (n=12) and old out-of-lay (n=15) hens. Means ± SEM. Different letters above bars, within each mRNA group, indicate a statistically significant difference between groups, $p<0.05$, by least significant difference of one way ANOVA after logarithmic transformation of data.
3.5 Hypothalamic GnIH and GnRH-I mRNAs

There were no differences in hypothalamic chGnRH-I nor GnIH mRNAs between young and ageing hens irrespective of whether they were laying or out-of-lay (Fig 3.7).

![Figure 3.7](image_url) Comparison of amounts of hypothalamic GnRH-I and GnIH mRNAs in young laying (n=15), old laying (n=12) and old out-of-lay (n=15) hens. Means ± SEM.

3.6 Discussion

This study shows that reduced numbers of yellow yolky ovarian follicles, and by inference, reduced persistency of lay in ageing, laying hens is associated with reduced plasma LH and FSH. This reduction in plasma LH agrees with previous studies in ageing chicken (Sharp et al., 1992) and turkey hens (Guemene and Williams, 1999), but a reduction in plasma FSH in ageing laying hens has not been previously reported. It is therefore likely that reduced ovarian function in ageing hens is caused by a reduction in gonadotrophin secretion. The finding that
there were no differences in chGnRH-I and GnIH mRNAs between young and old laying hens suggests that changes in the synthesis of these neuropeptides are not the primary cause of decreased gonadotrophin secretion in old laying hens.

The absence of a decrease in hypothalamic chGnRH-I mRNA in old laying hens is consistent with an earlier finding that hypothalamic GnRH-I peptide content does not decrease with decreased egg laying in ageing dwarf broilers (Sharp et al., 1992), although, in the incubating hen, decreased LH secretion is associated with decreased chGnRH-I mRNA but not chGnRH-I peptide (Dunn et al., 1996). The absence of a decrease in chGnRH-I mRNA in ageing broiler hens (Fig 3.7) provides no evidence for or against the hypothesis that changes in chGnRH-I release are responsible for decreased gonadotrophin secretion. This is supported by an observation in a mammalian GnRH cell line (GT1) where steady state levels of mGnRH-I mRNA do not correlate with mGnRH-I release (Pitts et al., 2001).

In ageing female rats and women, mGnRH-I pulse frequency is reduced (Hall et al., 2000; Rubin and Bridges, 1989; Rossmanith et al., 1991) and a similar decrease in chGnRH-I pulse frequency may explain the changes in plasma FSH and LH in old out-of-lay hens. The observation that plasma FSH, but not LH increases in old out-of-lay hens (Fig 3.3) is consistent with the finding that ovarian atrophy induced in hens by food deprivation is associated with increased plasma FSH but not LH (Lovell et al., 2000; Vanmontfort et al., 1994). Changes in GnRH-I pulse frequency with advancing age may also explain why ovarian regression was associated with increased plasma FSH but not LH. In mammals, a fast mGnRH-I pulse frequency preferentially stimulates LH release whereas a slow mGnRH-I pulse frequency
stimulates FSH release (Dalkin et al., 1989; Kaiser et al., 1997). In postmenopausal women reduced GnRH pulse frequency (Rossmanith et al., 1991) may explain why serum FSH levels are elevated (Rubin, 2000) while LH levels decrease (Matt et al., 1998). The same mechanism may apply to ageing out-of-lay hens. In support of this view, exogenous chGnRH-I administration into out-of-lay turkey (Guemene and Williams, 1999) and chickens (Sharp et al., 1992; Sharp and Lea, 1981; Williams and Sharp, 1978) increases plasma LH concentrations. These findings demonstrate that the pituitary gland of an out-of-lay bird is able to respond to chGnRH-I and the observed reduction in LH secretion could be due to a reduction in chGnRH-I pulse frequency and/or amplitude. This leaves the question of why plasma FSH is not elevated in ageing laying hens. It is suggested that this may be a consequence of high circulating concentrations of plasma oestrogen, which exert a greater inhibitory effect on FSH than LH pituitary content (Dunn et al., 2003) resulting in FSH secretion being more sensitive to the inhibitory action of oestrogen than LH secretion.

The present study shows that both pituitary content and concentration of FSHβ subunit mRNA are higher in out-of-lay than in-lay hens (Fig 3.4a, b) and this may also be a consequence of reduced chGnRH-I pulse frequency and plasma oestrogen.

The inhibitory effects of oestrogen on gonadotrophin secretion could be either at the level of the anterior pituitary or the hypothalamus. There is evidence in birds that the hypothalamus is a site of the inhibitory action of oestrogen on gonadotrophin
secretion, as demonstrated in adult male Japanese quail, when oestrogen treatment reduces basal release of GnRH-I from the hypothalamus in vitro (Li et al., 1994). Further, in the cockerel, hypothalamic chGnRH-I peptide is increased by treatment with the anti oestrogen, tamoxifen (Rozenboim et al., 1993) while chGnRH-I peptide (Wilson et al., 1990) and chGnRH-I mRNA (Dunn and Sharp, 1999) is suppressed in juvenile birds after treatment with oestrogen. At the level of the anterior pituitary, in mammals, FSHβ subunit gene transcription is highly sensitive to the inhibitory effect of oestrogen (Phillips et al., 1988; Miller and Miller, 1996) and this may be the case in the chicken since in the juvenile hen, pituitary FSH content is more responsive to the depressive action of oestrogen than is LH content (Dunn et al., 2003). The observation that LHβ mRNA was increased in out-of-lay compared to laying hens (Fig 3.4a, b) may also be ascribed to reduced circulating oestrogen, since oestrogen depresses avian LHβ mRNA in vivo (Terada et al., 1997). In out-of-lay birds a reduction in oestrogen may remove an inhibitory effect on LHβ mRNA. Therefore, the effect of oestrogen on LHβ mRNA maybe direct, by effecting the transcripts stability or rate of transcription, or indirect, by reducing pituitary responsiveness to chGnRH-I through reduction of GnRH receptor concentration on gonadotrophs. In support of this view, oestrogen suppresses GnRH receptor mRNA in the cockerel (Sun et al., 2001).

Due to a fall in chGnRH-I release and plasma oestrogen, a shift in the secretion of less acidic FSH isoforms in young broiler hens to more acidic FSH isoforms in old broiler hens is conceivable. FSH exists as a family of related isoforms that vary in
their charge, metabolic clearance rates, and in vitro biological activities (Ulloa-Aguirre et al., 1995). For example, in mammals, FSH isoforms vary over the ovulatory cycle and during puberty (Padmanabhan et al., 1992; Wide and Bakos, 1993). GnRH and oestrogen stimulate the secretion of less acidic FSH isoforms that, although having shorter half lives, have increased bioactivity (Ulloa-Aguirre et al., 1995). This view is supported by the finding that in menopausal women the majority of circulating FSH is more acidic and glycosylated to a lesser extent than in cycling women (Anobile et al., 1998). Therefore, the FSH isoforms preferentially secreted from the old out-of-lay hen pituitary gland may not be as effective in causing ovarian development.

The increase in pituitary FSHβ mRNA and plasma FSH in old out-of-lay hens may not only be due to decreasing oestrogen and chGnRH-I pulse frequency but also to a fall in circulating ovarian inhibin. The hierarchical YYF are a major source of ovarian inhibin in hens (Johnson, 1993), evidence for this comes from the inverse relationship between the presence of YYF and, therefore, inhibin and circulating FSH (Lovell et al., 2000; Vanmontfort et al., 1994; Johnson et al., 1993). In primary rat cultures inhibin reduces pituitary follistatin mRNA levels within 2 hours and by 80% at 24 hours (Bilezikjian et al., 1996). Further, passive immunoneutralization of circulating inhibin in rats increases follistatin mRNA expression, but not activin βB mRNA (Dalkin et al., 1998) and there is evidence that inhibin suppresses follistatin transcription (Prendergast et al., 2004). It is possible that the increase in pituitary follistatin and FSHβ mRNA (Fig 3.6) and plasma FSH seen in old out-of-lay
hens is due to a fall in circulating ovarian inhibin. Due to a reduction in chGnRH-I release, as well as a reduction in plasma inhibin, pituitary activin B secretion may have increased, regardless of any change in activinβB mRNA (Fig 3.6a, b).

This study shows that FSHβ mRNA is positively correlated with plasma FSH (Fig 3.5a) and supports the view that avian FSH (Hattori et al., 1986), like mammalian FSH (Farnworth, 1995) is in large part, constitutively released. Hattori and colleagues (1986) showed in quail that pituitary glands treated with hypothalamic extracts released more FSH than was initially present. Further, FSH but not LH secretion continued after the glands were collected and incubated in vitro while, in vivo, FSH and LH secretion is asynchronous in broiler breeder cockerels (Vizcarra et al., 2004). Similar observations have been reported in mammals (Kartun and Schwartz, 1987). These studies demonstrate that LH and FSH are differentially controlled and that LH secretion is more immediately dependent on the stimulatory action of GnRH-I than is FSH. This raises the possibility that an unidentified hypothalamic factor differentially controls FSH and LH secretion. The recently discovered avian hypothalamic RFamide gonadotrophin inhibitory hormone, GnIH (Tsutusi et al., 2000) is a potential candidate for this undiscovered factor in birds. An increase in GnIH mRNA in ageing laying hens might, therefore, explain the decrease in plasma LH and FSH. However, since there was no change in GnIH mRNA between young and old or laying and out-of-lay hens this seems unlikely. However, it is possible that an increase in GnIH secretion may occur in ageing hens independent of a change in GnIH mRNA.
A regression analysis of all data collected in the study showed that common α-subunit (Fig 3.5d), but not LHβ subunit mRNA (Fig 3.5c), was correlated with concentrations of plasma LH, explaining 52% of the variation in plasma LH. A direct relationship between common α-subunit mRNA and plasma LH has been observed previously in quail deprived of food (Kobayashi and Ishii, 2002) or after transfer from long to short days (Kobayashi et al., 2004). Further, exposure of cockerel pituitary fragments to pulses of chGnRH-I causes significant increases in LH secretion which is associated with an increase in common α-subunit mRNA but not LHβ subunit mRNA (Foster et al., 1992; Marsden et al., 1994).

In conclusion, this study demonstrates that the decline in reproductive performance in ageing laying broiler breeders is correlated with a decrease in both LH and FSH secretion. Observations on the relationship between plasma FSH and amounts of FSHβ subunit mRNA supports the view that FSH secretion is largely constitutive in birds. Interestingly, the expression of common α-subunit mRNA, rather than LHβ mRNA is associated with LH secretion, this raises the possibility that pituitary α-subunit mRNA could be a rate limiting step in LH release in birds. The regressed ovary in old out-of-lay hens is associated with increased FSHβ mRNA and FSH release which is ascribed to lifting inhibitory ovarian feedback, possibly oestrogen and/or inhibin.
CHAPTER 4. Effects of lifting food restriction on the broiler breeder hen reproductive axis

4.1 Introduction

Food restriction depresses LH and/or FSH secretion in mammals (Bronson, 1986; Sisk and Bronson, 1986; Foster et al., 1989; Landefeld et al., 1989, Bergendahl et al., 1991) and birds (Tanabe et al., 1981; Lal et al., 1990; Lovell et al., 2000; Kobayashi and Ishii, 2002), although a depressive effect on FSH secretion is not always observed (Sisk and Bronson, 1986; Kobayashi and Ishii, 2002; Lovell et al., 2000). This depression in LH and, less consistently, FSH secretion is associated with decreased pituitary gonadotrophin subunit mRNAs (Landefeld et al., 1989; Bergandahl et al., 1991; Kobayashi and Ishii, 2002) and decreased hypothalamic GnRH-I release (Berghandal et al., 1991) rather than to a decreased responsiveness of anterior pituitary gland to GnRH-I (Foster et al., 1989; Bruggerman et al., 1998). Changes in GnRH-I release induced by changes in food intake are likely to affect LH and FSH secretion in different ways since GnRH-I controls LH secretion directly (Kartun and Schwartz, 1987; Culler and Negro-Vilar, 1987; Shupnik, 1990; Dalkin et al., 1989) while it controls FSH by differential control of activin βB and follistatin mRNAs to stimulate FSHβ mRNA and constitutive release (Besecke et al., 1996, Dalkin et al., 1999, Burger et al., 2002).

It has not been established in breeding animals, whether changes in food intake modulate GnRH-I release only at the level of the median eminence or whether there is an effect at the level of GnRH-I gene transcription or GnRH-I mRNA stability.

A candidate providing a link between metabolic signals and the reproductive
system at the level of GnRH-I release is the neurotransmitter neuropeptide Y (NPY). The release of NPY secretion increases in response to food restriction in both mammals (Kalra et al., 1991) and birds (Boswell et al., 1999) and could therefore decrease in response to increased food intake. Changes in GnRH-I and LH secretion associated with a change in food intake may be mediated by NPY neurones (McShane et al., 1993). A further link between nutrition and gonadotrophin secretion, at least in birds, could be provided by the recently identified avian RFamide peptide, gonadotrophin inhibitory hormone (GnIH) (Tsutsui et al., 2000). RFamides have been implicated as neurotransmitters involved in the control of feeding behaviour in a wide range of species, including coelenterates and molluscs as well as mammals (Dockray, 2004).

The food restricted laying broiler provides a model to identify neuroendocrine factors controlling changes in reproductive function in a breeding animal in response to increased nutrition. The objective of the research presented in this chapter was to test the hypothesis that, in the food restricted laying broiler breeder hen, *ad libitum* feeding will increase the secretion of chGnRH-I at the level of the median eminence and/or increase chGnRH-I gene transcription or chGnRH-I mRNA stability. The possible increase in chGnRH-I neuronal activity could be mediated by altering hypothalamic NPY gene expression, resulting in altered pituitary gonadotrophin subunit, activin \( \beta_B \) and follistatin mRNAs and gonadotrophin secretion. This study aimed to test this hypothesis by measuring changes in hypothalamic GnRH-I, NPY and GnIH mRNAs and pituitary gonadotrophins, follistatin and activin \( \beta_B \) mRNAs in
35 week old laying, broiler breeder hens. These birds were ranked and randomised by body weight, 5-6 weeks after the onset of egg laying, and re-housed into 2 groups one week prior to the start of the experiment. Control hens were maintained on a restricted diet (energy 11.4 mJoules/kg) provided at 136 g/food/hen/day (n=6) or fed ad libitum (n=7) for 7 days. Food was restricted to around 50% of ad libitum intake (Hocking et al., 1993).

4.2 Body, pituitary weight and ovarian morphology

Seven days after lifting food restriction, body and oviduct weights and numbers of large rapidly maturing preovulatory (>8mm) yellow yolky ovarian follicles increased (Table 4.1), while residual ovarian weight and the numbers of smaller 5-8mm ovarian follicles did not change (Table 4.1). Pituitary gland weight did not change although there was an increase after lifting food restriction that approached significance (Table 4.1).
Table 4.1. Body, pituitary, residual ovary and oviduct weight, number of yellow yolky follicles (YYF) >8 mm, number of 5-8 mm follicles in feed restricted and broiler breeder hens released from restriction for 7 days. (Means ± SEM).

<table>
<thead>
<tr>
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<th>Feed Restricted (n=6)</th>
<th>ad libitum (n=7)</th>
<th>ANOVA p value</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td>4.24 ± 0.15</td>
<td>4.82 ± 0.15</td>
<td>0.04</td>
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<tr>
<td>Pituitary weight (mg)</td>
<td>10.35 ± 3.35</td>
<td>13.85 ± 2.94</td>
<td>0.08</td>
</tr>
<tr>
<td>No. YYF (&gt;8 mm)</td>
<td>6.00 ± 0.26</td>
<td>8.85 ± 0.64</td>
<td>0.002</td>
</tr>
<tr>
<td>No. follicles (5-8mm)</td>
<td>15.50 ± 2.00</td>
<td>13.85 ± 2.07</td>
<td>0.58</td>
</tr>
<tr>
<td>Residual ovary weight (g)</td>
<td>7.48 ± 0.60</td>
<td>8.38 ± 0.96</td>
<td>0.40</td>
</tr>
<tr>
<td>Oviduct weight (g)</td>
<td>62.42 ± 2.70</td>
<td>73.52 ± 2.97</td>
<td>0.02</td>
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</tbody>
</table>

4.3 Plasma LH and FSH

After 7 days of ad libitum feeding the pituitary gonadotrophins were differentially secreted with an increase in plasma LH and a decrease in FSH (Figure 4.1).
Figure 4.1 Comparison of plasma LH and FSH in food restricted broiler breeder hens and hens in which food restriction was lifted for 7 days. Values are mean ± SEM. One-way ANOVA was performed on log transformed data. *p < 0.05 compared to feed restricted hens.

4.4 Gonadotrophin subunits, activin βB and follistatin mRNAs

At the level of the anterior pituitary gland, lifting food restriction for 7 days increased glycoprotein α-subunit and follistatin mRNAs, but did not affect FSHβ, LHβ nor activin βB mRNAs (Fig 4.2a, b).
4.5 Hypothalamic NPY, GnRH-I and GnIH mRNAs

The increase in yellow yolky ovarian follicles observed in hens fed *ad libitum* was associated with an increase in hypothalamic chGnRH-I mRNA, but there was no change in GnIH nor NPY gene expression (Fig 4.3).
Chapter 4  
Food intake and reproduction

**Figure 4.3** Comparison of hypothalamic mRNA expression of GnRH-I, GnIH and NPY in food restricted hens and hens in which food restriction was lifted for 7 days. Values are mean ± SEM. One-way ANOVA was performed on data. ***p< 0.001 compared with ad libitum hens.

### 4.6 Discussion

The increase in hypothalamic GnRH-I mRNA in laying food-restricted broiler breeder hens after 7 days of *ad libitum* feeding (Fig 4.3) contrasts with studies in sheep (McShane *et al.*, 1993) and in male (Leonhardt *et al.*, 1999) and female cycling rats (Nappi and Revist, 1997) where food restriction did not affect hypothalamic
mGnRH-I mRNA, but is consistent with another study on the male rat, suggesting that food restriction does depress hypothalamic mGnRH-I mRNA (Gruenewald and Matsumoto, 1993). The view that metabolic signals generated by changes in food intake may directly affect hypothalamic GnRH-I mRNA is supported by the observation that mGnRH-I mRNA increases in cultures of mouse hypothalamic neuronal cells after incubation with insulin and leptin, which are key hormones involved in metabolic signaling (Burcelin et al., 2003). It therefore concluded that the effects of changes in food intake on gonadotrophin secretion, mediated by the actions of metabolic signals on the GnRH pulse generator (I’Anson et al., 2000; Blache et al., 2003), may also involve direct effects on GnRH-I gene transcription or GnRH-I mRNA stability.

The observation that NPY mRNA did not decrease after feeding ad libitum for 7 days (Fig 4.3) was unexpected because juvenile broiler hens reared on a food restricted diet have more hypothalamic NPY mRNA than in control birds fed ad libitum (Boswell et al., 1999). However, it is possible that 7 days ad libitum feeding in the present study was not sufficient to allow NPY gene expression to attain its normal ad libitum-fed level. Although increased NPY peptide has been implicated in the control of pre-ovulatory release of LH and the onset of puberty in the chicken (Kuenzel and Fraley, 1995), the results of the present study do not support a role for NPY in mediating the stimulatory effect of ad libitum feeding of food-restricted broilers on GnRH-I mRNA.
The avian RFamide GnIH has been shown to inhibit LH secretion in vitro (Tsutsui et al., 2000). In the present study GnIH mRNA was not changed after 7 days of ad libitum feeding (Fig 4.3), it is concluded that decreased GnIH neuronal function is not a primary cause in increased gonadotrophin function. However, it cannot be ruled out that a change in GnIH secretion may occur independent of a change in GnIH mRNA.

Observations in the rat and sheep suggest that supplementary nutrition results in a transitory increase in LH (Bronson, 1986; Sisk and Bronson 1986; Foster et al., 1989) that may reflect an acute release of mGnRH-I from the median eminence. In the sheep this initial transitory increase in LH is followed after 2 days by a substantial increase in the frequency of LH discharges. Therefore, it is possible that, after re-feeding, the GnRH-I pulse generator is controlled primarily at the level of GnRH-I release, but secondarily by increased production of GnRH-I peptide mediated by an increase in GnRH-I mRNA. In support of this view in the chicken, chronic changes in gonadotrophin secretion are associated with changes in chGnRH-I mRNA. For example, depressed plasma LH in incubating hens (Dunn et al., 1996) and in castrated cockerels treated with oestrogen for two weeks (Sun et al., 2001) is associated with decreased chGnRH-I mRNA, while conversely, in hens, increased plasma LH observed after 7 days photostimulaton is associated with increased chGnRH-I mRNA (Dunn and Sharp, 1999).

The increase in chGnRH-I mRNA in broiler hens after ad libitum feeding for 7 days may have affected the GnRH-I pulse generator (I’Anson et al., 2000; Blache et
al., 2003) by increasing GnRH-I synthesis that in turn, resulted in a change the pulsatile pattern of GnRH-I release. A change in the pulsatile pattern of GnRH-I release may explain the observed increase in plasma LH and decrease in plasma FSH (Fig 4.1).

In mammals, a high pulse mGnRH-I frequency preferentially stimulates more LH than FSH production and release, while a low mGnRH-I pulse frequency preferentially stimulates more FSH than LH (Wildt et al., 1981; Kaiser et al., 1997; Jayes et al., 1997). It is therefore suggested in the present study, that ad libitum feeding for 7 days may have increased chGnRH-I pulse frequency that in turn, affected the secretion of gonadotrophins to increased plasma LH and decreased plasma FSH. This interpretation of the data is consistent with the stimulatory effects of increased mGnRH-I pulse frequency on LHβ subunit mRNA and LH secretion in sheep and rats (Landefeld et al., 1989; Bergendahl et al., 1991). However, there was no evidence of an increase of LHβ mRNA associated with increased plasma LH in ad libitum hens, although pituitary α-subunit mRNA was elevated. This observation is consistent with the observation in the quail where plasma LH is highly correlated with α-subunit mRNA rather than LHβ subunit mRNA (Kobayashi and Ishii, 2002; Kobayashi et al., 2004).

In hens, plasma FSH is inversely related to ovarian development since plasma FSH increases after induction of ovarian atrophy induced by food deprivation (Vanmontfort et al., 1994; Lovell et al., 2000) or with reduced ovarian function.
associated with reproductive ageing (chapter 3). The decrease in plasma FSH, after feeding *ad libitum* for 7 days, is associated with increased ovarian development and is consistent with these earlier observations. However the decrease in plasma FSH after *ad libitum* feeding for 7 days was not associated with a change in FSHβ subunit mRNA (Fig 4.3). As in mammals (Farnworth, 1995), avian FSH is thought to be released constitutively (Hattori *et al.*, 1986), and consequently, plasma FSH is predicted to be directly related to the rate of FSH synthesis and FSHβ mRNA (chapter 3). The absence of a relationship between plasma FSH and FSHβ subunit mRNA in the present study may be related to the way in which FSHβ mRNA is regulated through interactions between the pulsatile release of GnRH-I and pituitary follistatin and activin βB (Bilezikjian *et al.*, 2004; Winters and Moore, 2004). In rats, the regulation of FSHβ mRNA by mGnRH-I is mediated by a follistatin/activin paracrine loop within the anterior pituitary gland (Bilezikjian *et al.*, 2004; Winters and Moore, 2004). This mechanism may also occur in the chicken since both follistatin and activin βB mRNAs were expressed in the anterior pituitary gland (Fig 4.4). In the rat, fast pulses of mGnRH-I stimulate follistatin mRNA while slow pulse frequency suppresses follistatin mRNA and increases both activin βB and FSHβ mRNAs implying that follistatin attenuates the FSH response by bio-neutralising the effect of activin B in increasing FSHβ mRNA and constitutive FSH release (Bilezikjian *et al.*, 2004). When mGnRH-I release is increased in rats, follistatin mRNA is increased, for example in gonadectomised males (Kasier and Chin, 1993) and females during the preovulatory gonadotrophin surge (Halverson *et al.*, 1994).
In contrast, in primates, follistatin is unaffected by castration and *in vitro* GnRH does not stimulate follistatin mRNA in primate pituitary cells (Winters and Moore, 2004). The present study supports the view that GnRH-I stimulates follistatin mRNA (Fig 4.2b) since pituitary follistatin mRNA content increased after lifting food restriction for 7 days (Fig 4.4a) associated with an increase in chGnRH-I mRNA. The expression of chicken activin βB mRNA pituitary content did not change (Fig 4.2b). The observations on follistatin and activin βB mRNAs in broiler breeder hens after feeding *ad libitum* for 7 days suggests that chGnRH-I regulates pituitary follistatin gene expression in the chicken as has been reported in the rat. It is therefore predicted that the increase in follistatin mRNA in broiler hens fed *ad libitum* for 7 days should have depressed FSH secretion by increasing follistatin which in turn would bind to activin B protein to depress FSHβ mRNA and hence, FSH secretion. It is therefore uncertain why plasma FSH was depressed in the absence of a depression in FSHβ mRNA, but it has been proposed that mGnRH-I induced increases in follistatin may serve to delay FSHβ mRNA responses rather than completely prevent them (Kirk *et al.*, 1994). It may also be some other factors possibly associated with increased ovarian growth observed after 7 day *ad libitum* feeding, depresses FSH secretion independently of a change in pituitary FSHβ mRNA.

Secreted follicular ovarian factors most likely increase after lifting food restriction are oestrogen and inhibin. Plasma oestrogen is higher in laying broilers fed *ad
libitum than fed a restricted diet (Bruggerman et al., 1998). In the juvenile female chicken, pituitary FSH content is more responsive to the depressive action of oestrogen than is LH content (Dunn et al., 2003) and consequently it is possible that decreased plasma FSH in laying broiler hens fed ad libitum for 7 days may have been a consequence of an inhibitory effect of oestrogen which is independent of a change in FSHβ mRNA. The increase in oviduct weight is an indication that serum levels of oestrogen in ad libitum hens have increased (Klandorf et al., 1992). It is also likely that the increase in ovarian development, which occurred after lifting food restriction for 7 days, resulted in an increase in plasma inhibin. This deduction is consistent with the observation that inhibin is produced by the largest yellow yolky ovarian follicles (Johnson, 1993) and is present in the circulation of laying hens (Lovell et al., 2001). However attempts to demonstrate, in the hen, that circulating inhibin regulates plasma FSH secretion are inconclusive (Lovell et al., 2001). Nonetheless, it is possible that during 7 days of ad libitum feeding plasma FSH levels increased, to facilitate the recruitment of additional follicles into the yellow yolky follicular hierarchy and the first release of stored chGnRH-I from the median eminence may have mediated this rise in FSH. As the ovary developed, during 7 days of ad libitum feeding, the increasing levels of oestrogen and inhibin may have inhibited FSH secretion by a negative feedback loop. This prediction is not mutually exclusive to a proposed effect on activin stimulated signalling as one mechanism by which inhibin may effect pituitary FSH secretion is by interrupting activin signalling (See chapter 1; section 1.6).
In conclusion, this study provides evidence that acute *ad libitum* feeding for 7 days of chronically food-restricted laying broiler hens increases hypothalamic chGnRH-I mRNA which may be a consequence or cause of increased chGnRH-I release, resulting in increased pituitary gonadotrophin α-subunit and follistatin mRNAs. An increase in pituitary gonadotrophin α-subunit but not LHβ mRNA is associated with an increase in plasma LH raising the possibility that α-subunit biosynthesis, and not LHβ subunit, is rate limiting LH secretion. The increase in pituitary follistatin mRNA content and decrease in pituitary activin βB mRNA concentration may explain the associated decrease in FSH secretion in spite of no associated depression in pituitary FSHβ mRNA. The depression in plasma FSH may also have been a consequence of increased secretion of ovarian hormones such as oestrogen and inhibin from increased numbers of yellow yolky ovarian follicles stimulated by *ad libitum* feeding.
CHAPTER 5. Effects of inhibiting egg laying by inducing incubation behaviour on neuroendocrine mRNAs

5.1 Introduction

Ovarian regression and cessation of egg laying can be induced by manipulation of the environment. Two such manipulations are explored in this Thesis to induce ovarian regression: induction of incubation behaviour (this chapter) and transfer from long to short photoperiods (chapter 6). Such environmental manipulations make it possible to investigate changes in neuroendocrine function associated with ovarian regression, which may contribute to increasing understanding of the neuroendocrine mechanisms underlying reduced ovarian function in ageing broiler breeders.

Figure 5.1. a. Laying and b. incubating hens provide a good model to further understand neuroendocrine mechanisms responsible for reduced ovarian function in adult hens
Incubation behaviour is induced by tactile stimulation from eggs and by visual information provided by an adequate nest site (Sharp, 1988; personal observation). The onset of incubation behaviour is characterised by changes in behaviour and by regression of the ovary and oviduct (Fig 5.1), cessation of egg laying, increased prolactin secretion and decreased LH and oestrogen secretion (Richard-Yris et al., 1998; Sharp et al., 1988; Sharp et al., 1979), decreased hypothalamic GnRH-I mRNA (Dunn et al., 1996), and decreased LHβ subunit mRNA (Wong et al., 1992). The inhibitory effect of incubation behaviour on LH secretion has been ascribed to decreased hypothalamic chGnRH-I release rather than to a decreased responsiveness of the anterior pituitary gland (Sharp and Lea, 1981). If an incubating hen is deprived of its nest and eggs hypothalamic chGnRH-I mRNA and plasma LH increase (Dunn et al., 1996), circulating prolactin decreases (Richard-Yris et al., 1998) and the ovary begins to develop, which facilities a return to lay (Sharp et al., 1988).

The objective of the research presented in this chapter was to test the hypothesis that incubation behaviour depresses both FSH and LH secretion by inhibiting chGnRH-I gene transcription or chGnRH-I mRNA stability, which, in turn, inhibits gonadotrophin subunit mRNAs. A further objective was to establish whether gonadotrophin inhibitory hormone (GnIH) mRNA increases in incubating hens and thereby provide an additional previously undescribed mechanism to inhibit constitutive gonadotrophin release. This study aimed to test these hypotheses determining whether correlating hypothalamic GnRH-I, and GnIH and pituitary
gonadotrophin subunit mRNAs were correlated with plasma LH and FSH in incubating and laying hens.

5.2 Body, pituitary and ovary weights and ovarian morphology

Laying hens were heavier than incubating hens (Table 5.1). In contrast, incubating hens had significantly heavier pituitary glands. Incubating hens had no yellow yolky follicles (YYF) >8mm in diameter while laying hens had 4-6 YYF. Residual ovary weights, recorded after removal of YYF larger than 8mm, were significantly higher in laying hens.

<table>
<thead>
<tr>
<th></th>
<th>Incubating (n=5)</th>
<th>Laying (n=5)</th>
<th>ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>0.896 ± 0.05</td>
<td>1.36 ± 0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pituitary weight (mg)</td>
<td>7.84 ± 0.57</td>
<td>6 ± 0.56</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>YYF (&lt;8 mm)</td>
<td>0</td>
<td>5.33 ± 0.34</td>
<td>n/a</td>
</tr>
<tr>
<td>Residual ovary (g)</td>
<td>1.53 ± 0.09</td>
<td>4.33 ± 0.41</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

5.3 Plasma LH and FSH

Circulating LH was significantly higher in laying than in hens which had been incubating for 4-21 days, with no difference in plasma FSH (Fig 5.2).
Figure 5.2 Comparison of plasma LH and FSH in incubating and laying bantam hens. The hens had been incubating for more than a week. Values are mean ± SEM. n=5. ANOVA performed on log transformed data. **p<0.001.

The absence of a difference in plasma FSH might be related to the time elapsed since the onset of incubation. To test this hypothesis a comparison was made between plasma FSH in hens that had been incubating for 1-4 days with hens that had been incubating for 4-21 days.
Figure 5.3 Comparison of plasma FSH in a) hens which had been incubating for a short period (<4 days) and laying hens, and b) in hens which had been incubating for a longer period (4-21 days) and laying hens. (n=8). Values are mean ± SEM. ANOVA performed on log transformed data.

Although plasma FSH was higher in hens that had been incubating for 1-4 days than in laying hens, this did not reach statistical significance (3.4 ± 0.3 vs. 2.55 ± 0.4 ng/ml, Fig 5.3a). Concentrations of plasma FSH in hens that had been incubating for 4-21 days was not different from plasma laying hens (Fig 5.3b).
5.4 Effect of nest deprivation on plasma gonadotrophins in incubating hens

Nest depriving incubating hens significantly increased plasma LH within 5.5 hours confirming a previous observation (Sharp et al., 1988). Nest removal also causes an increase in plasma FSH in incubating hens but the increase was less rapid than LH occurring approximately 7.5 hours after nest deprivation (Fig 5.3).

![Graph showing changes in plasma LH and FSH in incubating hens after nest deprivation. n=6. *p<0.05 compared to plasma gonadotrophin at time zero. One way ANOVA and least significant difference performed on Log transformed data using bird as a block.](image)

**Figure 5.4** Changes in plasma LH and FSH in incubating hens after nest deprivation. n=6. *p<0.05 compared to plasma gonadotrophin at time zero. One way ANOVA and least significant difference performed on Log transformed data using bird as a block.
5.5 Gonadotrophin subunit mRNAs

Pituitary concentration and content of common α and LHβ subunit mRNAs were lower in incubating than in laying hens (Fig. 5.5a-b). No significant difference in the amount of FSH β mRNA was observed between incubating and laying hens (Fig. 5.4a-b).

Figure 5.5 Comparison of a) pituitary content and b) pituitary concentrations of gonadotrophin subunit mRNAs in incubating (n=5) and laying (n=5) bantam hens. ANOVA was performed on log-transformed data. **p < 0.01, ***p < 0.001 compared with laying hens.
5.6 Hypothalamic GnIH and GnRH-I mRNAs

Compared with laying hens, hypothalamic chGnRH-I mRNA was lower in incubating hens and GnIH mRNA was higher (Fig 5.6).

![Graph comparing hypothalamic GnRH-I and GnIH mRNAs in incubating and laying hens. Values are mean ± SEM. n=5. ANOVA was performed on log-transformed data.](image)

**Figure 5.6** Comparison of hypothalamic GnRH-I and GnIH mRNAs in incubating and laying hens. Values are mean ± SEM. n=5. ANOVA was performed on log-transformed data.
A second set of hypothalami from laying (n=6) and incubating (n=6) hens were collected on a separate occasion and sent for GnIH peptide quantification in the laboratory of Prof Kazu Tsustui (University of Hiroshima). He found that the concentration of hypothalamic GnIH peptide was greater in incubating than in laying hens (0.394 ± 0.022 vs. 0.326 ± 0.021 ng/mg hypothalamus, p< 0.05)

5.7 Effect of GnIH injections on plasma gonadotrophins in nest deprived hens

The objective was to establish whether GnIH inhibits LH and FSH in vivo as was demonstrated in vitro in adult cockerels (chapter 7). The experiment used nest-deprived incubating hens. Nest removal results in an increase in plasma LH (Sharp et al., 1988) and could be a consequence of reduced GnIH release which was predicted to be reversed by exogenous administration of GnIH. Quail GnIH, synthesised in the laboratory of Prof Kazu Tsutsui was dissolved in physiological saline and injected (100μl) into the brachial veins of nest deprived incubating hens. The hens were given three injections of 50μg GnIH/kg body weight or saline vehicle at 5.5, 6.5 and 7.5 hours after nest deprivation. The dose of GnIH chosen was based on analogous experiments in the incubating hens using GnRH analogues, which suggested that injections of 50μg/kg GnRH stimulate gonadotrophin secretion (Sharp et al., 1986). This is similar to the dose of GnIH shown to be effective in depressing LH, in vivo, in the White Crowned sparrow (Osugi et al., 2004). Further, the
molecular weights of GnRH (1245) and GnIH (1392) are similar and equivalent
doses might be expected to exert a biological effect on LH secretion. Three
injections of GnIH were given at hourly intervals to cover the possibility that an
inhibitory effect of the peptide on LH release may be secondary to a longer term
inhibitory affect on synthesis. Blood samples (~1ml) were taken from a brachial
vein before nest deprivation at 0 hours, and at 5.5, 6.5, 7.5 and 8.5 hours thereafter
for LH and FSH assays. FSH was also measured since GnIH inhibits FSH in vitro
(chapter 7). GnIH or saline injections were given immediately after withdrawing
blood samples at 5.5, 6.5 and 7.5h after nest deprivation.

Three intravenous injections of 50µg GnIH/kg given at hourly intervals after nest
deprivation of incubating hens for 5.5 hours failed to attenuate or block the increase
in plasma LH (Fig 5.7b). Similarly, within the time frame of the experiment, GnIH
injections did not effect the increase in plasma FSH (Fig 5.7a).
5.8 Effects in vitro of GnIH on gonadotrophin subunit mRNAs and gonadotrophin secretion using pituitary fragments from incubating and laying hens

The objective was to determine whether short term in vitro exposure to GnIH depresses gonadotrophin subunit mRNAs and FSH and LH secretion in incubating and laying hens, as was observed in adult cockerels (chapter 8). Pituitary glands were dissected from eight laying and eight incubating hens. The ovarian condition of each bird was checked on dissection. Pituitaries were allocated to separate pools for
incubating and laying hens. The pituitaries were diced into fragments and randomly distributed into the wells of 24 well culture plate (Corning Costa, Amsterdam, Netherlands). Each well contained two pituitary fragments, 1ml DMEM culture medium with phenol red (Invitrogen Life Technologies, Paisley, UK) supplemented with 3.75% foetal calf serum (FCS), 6% horse serum (HS) and antibiotics: 100u/ml streptomycin and 100μg/ml penicillin. The culture plates were placed in an airtight container, and after equilibration with 95% O₂ and 5% CO₂ transferred to an incubator maintained at 37°C and shaken gently on an orbital platform (60 rev/min.) for a 90 minute pre-incubation. The culture medium was then removed and 1ml of fresh medium was added to each well containing 1x10⁻⁷ M quail GnIH peptide (courtesy of Prof. Kazu Tsutsui) or no peptide (control) in a Latin square design. The culture plates were re-equilibrated with 95% O₂ and 5% CO₂ and replaced in the orbital incubator for a 120 minute incubation. Pituitary fragments were harvested and snap frozen in liquid nitrogen and stored at -80°C before RNA extraction and culture media stored for LH and FSH quantification.

Incubation of pituitary fragments from incubating and laying hens with GnIH had no effect on gonadotrophin subunit mRNAs (Fig 5.8a-c). Although LHβ and α-subunit mRNAs were lower in incubating than in laying pituitary fragments while FSHβ mRNA was not different confirming the previous experiment (Fig 5.5). There was no difference in LH or FSH released between control and GnIH treated pituitary fragments (Fig 5.8 d-e). LH release was lower from pituitary fragments from
incubating than from laying hens while there was no difference in FSH release (Fig 5.8 d-e) confirming the previous experiment (Fig 5.2).

Figure 5.8 Lack of 10^-7 M GnIH effect on gonadotroph function in pituitary fragments from incubating and laying bantam hens. Two way ANOVA was performed using incubating or laying hens and GnIH or control as factors. Data was log transformed. Different letters above bars indicate a statistically significant difference between factors, a vs. b, p<0.001; a vs. c, p<0.01.

5.9 Effects in vitro of GnRH-I on gonadotrophin subunit mRNAs using pituitary fragments from incubating hens

The objective was to investigate the effects of short term exposure to chicken GnRH-I (Bachem, St Helens, UK) on pituitary gonadotroph function. Incubating
hen pituitary glands (n=6) were diced into fragments and distributed into the wells of 24 well culture plate (Corning Costa, Amsterdam, Netherlands). Each well contained two pituitary fragments and the pre-incubation was as described in section 5.2.3. The culture medium was removed after the 90 minute pre-incubation and 1ml of fresh medium was added to each well containing GnRH-I peptide (Bachem, St Helens, UK) at $1 \times 10^{-8}$, $1 \times 10^{-7}$, $1 \times 10^{-6}$ log M or no peptide (control) in a Latin square design. The culture plates were re-equilibrated with 95% O$_2$ and 5% CO$_2$ and replaced in the orbital incubator for a 120 minute incubation. Pituitary fragments were harvested and snap frozen in liquid nitrogen and stored at $-80^\circ$C before RNA extraction.

Incubation of pituitary fragments from incubating hens with a range of concentrations of GnRH-I had no effect on FSH$\beta$ nor $\alpha$-subunit mRNAs (Fig 5.9).
Figure 5.9 Lack of effect of a 120 minute incubation of GnRH-I on FSHβ and α-subunit mRNAs in incubating hen pituitary fragments. Data was log transformed. ANOVA was performed using treatment and position in culture plate as factors.

5.10 Discussion

The regression of the ovary (Table 5.1) and the cessation of egg production in incubating hens were associated with changes in reproductive neuroendocrine function. At the level of the hypothalamus, confirming observations by Dunn et al. (1996), inhibition of reproductive function induced by incubation behaviour was associated with a reduction in chGnRH-I mRNA (Fig 5.6). The increase in GnIH mRNA (Fig 5.6) has not been observed previously and appears to be translated since
GnIH peptide concentration is higher in the hypothalamus of incubating than laying hens. The increase in both GnIH mRNA and GnIH peptide indicates an increase in GnIH neuronal activity in the incubating hen that may be associated with an increase in GnIH release.

The inverse relationship between hypothalamic chGnRH-I and GnIH mRNAs in incubating and laying hens was associated with depressed pituitary concentration and content of common α and LHβ subunit mRNAs (Fig. 5.5a, b) and plasma LH (Fig. 5.2) in the incubating hen, but not FSHβ mRNA (Fig. 5.4a, b) or plasma FSH (Fig 5.2 and 5.3).

This cascade of neuroendocrine gene expression is consistent with a predicted (see section 5.1) increase in GnIH release into the hypophysial portal vasculature resulting in a depression in LH synthesis and constitutive release (chapter 8). However, there is no evidence in vivo (Fig 5.7) or in vitro (Fig 5.8) to suggest that GnIH suppresses pituitary gonadotroph function in the incubating hen. Repeated injections of GnIH failed to suppress the increase in plasma LH that occurs in incubating hens after nest deprivation (Fig. 5.7b). These observations contrast with a study in the White Crowned sparrow where an intravenous injection of GnIH transiently suppressed plasma LH after 2 but not 10 minutes, and an injection of 1000ng GnIH antagonised the stimulatory effect of 10ng GnRH on LH release (Osugi et al., 2004). The sampling procedure used in the present study would not have detected a transient depression in plasma LH after 2 minutes. The failure to
demonstrate a long term inhibitory effect of GnIH in vivo in nest deprived incubating hens (Fig.5.7b) may be explained by an inappropriate dose or timing of GnIH administration. Alternatively, it is possible that the quail GnIH used in the study, which differs from the predicted sequence of chicken GnIH by a conservative substitution of lysine by arginine at position three, may be inactive in the chicken. This is unlikely because quail and White Crowned sparrow GnIH are equally effective in depressing plasma LH in the White Crowned sparrow (Osugi et al., 2004) and the predicted sequence of White Crowned sparrow GnIH (SIKPSNLPLRF-NH$_2$) differs from quail GnIH at positions 5, 6 and 7 (Osugi et al., 2004). It therefore appears that the key sequence needed for the depressive effect of GnIH on plasma LH is the terminal LPLRF-NH$_2$, which is conserved in the chicken GnIH sequence. In support of this view, GnIH and related GnIH peptides, as well as the mammalian and amphibian GnIH orthologues, which contain the LPLXF-NH$_2$ motif, bind to the GnIH receptor with high affinity (Yin et al., 2005). On the other hand, LPLRF-OH or other amidated neuropeptides that lack the LPLXF motif, such as galanin and neuropeptide Y, fail to bind (Yin et al., 2005). For this reason it is predicted that quail GnIH should be biologically active in the hen.

It is possible that GnIH secretion into the hypophysial portal vasculature is pulsatile as has been demonstrated for mGnRH-I and is required for continued pituitary gonadotrophin responsiveness (see section 1.3.3). Therefore, it is possible that the 120 minute exposure of GnIH to incubating and laying hen pituitary fragments may have caused gonadotroph desensitisation which
may explain the lack of GnIH effect on gonadotrophin mRNAs and gonadotrophin secretion (Fig 5.8). However, this is not consistent with the observation that exposure of cockerel pituitary fragments to GnIH for 120 minutes suppresses gonadotroph function (chapter 7). The fact that GnIH inhibits in vitro LH secretion in adult cockerels and male quail (Tsustui et al., 2000) but not in laying or incubating hen pituitary glands could be due to a sex difference in the mode of GnIH action. This is possible as, by analogy, the sex difference in anterior pituitary growth hormone (GH) release in rats is ascribed to the asynchronous release and differential control of GH-releasing hormone and somatostatin into the hypophyseal portal vasculature (Plotsky and Vale, 1985; Argente et al., 1991). Another reason for the absence of an effect of GnIH on gonadotroph function in the hen may be an inappropriate GnIH concentration (1 x 10^{-7} M) was used, although the same concentration significantly reduces LH secretion from male Japanese quail pituitary glands (Tsutsui et al., 2000) and cockerel pituitary fragments (chapter 7) in vitro.

In the incubating hen, the increase in hypothalamic GnIH mRNA is also associated with a voluntary reduction in food intake (Zadworny et al., 1988) resulting in a decrease in body weight (Table 5.1). Therefore, GnIH may play a role in modulating appetite in incubating hens. This view is supported by the finding that RFamide peptides are involved in the control of feeding behaviour in a wide range of species, including coelenterates, molluscs and mammals (Dockray, 2004). Further, it is well established that neuropeptides, such as NPY, that control feeding also have effects on reproductive axis (Kuenzel and Fraley, 1995). Taken together there is a
potential role for avian GnIH in controlling feeding behaviour, which may interface with the reproductive axis.

In contrast to an increase in GnIH neuronal function in incubating hens, chGnRH-I mRNA was reduced. This reduction in chGnRH-I mRNA is likely to result in reduced chGnRH-I release, although a reduction in GnRH-I peptide is not consistently seen in chickens (Burke et al., 1990; Dunn et al., 1996) and turkeys (El Halawani et al., 1993; Rozenboim et al., 1993; Millam et al., 1989).

It has been suggested that decreased chGnRH-I neuronal activity in the incubating hen is a result either of increased prolactin or of a direct inhibitory neuronal input from the brood patch to chGnRH-I neurones (Sharp, 2004). A further mechanism is suggested by the observation in the house sparrow (Passer domesticus) that GnIH fibers come into close contact with, and may synapse on, chGnRH-I neurones and fibers. Consequently, Bentley et al. (2003) suggested that GnIH could be acting at the level of the hypothalamus to indirectly inhibit GnRH release as well as acting directly at the level of the pituitary gland.

A decrease in chGnRH-I release into the hypophysial portal vasculature in incubating hens may adequately explain decreased plasma LH without evoking a role for GnIH. This view is supported by the finding that injections of chGnRH-I increase LH release in incubating hens (Sharp and Lea, 1981) and demonstrates that decreased plasma LH is not due to a loss of anterior pituitary responsiveness to chGnRH-I. Associated with a reduction in plasma LH in the incubating hen is a decrease in LHβ subunit mRNA. The reduction in LHβ subunit mRNA has also
been observed in the incubating turkey hen (Wong et al. 1992). The reduction in LHβ mRNA in the incubating hen could be ascribed to a reduction in GnRH-I release from the median eminence or due to an inhibitory effect of increased plasma prolactin at the level of the anterior pituitary gland as has been demonstrated in the turkey (You et al., 1995). A reduction in α-subunit mRNA in incubating hens has not been reported previously but is consistent with the observation in ageing broilers where ovarian regression is correlated with a decrease in α-subunit mRNA (chapter 3).

The finding that plasma FSH increases after 6.5 hours after nest deprivation in incubating hens (Fig 5.3) has not been reported previously. The rise in plasma FSH follows the more rapid increase in LH and supports the idea that, in birds, the secretion of gonadotrophins are differentially regulated (Hattori et al., 1986). A direct stimulatory effect on chGnRH-I followed secondarily by increased chGnRH-I gene transcription (Dunn et al., 1996) stimulates the release of stored pituitary LH while FSH secretion lags because of the time for chGnRH-I to stimulate FSHβ mRNA and constitutive FSH release (McNeilly et al., 2003). However, there was no change in FSHβ mRNA (Fig 5.5) nor plasma FSH (Fig 5.2, 5.3) in incubating and laying hens. A possible reason for this is that the effect of chGnRH-I on FSHβ mRNA and plasma FSH in the nest-deprived hen is negated by increased oestrogen secretion from the reactivated ovary as laying is resumed. In support of this view, oestrogen has a greater inhibitory effect on FSH than LH. For example, pituitary FSH content is more sensitive to oestrogen than LH in both birds (Dunn et al., 2003).
and mammals (Miller et al., 1986). Taking into account a reduction in ovarian weight (Table 5.1) and plasma oestrogen in incubating hens (Bedrak et al., 1981) it is possible that FSHβ mRNA and plasma FSH is not decreased in incubating hens because the reduction in stimulatory hypothalamic chGnRH-I is counterbalanced by a loss of the inhibitory feedback effect of ovarian oestrogen to the pituitary gland. A reduction in ovarian inhibin could also explain why plasma FSH or FSHβ mRNA is not decreased in the incubating hen. There is an indication that plasma FSH increases during the first 4 days of incubation (Fig 5.3a) and this may be mediated by the regression of the ovary and a reduction in the secretion of ovarian hormones. However, in vitro chGnRH-I failed to increase FSHβ or α-subunit mRNAs from cultured incubating hen pituitary fragments (Fig 5.9). A reason for this is could be that the gonadotrophs had become desensitised to chGnRH-I during the 120 minute incubation. This is possible as the internalisation rate of the chicken GnRH type I receptor is 12 fold more rapid than the human GnRH type I receptor (Pawson et al., 1998) and this could be why α-subunit mRNA was not increased. The lack of a stimulatory chGnRH-I effect on gonadotrophin subunit mRNAs suggests that a pulsatile mode of chGnRH-I release is important in maintaining gonadotroph responsiveness. Furthermore, FSHβ mRNA was lower in incubating hen pituitary fragments exposed to 1 x 10^-8 and 1 x 10^-7 M compared to control values. Statistical analysis of 1 x 10^-7 M GnRH-I exposure and control FSHβ values alone shows that this is almost significant (p= 0.057). This counterintuitive finding could be explained by an increase in chGnRH-I stimulated follistatin protein. In rats, high
pulse frequency or continuous mGnRH-I has major stimulatory effects on follistatin mRNA (Besecke et al., 1990). Follistatin has been implicated in the intrapituitary inhibition of FSHβ mRNA (Attardi and Winters, 1993) and constitutive release presumably by its ability to neutralise activin protein (chapter 1, section 1.5; Bilezikijian et al., 1993a).

In conclusion, this study provides evidence that induced ovarian regression and cessation of egg laying in the incubating hen is associated with decreased pituitary common α and LHβ mRNAs associated with decreased plasma LH. This cascade of reduced neuroendocrine gene expression is can be ascribed to a reduction in chGnRH-I and an increase in GnIH release into the hypophysial portal vasculature. The observation that FSHβ mRNA and plasma FSH does not change between incubating and laying hens may be a consequence of reduced secretion of ovarian hormones such as oestrogen and inhibin from the regressed ovary of the incubating hen that negates the reduction in chGnRH-I release.
CHAPTER 6. Effects of inhibiting egg laying by decreasing daylength on reproductive neuroendocrine mRNAs

6.1 Introduction

The reproductive system in the domestic chicken is responsive to daylength (Dunn and Sharp, 1990) and ovarian regression and cessation of egg laying can be induced by reducing daylength (Sharp et al., 1992). An understanding of the neuroendocrine changes associated with photoinduced ovarian regression may contribute to increasing understanding of the neuroendocrine mechanisms underlying reduced ovarian function in ageing broiler breeders.

The induction of ovarian regression in the chicken by reducing daylength depends, in part, on the duration of prior exposure to long days and age (Sharp et al., 1992; Dunn and Sharp, 1992). In the absence of baseline information on the inhibitory effects of reducing daylength on ovarian function in broiler breeders it was considered preferable to investigate the effect of short day inhibition on reproductive neuroendocrine function in female Japanese quail since much more information is available for this species (Follett and Pearce-Kelly, 1990).

An increase in photoperiod results in increased hypothalamic GnRH-I mRNA in the chicken (Dunn and Sharp, 1999) and the quail (Baines, 2001) but, there is no information available on the effects of reducing photoperiod on GnRH-I mRNA. There is limited information on changes in gonadotrophin subunit gene expression associated with changes in photoperiod in birds. An increase in pituitary LHβ
subunit mRNA is associated with photostimulation in male white-crowned sparrows (Kubokawa et al., 1994) while in male Japanese quail FSHβ subunit mRNA pituitary content is higher during the breeding than the non-breeding season (Kikuchi et al., 1998). Transfer of breeding male Japanese quail from long to short days results in a decrease in testicular weight and plasma LH and in a reduction in α-subunit mRNA with no change in LHβ mRNA (Kobayashi et al., 2004).

The objective of the research presented in this chapter was to investigate in Japanese quail the effects of photo-induced ovarian regression on reproductive neuroendocrine genes encoding hypothalamic quail (q)GnRH-I mRNA and pituitary gonadotrophin subunit mRNAs. The experiment was carried out as a collaborative study with a scientist visiting the laboratory (R. M. Karlsson) and Drs T. Boswell and I. C. Dunn.

It was hypothesised that transfer to short daylengths would inhibit the reproductive axis by suppressing gonadotrophin subunit mRNAs as a consequence of reduced qGnRH-I release and qGnRH-I mRNA.

6.2 Experimental design

Female Japanese quail were transferred from short days (8L:16D) to long days (18L:6D) for 8 weeks to induce full ovarian development. The experimental design is summarised in Fig 6.1. Sixteen birds were transferred to short days (8L:16D) for 5 weeks (LSS) and eight of these birds were transferred back to 18L:6D for 3 days.
(LSL). A further sixteen females were retained on long day lengths for 5 weeks, and eight of these females were transferred to short days for 1 week prior to the end of the experimental period (LLS). A group of eight control quail remained on a long daylength for the entire experiment (LLL).

**Figure 6.1** Experimental design used to investigate in female quail the effects of inducing ovarian regression by decreasing photoperiod and the expression of reproductive neuroendocrine genes.

### 6.3 Body, pituitary weight and ovarian morphology

After exposure to long days for 13 weeks, LLL females had the greatest ovarian weights and larger number of yellow yolky follicles (YYF) of all experimental groups. Compared with other groups in the experiment the LSS females transferred from long days to short days for five weeks had the smallest ovarian weight and no
YYF. The LLS group had the second heaviest ovaries, whereas the LSL group had the second lightest ovary. Follicle number reflected differences in ovarian weights between experimental groups. Body weight showed a positive correlation with ovarian weight ($p < 0.001, r^2 = 0.68$). Pituitary gland weights were the same in all groups of birds irrespective of photoperiodic treatment.

Table 6.1 Body, pituitary weight and ovarian morphology in female Japanese quail exposed to the combinations of long (L, 18L:6D) and short (S, 8L:16D) days (n=8) shown in figure 6.1.

<table>
<thead>
<tr>
<th></th>
<th>LSS (n=8)</th>
<th>LLS (n=8)</th>
<th>LSL (n=8)</th>
<th>LLL (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>123.7 ± 2.5$^a$</td>
<td>136.7 ± 5.4$^{b,c}$</td>
<td>130.9 ± 4.4$^{a,c}$</td>
<td>145.5 ± 3.9$^b$</td>
</tr>
<tr>
<td>Pituitary (mg)</td>
<td>1.3 ± 0.13$^a$</td>
<td>1.7 ± 0.18$^a$</td>
<td>1.8 ± 0.12$^a$</td>
<td>1.6 ± 0.07$^a$</td>
</tr>
<tr>
<td>Ovary (mg)</td>
<td>87.4 ± 15.4$^d$</td>
<td>3780.3 ± 784.8$^e$</td>
<td>800.3 ± 544$^d$</td>
<td>5589.6 ± 472$^f$</td>
</tr>
<tr>
<td>YYF Follicles</td>
<td>0$^d$</td>
<td>2.6 ± 0.3$^e$</td>
<td>0.25 ± 0.2$^d$</td>
<td>3.6 ± 0.1$^f$</td>
</tr>
</tbody>
</table>

Means within row followed by different superscripts are significantly different by analysis of variance and least-significant differences. $a$ vs. $b$, $b$ vs. $c$; $p < 0.01$, $d$ vs. $e$, $e$ vs. $f$, $e$ vs. $f$; $p < 0.001$. YYF = Yellow Yolky Follicles. Raw data collected by R-M. Karlsson, I. C. Dunn and T. Boswell.

6.4 Plasma LH and FSH

Only plasma LH was measured since it was established that the Krishnan FSH radioimmunoassay (see chapter 2, section 2.4.3) did not cross react with quail plasma FSH (Fig 6.2).
Plasma LH was depressed after transfer to short days for 5 weeks (LSS), but not for 1 week (Fig 6.3). Plasma LH increased within 3 days of transfer from short to long days (LSL) to values which were not significantly different from long day control birds (LLL) (Fig 6.3).
6.5 Gonadotrophin subunit mRNAs

When developing QC RT-PCR assays for chicken gonadotrophin subunits (section 2.7) it was established that the assays were suitable for the quantification of quail gonadotrophin subunit cDNAs. This was predicated because quail and chicken gonadotrophin subunit mRNA sequences are >90% homologous (Ishii, 1993; Shen and You, 2002).

The pituitary contents of the three gonadotrophin subunit mRNAs were lower in quail transferred to short days for 5 weeks (LSS) than in the long day control (LLL) group (Fig 6.4). Transfer of short day quail to 3 long days (LSL) increased common
α and FSHβ subunit mRNAs, but not LHβ mRNA compared to short day control birds (LSS). The pituitary contents of LHβ and FSHβ mRNAs but not α-subunit mRNAs were higher in females transferred from long to short days for 1 week (LLS) than in females transferred to short days for 5 weeks (LSS) (Fig 6.4).

There was no difference between FSHβ or α-subunit mRNAs between females transferred from short to long days for 12 weeks (LLL) or 3 days (LSL) while LHβ mRNA was higher in females transferred from short days to 12 weeks long days (LLL) and females transferred from long days to 5 weeks short days (LLS).

Figure 6.4 Comparison of pituitary content of gonadotrophin subunit mRNAs between adult female Japanese quail after exposure to various combinations of long (18L:6D) and short (8L:16D) days described in Fig 6.1. a vs. b; p < 0.05, c vs. b; p < 0.05, d vs. e; p < 0.005, g vs. f; p < 0.001. b vs. c p < 0.001, d vs. e; p < 0.001, e vs. f; p < 0.001. n = 8. One way ANOVA and least significant difference analyses was performed on log transformed data.
Regression analysis of LHβ or α- subunit mRNAs on plasma LH showed that α-subunit mRNA was significantly correlated with plasma LH accounting for 48% of the observed variation in circulating plasma LH (Fig 6.5a). Plasma LH was not significantly correlated with LHβ mRNA (Fig 6.5b).

![Figure 6.5](image.png)

**Figure 6.5** Regression analysis of a), plasma LH and pituitary α-subunit mRNA content and b), plasma LH and pituitary LHβ mRNA content. Data was log transformed. Statistics shown in graph.
6.6 Hypothalamic GnRH-I mRNA

Hypothalamic qGnRH-I mRNA was significantly lower in females transferred from long days for 5 weeks (LSS) but not for 1 week (LLS) short days than in the long day control group (LLL) (Fig 6.7). Hypothalamic GnRH-I mRNA in quail transferred from short days to 3 long days (LSL) was not significantly different from hypothalamic qGnRH-I mRNA in control quail transferred from short days to 12 weeks of long days (LLL) (Fig 6.7).

![Graph comparing hypothalamic GnRH-I mRNA content](image)

**Figure 6.7** Comparison of hypothalamic GnRH-I mRNA content in adult female Japanese quail after exposure to various combinations of long (18L:6D) and short (8L:16D) days as described in Fig 6.1. Different letters above bars indicate a statistically significant difference between groups, p < 0.001, by least significant difference of one way ANOVA after logarithmic transformation of experimental data. Raw data provided by R-M. Karlsson.
6.7 Discussion

This study, in laying Japanese quail, established the correlated effects of decreasing daylength on plasma LH, gonadotrophin subunit mRNAs, qGnRH-I gene expression and ovarian regression. The reduction in ovarian weight and a loss of YYFs induced by prolonged exposure to short days (LSS) was correlated with decreased hypothalamic qGnRH-I mRNA indicating that a withdrawal of stimulatory long daylengths reduces qGnRH-I neuronal activity and qGnRH-I release from the median eminence. As a consequence of this reduction in qGnRH-I release, pituitary content of LHβ, FSHβ and α-subunit mRNAs was also reduced which in turn, resulted in a reduction in plasma LH secretion. In the pituitary, α-subunit mRNA but not LHβ mRNA content was significantly correlated with plasma LH, and accounted for 48% of the observed variation in plasma LH (Fig 6.5). The finding that pituitary content of α-subunit mRNA was a better predictor of plasma LH than LHβ mRNA in quail transferred to an inhibitory photoperiod confirms a similar study in male Japanese quail (Kobayashi et al., 2004). The positive correlation between α-subunit mRNA and LH secretion was particularly clear in quail transferred from short to 3 long days (LSL). This resulted either in an increase in hypothalamic qGnRH-I mRNA and, subsequently an increase in GnRH-I release from the median eminence, or an increase in qGnRH-I release which triggered an increase in qGnRH-I mRNA. This increase in qGnRH-I function resulted in the increase in pituitary α-subunit mRNA but not LHβ mRNA. The increase in qGnRH-I and α-subunit mRNAs was associated with increased plasma LH and suggests that GnRH-I in birds stimulates α-
subunit biosynthesis and LH secretion but not LHβ subunit biosynthesis suggesting that α-subunit mRNA could be a rate limiting step in LH release in quail. This view is supported by observations in male Japanese quail (Kobayashi et al., 2004; Kobayashi and Ishii, 2002; Kobayashi et al., 2002). Transfer from long to short days for 5 weeks reduced qGnRH-I mRNA and, in addition to reducing LHβ and α-subunit mRNAs also reduced FSHβ mRNA. This observation suggests that qGnRH-I, at least in part, stimulates FSHβ transcription or mRNA stability that may be relevant in increasing constitutive FSH release, which has been shown to be a major component of FSH secretion in Japanese quail (Hattori et al., 1986). Transfer from short to three long days increased qGnRH-I and FSHβ mRNAs providing further evidence that qGnRH-I, at least in part, controls FSHβ mRNA.

In conclusion, the reduced function of the quail hypothalamic-pituitary-ovarian axis induced by reducing daylength was the result of a reduction in mRNAs encoding hypothalamic qGnRH-I and pituitary gonadotrophin subunits, leading to decreased plasma LH and presumably plasma FSH associated with ovarian regression. The photo-induced ovarian regression was related to decreased qGnRH-I mRNA, but this is only seen after prolonged exposure to short days. A significant decrease in ovarian YYFs is seen after only 3 days of transfer to short days and this was not associated with a reduction in either qGnRH-I mRNA or plasma LH but was associated with a decrease in FSHβ mRNA. This transient state during the onset of ovarian regression in response to a short period of exposure to short days may be similar to the reproductive neuroendocrine status of ageing broilers where ovarian regression was
not associated with decreased chGnRH-I mRNA but was associated with decreased plasma FSH. It is therefore possible that a reduction in plasma FSH is the initial key factor responsible for reduced ovarian function in ageing broiler breeders similar to that in laying quail transferred to short days.
Chapter 7. Effects of GnRH-I, GnRH-II and GnIH *in vitro* on gonadotrophin subunit mRNAs and gonadotrophin release

7.1 Introduction

The objective of the experiments described in this chapter was to determine whether changes in pituitary gonadotrophin subunit gene expression observed in the ageing broiler breeder hen could be accounted for by changes in chGnRH-I, GnRH-II and/or GnIH secretion. An *in vitro* approach to studying pituitary gonadotrophin gene expression has the advantage of isolating the anterior pituitary gland from circulating inhibitory or stimulatory feedback effects on gonadotroph function (Chapter 1 section 1.4.1).

It remains unclear whether GnRH-II plays a physiological role in controlling gonadotrophin release in either mammals or birds (chapter 1, section 1.3.1). Nonetheless, GnRH-II is more potent than GnRH-I in stimulating LH release in the hen (Sharp et al, 1987; Wilson et al, 1989). Furthermore, GnRH-II is reported to be more than ten fold more potent than GnRH-I in stimulating chicken FSH release *in vitro* (Millar *et al*., 1986) although other studies found no effect of GnRH-I on FSH secretion *in vivo* (Dunn *et al* 2003; Bruggeman *et al*., 1998; Krishan *et al*., 1993).

Although chGnRH-I may not stimulate FSH release directly it may do so indirectly by stimulating FSHβ synthesis resulting in a constitutive release of FSH. This possibility was first suggested by studies in the Japanese quail where it was found that the amount of FSH released from pituitary glands cultured with hypothalamic extract was
three times more than the original content (Hattori et al., 1986). The factors that control FSH synthesis in birds have not been extensively investigated. In rats, FSH synthesis is controlled by interactions between mGnRH-I and a pituitary activin/follistatin paracrine loop (Chapter 1, section 1.4) although this intra-pituitary mechanism has not been investigated in birds.

A pulsatile pattern of exposure to chGnRH-I in vitro was chosen for the experiments presented in this chapter because, first, the activin/follistatin loop in the rat is mGnRH-I pulse frequency dependent (Besecke et al., 1996) and, secondly, prolonged incubation in the presence of chGnRH-I does not stimulate gonadotrophin subunit mRNAs in pituitary fragments of incubating hens (chapter 5, section 5.2.4). This lack of response to chGnRH-I may be due to the desensitization of the chicken gonadotroph to chGnRH-I that is more rapid than observed in mammals (Pawson et al., 1998).

In addition to a regulatory role for chGnRH-I on gonadotrophin secretion, the novel RFamide neuropeptide, gonadotrophin inhibitory hormone (GnIH, Tsustui et al., 2000) may also act on the anterior pituitary gland to depress gonadotrophin subunit gene expression and secretion; this is possible as GnIH suppresses in vitro LH release from the male Japanese quail (Tsustui et al., 2000).

The objective of the research presented in this chapter was to test the hypothesis that pulsatile chGnRH-I stimulates LH release from cultured adult cockerel pituitary fragments and is associated with increased gonadotrophin LHβ and α-subunit mRNAs. Pulsatile chGnRH-I is also hypothesised to effect follistatin and activin βB mRNAs from cultured adult cockerel pituitary fragments that would have effects on FSHβ mRNA. Taking into consideration the more potent effect of GnRH-II than
chGnRH-I on LH release (Sharp et al., 1897), it was predicted that GnRH-II may
stimulate gonadotrophin subunit mRNAs to a greater extent than chGnRH-I. A
second objective of the research presented in this chapter was to determine whether
GnIH suppresses constitutive gonadotrophin secretion from cultured adult cockerel
pituitary fragments by reducing gonadotrophin subunit mRNAs.

**7.2 Effects of pulsatile GnRH-I using adult cockerel pituitary fragments**

The aim of this experiment was to determine, *in vitro*, whether changes in pituitary
gene expression observed in ageing (chapter 3, section 3.4) and *ad libitum* fed
(chapter 4, section 4.4) broiler breeder hens can be accounted for by exposure to a
pulsatile pattern of chGnRH-I. Pituitary glands were dissected from cockerels and
pooled in chilled phosphate buffered saline. They were then diced and distributed
equally into a 6 x 4 array of plastic cups (1 mm diameter, 2 mm deep, 2-3
fragments/cup) with nylon mesh bottoms which fitted into a 24 well cell culture plate
(Corning Costa, Amsterdam, Netherlands). The nylon mesh allowed the pituitary
gland fragments to be exposed to culture medium alone or medium containing
chicken GnRH-I (Try-His-Trp-Ser-Try-Gly-Leu-Gln-Pro-Gly-NH₂, Bachem, St.
Helens, UK) to a stimulated pulsatile pattern of exposure to GnRH-I. The
experimental conditions were as described in chapter 2 section 2.10. Pituitary
fragments were transferred from medium alone to medium containing 1 x 10⁻⁷ M
chicken GnRH-I or vehicle, for five minutes every 120 minutes according to a Latin
square design. After a 5 minute exposure to GnRH-I, the pituitary gland fragments were re-equilibrated with 95% O₂ and 5% CO₂ and fresh media alone until the next pulse treatment. The times and dose chosen for GnRH treatments were based on the observations that pulses of LH occur about every 90 minutes in adult chickens (Wilson and Sharp, 1975) and that pituitary cell responsiveness to GnRH-I is maintained in dispersed chicken pituitary cell preparations perifused with 1 x 10⁻⁷ M GnRH-I at 5 minute intervals (King et al., 1986). At the end of the experimental period pituitary fragments were harvested, snap frozen in liquid nitrogen and stored at -80°C before RNA extraction and media was stored at -20°C before LH assays.

Exposure of cultured pituitary cell fragments to 5 minute pulses of 1 x 10⁻⁷ M GnRH-I at a 90 minute frequency for 24 hours increased α-subunit and follistatin mRNAs, but did not affect FSHβ, LHβ nor activin β₃ mRNAs (Fig 7.1).
Chapter 7  
Studies in vitro

Figure 7.1 Effect of in vitro exposure to 16 x 5 minute pulses of 1 x 10^{-7} M GnRH for 24 hours on a) gonadotrophin subunit and b) follistatin and activin B mRNAs in adult cockerel pituitary fragments. Values are mean ± SEM. ANOVA was performed on log transformed data. *p <0.05, **p <0.01 compared to controls.

The effect of GnRH-I on LH release became less pronounced during the course of the experiment until LH within the media was undetectable in the incubation media of the last five GnRH-I pulses (Fig 7.2).
Figure 7.2 The effect of in vitro exposure of cockerel pituitary fragments to 16 x 5 minute pulses of $1 \times 10^{-7}$ M GnRH-I for 24 hours on LH release in adult cockerel pituitary fragments. Values are mean ± SEM.

7.3 Effects of pulsatile GnRH-I and GnRH-II using broiler breeder hen pituitary fragments

The objective of this experiment was to compare the effects of chGnRH-I and GnRH-II administrated in a pulsatile mode on gonadotrophin subunit mRNAs in laying, food restricted broiler breeder hens. Hens were 45 weeks old at the time of the experiment and were culled in the evening to avoid the descending slope of the preovulatory surge of LH (Sharp, 1983) that is known to be associated with a
desensitisation of the anterior pituitary gland to GnRH. The experimental design was as described in section 7.2.1.

Both chGnRH-I and GnRH-II administered in a pulsatile manner significantly increased gonadotrophin α-subunit mRNA (Fig 7.3a) without effecting LHβ subunit mRNA (Fig 7.3c). Pulsatile chGnRH-I, but not GnRH-II, stimulated FSHβ mRNA (Fig 7.3b), while GnRH-II, but not GnRH-I, stimulated follistatin mRNA (Fig 7.3d). Unfortunately activin βB mRNA levels were too low in this experiment to quantify accurately.

**Figure 7.3** Comparison of the in vitro effect of 16 x 5 minute pulses of 1 x 10^{-7} M GnRH-I and GnRH-II for 24 hours on a). α-subunit, b). FSHβ, c). LHβ and d). follistatin mRNAs in laying broiler breeder hen pituitary fragments. Values means ± SEM. Statistical analysis was performed on log transformed data using treatment and plate position as a factor in ANOVA. *p< 0.05; **p< 0.01; ***p< 0.005 compared to control values.
7.4 Calculation of half lives of gonadotrophin subunit mRNAs

An important factor to consider when evaluating the predicted inhibitory effect of GnIH on gonadotrophin subunit mRNAs in a short term pituitary gland culture, is the half lives of the mRNAs. The objective of this experiment was to measure the half life of each gonadotrophin subunit mRNA using the transcriptional inhibitor actinomycin D (Loflin et al., 1999). The experimental conditions were as described in chapter 2, section 2.10.1. Cockerel pituitary fragments were incubated without (control) or with 8μM actinomycin D (Sigma-Aldrich, Poole, Dorset) in a Latin square design and harvested for gonadotrophin subunit mRNA assay at 1, 3, 6 and 9 hours. Observations were made at each time point in triplicate. Estimation of gonadotrophin subunit mRNA half lives, degradation of each respective transcript was calculated by subtraction of the control values from the actinomycin D values to give the change in the concentration of mRNA due to degradation. The value for change in the concentration of mRNA was log₂-transformed and plotted against time. Half lives were calculated by taking the reciprocal of the slope of the regression between the change in the concentration of mRNA and time, since a change of 1 in log₂ transformed data equals a decrease in concentration of a half.

The half lives of the gonadotrophin α, FSHβ and LHβ subunit mRNAs were calculated to be respectively, 8.6, 4.1 and 13 hours although the error estimates are relatively large (Fig 7.4).
7.5 Effects of short term incubation with GnIH using adult cockerel pituitary fragments

The objective of this experiment was to confirm, using adult cockerel pituitary fragments, the observation that GnIH inhibits LH secretion from male quail pituitary glands incubated in vitro for 120 minutes (Tsutsui et al., 2000).
Pituitary glands were dissected from cockerels (n=8) and pooled into chilled phosphate buffered saline. The pituitaries were collectively diced into fragments and distributed randomly into the wells of 12 well cell culture plates, (Corning Costa, Amsterdam, Netherlands). The experimental conditions were as described in chapter 2, section 2.10.1. The culture medium was removed after a 90 minute pre-incubation and 1ml of fresh medium was added to each well containing GnIH peptide (1x10^{-7}, 1x10^{-6}, 1x10^{-5} log M) or no peptide (control) in a Latin square design. The culture plates were re-equilibrated with 95% O_2 and 5% CO_2 and replaced in the orbital incubator for a 120 minute incubation. Pituitary fragments were harvested and snap frozen in liquid nitrogen and stored at −80°C before RNA extraction. The experiment was replicated on a separate occasion. GnIH used in the experiments presented in this chapter was a kind gift from Prof. Kazu Tsutsui.

Incubation of cockerel pituitary fragments for 120 min with GnIH at doses of 1 x 10^{-7} and 1 x 10^{-6} M but not at 1 x 10^{-5} M depressed the concentration of common α subunit mRNA (Fig. 7.5a). GnIH had a similar depressive effect on FSHβ mRNA although this effect was seen with doses of 1 x 10^{-7} and 1 x 10^{-5} but not 1 x 10^{-6} M (Fig 7.5b). In contrast, GnIH did not depress LHβ subunit mRNA (Fig. 7.5c). GnIH inhibited both FSH and LH release into the incubation medium at the lowest but not the highest doses (Fig. 7.5d,e). The experiment was repeated, and the combined results are shown in Fig. 7.5.
Figure 7.5 Effects of incubating anterior pituitary gland fragments from adult cockerels for 120 minutes with GnIH, on concentrations of gonadotrophin subunit mRNAs and of LH and FSH in the incubation medium. The experiment was repeated and showed similar effects; the data from the two experiments were combined. Values are shown for a) common α subunit mRNA, b) FSH β subunit mRNA c) LH β subunit mRNA d) FSH concentration and e) LH concentration. Values are means ± SEM; n=15 where “n” refers to the number of pituitary fragments assayed. ANOVA was performed on log-transformed data followed by Student’s t-test to test for significance between means. *p < 0.05, **p < 0.01, ***p< 0.005 compared with control samples not incubated with GnIH.
7.6 Effects of long continuous incubation with GnIH using adult cockerel pituitary fragments

The objective of this study was to determine the effects of a prolonged continuous (24h) GnIH exposure on gonadotrophin subunit mRNAs and gonadotrophin secretion. The experimental design was as described in section 7.2.4 apart from the incubation period which was 24 hours and the concentrations of GnIH used, which were $1 \times 10^{-9}$, $1 \times 10^{-7}$, $1 \times 10^{-5}$ log M.

Incubation of cockerel pituitary fragments for 24 hours with GnIH failed to inhibit gonadotrophin sunbunit mRNAs (fig 7.5a-c). There was no effect on FSH or LH secretion of GnIH at concentrations $1 \times 10^{-9}$ and $1 \times 10^{-7}$ M, while at the highest dose ($1 \times 10^{-5}$), GnIH stimulated both FSH and LH release (Fig 8.5d).
Figure 7.6 Effects of incubating anterior pituitary gland fragments from adult cockerels for 24 hours with continuous GnIH, on concentrations of gonadotrophin subunit mRNAs and of LH and FSH in the incubation medium. Values are shown for a) common α-subunit mRNA, b) FSH β subunit mRNA, c) LH β subunit mRNA, d) FSH concentration, and e) LH concentration. Values are means ± SEM; n=15 where “n” refers to the number of pituitary fragments assayed. ANOVA was performed on log-transformed data followed by Student’s t-test to test for significance between means. *p <0.05, **p <0.01 compared with control samples not incubated with GnIH.

7.7 Effects of pulsatile GnIH using cockerel pituitary fragments

It is possible that GnIH is released from the median eminence in a pulsatile manner. The objective of this study was to determine the effects of pulsatile administration of GnIH on gonadotrophin subunit mRNAs in vitro. The experimental design was as described in section 7.2.1 except for the duration of the experiment which was 12
hours. Pituitary fragments were exposed to eight pulses of $1 \times 10^{-7}$ M GnIH or vehicle, for five minutes every 120 minutes according to a Latin square design.

Eight 5-minute pulses of GnIH at intervals 120 minutes had no effect on LH$\beta$, FSH$\beta$ nor $\alpha$-subunit mRNAs (Fig 7.7).

![Graph showing effect of in vitro exposure of 8 x 5 minute pulses of 1 x 10^{-7} M GnRH for 12 hours on gonadotrophin subunit mRNAs in adult cockerel pituitary fragments. Values are mean ± SEM. One-way ANOVA was performed on log transformed data.](image)

**Figure 7.7** Effect of in vitro exposure of 8 x 5 minute pulses of $1 \times 10^{-7}$ M GnRH for 12 hours on gonadotrophin subunit mRNAs in adult cockerel pituitary fragments. Values are mean ± SEM. One-way ANOVA was performed on log transformed data.
7.8 Discussion

LH release became progressively less pronounced during the time course of pulsatile chGnRH-I-stimulation of cockerel pituitary fragments until, at the end of the experimental period, LH secretion was undetectable (Fig 7.2). This gradual reduction in LH release may be due to a depletion of the readily releasable LH pool. In support of this view, the preovulatory surge of LH in the sheep, which is caused by increased GnRH release, is associated with the depletion of up to 80% of total pituitary LH content in sheep (Brooks et al., 1993) resulting from the secretion of most, if not all, intracellular LH (Crawford et al., 2000). In addition, there was evidence that chGnRH-I also increases the baseline secretion of LH in the cultured cockerel pituitary fragments that would accelerate the reduction of readily releasable LH. An alternative but not mutually exclusive explanation is that the reduction in LH release is due to a desensitisation of the pituitary gland to the mode of chGnRH-I presentation used in the current study. However, this loss of LH release during the experimental period can equally be ascribed to the pituitary cells becoming necrotic. Nonetheless, pulsatile chGnRH-I stimulated LH secretion was associated with an increase in α-subunit but not LHβ subunit mRNA using both cultured cockerel (Fig 7.1a) and broiler breeder hen (Fig 7.3) pituitary fragments. These observations are consistent with findings in ad libitum broiler breeder hens that an increase in chGnRH-I mRNA in vivo was associated with an increase in α-subunit mRNA and plasma LH but not LHβ mRNA (chapter 4, section 4.4.4 and 4.4.5). Both chGnRH-I and GnRH-II stimulated α-subunit mRNA from cultured laying broiler breeder hen pituitary
fragments (Fig 7.3a) with no difference in potency, it is possible that the more potent LH release by GnRH-II than chGnRH-I (Sharp et al., 1987) may be mediated at the post-transcriptional level perhaps involving greater mobilisation of intracellular Ca\(^{2+}\) stores within gonadotrophs to facilitate greater LH release (Liu et al., 1995).

In rats, the regulation of FSH\(\beta\) mRNA by mGnRH-I is mediated by a follistatin/activin paracrine loop within the anterior pituitary gland (Bilezikjian et al., 2004; Winters and Moore, 2004). The present study supports the view that chGnRH-I stimulates follistatin mRNA since pulsatile chGnRH-I (Fig 7.1b) or GnRH-II (Fig 7.3d) stimulated follistatin mRNA in cultured cockerel and broiler breeder hen pituitary fragments. It is possible that the mode of pulsatile GnRH used in the current experiment was equivalent to fast mGnRH-I pulses that has been found, in rats, to preferentially stimulate LH biosynthesis and secretion, rather than FSH biosynthesis (Dalkin et al., 1989; Kasier et al., 1997). If the rise in follistatin mRNA in cockerel pituitary fragments is a reflection of increased pituitary follistatin protein then an activin/follistatin paracrine loop may also be operational to control FSH release in birds. In support of this view activin \(\beta_3\) mRNA was almost significantly decreased (p= 0.075 on log transformed data; p= 0.057 on un-transformed data) in chGnRH-I treated cockerel pituitary cells (Fig 7.1b) possibly due to an increase in secreted follistatin protein. The chGnRH-I stimulated increase FSH\(\beta\) mRNA in broiler breeder hen pituitary fragments (Fig 7.3b) was not associated with a change in follistatin mRNA (Fig 7.3d). In contrast, GnRH-II stimulated follistatin mRNA but not FSH\(\beta\)
mRNA in the same experiment. These observations agree with the observations of Kirk et al. (1994) who suggested an increase in pituitary follistatin serves to delay the FSHβ mRNA responses to mGnRH-I rather than to prevent it.

In agreement with the finding in the quail (Tsutusi et al., 2000), in the cockerel, GnIH inhibited LH release from pituitary fragments in short term culture (Fig 7.5e) and, further, also inhibited FSH release (Fig. 7.5d). The inhibitory effect of GnIH on FSH release has not been previously reported. This depressive effect of GnIH on LH and FSH release was correlated with a depression in the concentrations of common α and FSHβ subunit, but not LHβ subunit mRNAs (Fig 7.5a-c). It remains to be established whether this suppressive effect of GnIH is a consequence of an inhibition of α and FSHβ subunit gene transcription, or of decreased stability of the gonadotrophin subunit mRNAs. The possibility that GnIH also depresses LHβ mRNA cannot be excluded because the half life of chicken LHβ mRNA was calculated to be 13 hours (Fig 7.4c) which is much longer than the 120 minutes in vitro experiment. Mammalian LHβ mRNA also has a long half life, 44 hours in the rat (Bouamoud et al., 1992), while the shorter, 4-8 hours half lives of common α and FSHβ subunit mRNAs calculated for the chicken (Figs. 7.4a, b) are similar to those reported for the corresponding mammalian mRNAs (Bouamoud et al., 1992; Attardi and Winters, 1993; Carroll et al., 1991b; Chedrese et al., 1994).

The rapid decay of the FSHβ transcript allows FSH secretion to be controlled at the level of FSHβ mRNA stability. The relatively short t½ of FSHβ mRNA can be ascribed to the transcripts long 3' untranslated region (UTR) that contains numerous AUUUA motifs in a number of species. Ovine (Moutford et al., 1989) and bovine
(Esch et al., 1986) FSHβ mRNAs contain 5 and 6 AUUUA motifs in their 3' UTR respectively, and in the chicken (fig 9.2) and quail (Kikuchi et al., 1998) 5 AUUUA motifs are present in the 3' UTR of the transcript. In contrast, only one or no AUUUA motif exists in the mammalian and avian α-subunit mRNA (accession number human: XM 01144; bovine: X00050; ovine: X16977; possum: AF004520; quail: S70833; turkey: M33698) and no such motifs are present in LHβ mRNA (accession number human: BC006290; bovine: M10077; quail S70834). Studies indicate that AUUUA pentamers in 3' UTR serve as a cis-acting element for the rapid degradation of mRNAs for proto-oncogenes, cytokines and lymphokines (Malter, 1989). In support of the view that nucleotide sequences within the 3' UTR of the FSHβ mRNA contributes to accelerated degradation, removal of the 3' UTR greatly increases the transcripts half life of both ovine FSHβ mRNA in vitro (Mountford et al., 1992) and in transgenic mice in vivo (Brown et al., 2001). Factors that are known to influence FSH release in mammals mediate their effects by modulating FSHβ mRNA. For example, in rats, activin, follistatin and inhibin effect FSH secretion by modulating FSHβ mRNA stability (Carrol et al., 1991a; Attardi and Winters, 1993).
Figure 7.8 3’ UTR region of the chicken FSHβ cDNA (accession number: NM 204257) highlighting a putative TGA stop codon and five ATTTA pentamers that may contribute to the transcript’s rapid degradation.

It is possible that the suppressive action of GnIH on LH release is mediated by more than one mechanism: the first appears to be a short-acting effect, as suggested by the observations of Osugi et al. (2004), while a second, long acting mechanism is suggested by the observations in vitro reported in this chapter. The long acting mechanism may involve an inhibitory effect of GnIH on LH release as a consequence of decreased LH synthesis associated with a decrease in constitutive LH release. The possibility that LH is released without concurrent GnRH stimulation has been
reported in mammals (McNeilly et al., 1991; Crawford et al., 2002) where it has been observed that gonadotrophin subunit mRNA levels are maintained for at least 30 hours after mGnRH-I deprivation (Mercer and Clarke, 1989). In birds a similar mechanism is suggested by the observation in the male turkey, that increased baseline plasma LH during sexual maturation is not associated with a change in GnRH pulse frequency or amplitude (Yang et al., 1998). It is relevant to note that baseline concentrations of LH in laying hens are not pulsatile, further suggesting constitutive release of LH in the chicken (Wilson and Sharp, 1975). If GnIH inhibited LH release \textit{in vitro} is a consequence of an inhibition of synthesis, LH synthesis would have been depressed within 120 minutes duration of the pituitary fragment incubation experiment. There is no information on the rate of LH synthesis in birds, but in mammals, synthesis to processing and packaging of a mature LH dimer takes 1.5 hours (Blomquist and Baenziger, 1992). This was within the time-frame of the incubation experiment and supports the view that GnIH may suppress LH release secondarily to a reduction in synthesis.

The lack of an effect of a high dose of GnIH on LH release can be ascribed to a desensitisation of the gonadotrophs to GnIH. By analogy, the phenomenon of desensitisation to GnRH-I has been established in the chicken (King et al., 1986, Sharp et al., 1986) and mammals (Weiss et al., 1990; Mercer and Clarke, 1989; Jinnah and Conn, 1986; Baird et al., 1986). GnIH also inhibited FSH release \textit{in vitro} (Fig 7.5d), which has not been previously reported although this was suggested by a non-significant inhibitory effect on FSH release \textit{in vitro} in the quail (Tsutsui et al., 2000). Further, there was no effect of a 24-hour continuous exposure of GnIH on
pituitary gonadotrophin subunit mRNAs (Fig 7.6a-c) while both FSH and LH were increased at the highest does of GnIH (Fig 7.6d,e). This lack of a suppressive effect of GnIH on gonadotrophin subunit mRNAs and LH and FSH release could be ascribed to homologues desensitisation of the GnIH receptor. The GnIH receptor has been cloned and has been found to be a member of the of G protein coupled receptor super family (Yin et al., 2005), and the phenomenon of rapid desensitisation is a characteristic feature of many G protein-coupled receptors (Krupnick and Benovic, 1998) including the chicken GnRH type I receptor (Pawson et al., 1998).

In conclusion, in vitro chGnRH-I and GnRH-II stimulates α-subunit mRNA but not LHβ subunit mRNA in cultured broiler breeder hen pituitary fragments. Conversely, in cultured cockerel pituitary fragments, the in vitro inhibition of LH release by GnIH is associated with a reduction α-subunit mRNA, but not LHβ subunit mRNA. Collectively, these findings demonstrate that pituitary α-subunit mRNA is a better indicator of LH secretion and, furthermore, may be rate limiting LH release. GnIH also inhibits FSHβ mRNA and FSH secretion and may play a role in inhibiting constitutive gonadotrophin release in birds. The chGnRH-I stimulated increase in follistatin mRNA provides evidence that an intra-pituitary mechanism controlling FSH release may be operational in poultry as it is in the rat.
8.1 Summary

The aim of this Thesis was to gain further understanding of the underlying neuroendocrine mechanisms controlling reproduction in poultry with particular focus on the ageing broiler breeder hen. Decreased egg production in old laying broiler breeder hens was found to be related to reduced concentrations of plasma FSH and LH. The studies presented in this Thesis explore the neuroendocrine mechanisms that might be responsible for this reduction in gonadotrophin secretion. It was demonstrated that FSH and LH biosynthesis in the chicken is stimulated by chGnRH-I and inhibited by GnIH as inferred from measurements of gonadotrophin subunit mRNAs and secreted gonadotrophins. It was further concluded that reduced gonadotrophin biosynthesis is a major factor determining the reduction in gonadotrophin secretion in ageing broiler breeder hens. In contrast to mammals where LHβ subunit biosynthesis is rate limiting for LH release (Gharib et al., 1990; Shupnik, 1996; Nilson et al., 1983), in the broiler hen pituitary α-subunit biosynthesis was found to be a rate limiting step in the regulation of LH secretion. Evidence was obtained to show that FSHβ subunit biosynthesis is a rate limiting factor for FSH secretion which, as in mammals is predominantly constitutive (Farnworth, 1995; Muyan et al., 1994). It was further demonstrated, through measurements of their mRNAs in the anterior pituitary gland, that there is likely to be a paracrine activin/follistain interaction which controls chicken pituitary FSH secretion similar to that reported in mammals (Winters and Moore, 2004; Bilezikjian
et al., 2004). Continuous exposure to chGnRH-I in vitro did not stimulate gonadotrophin subunit mRNAs. However, pituitary gonadotrophin subunits, activin βB and follistatin mRNAs were found to be responsive to chGnRH-I administered in a pulsatile mode. This observation provided the first evidence in the chicken that a pulsatile pattern of chGnRH-I exposure is important in modulating gonadotrophin subunit biosynthesis and in maintaining pituitary responsiveness to chGnRH-I.

8.2 Main conclusions

The main conclusions from this Thesis related to reproductive ageing in the broiler breeder hen are summarised in Figure 8.1.

Reduced ovarian follicular growth in old compared with young laying broiler breeder hens was assumed to be causally related to decreased persistency of lay and associated with reduced plasma LH and FSH. The reduction in plasma LH confirms previous studies in ageing chicken (Sharp et al., 1992) and turkey hens (Guemene and Williams, 1999), but the reduction in plasma FSH in ageing laying hens has not been previously reported. The reduction in plasma LH and FSH was associated with reduced pituitary α-subunit and LHβ but not FSHβ mRNAs (Fig 8.1a,b). It is therefore likely that reduced ovarian function in old laying hens is caused, at least in part, by a reduction in gonadotrophin biosynthesis which is a consequence of reduced α-subunit gene transcription or α-subunit mRNA stability.
Figure 8.1 Differences within the hypothalamic-gonadotroph axis of a). young laying, b). old laying and c). old out-of-lay broiler breeder hens, that may contribute to a reduction in persistency of egg production with age. The thickness of the lines with arrowheads indicates amounts of synthesis/output of mRNAs and hormones indicated respectively. Note that reduced persistency of lay in a flock is considered to be causally related to a combination of a reduction in numbers of large yellow yolky follicles shown in b) and complete ovarian regression shown in c).
The decrease in α-subunit mRNA in old laying hens is suggested to be due to a loss of pituitary sensitivity to chGnRH-I (Sharp et al., 1992) resulting from decreased chGnRH-I release. The decrease in chGnRH-I release is suggested to be reflected in a reduction in the frequency of the pulsatile release of the neuropeptide (Fig 8.1a and 8.1b,c). The proposed decrease in chGnRH-I release in ageing laying broiler breeders is unlikely to be initiated at the level of chGnRH-I gene transcription or mRNA stability as a decrease in chGnRH-I mRNA was not associated with reduced numbers of yellow olky follicles (YYFs) in old laying hens (chapter 3). It would appear that the proposed decrease in chGnRH-I release in old laying broilers is a consequence of a change in the hypothalamic mechanisms controlling chGnRH-I release rather than amounts of chGnRH-I mRNA.

Both LHβ and FSHβ mRNAs increase in association with the regression of the ovary in the old out-of-lay hen while α-subunit mRNA is decreased (Fig 8.1c). This indicates a lifting of the inhibitory feedback effects of ovarian hormones on gonadotrophin subunit biosynthesis. The decrease in plasma LH in old out-of-lay broilers is associated with decreased α-subunit but not LHβ subunit mRNAs suggesting that α-subunit synthesis may be a rate limiting step for LH secretion. The increase in FSHβ mRNA in old out-of-lay hens is correlated with an increase in plasma FSH and indicates that FSHβ subunit synthesis may be a rate limiting step for FSH release is predominantly constitutive. In addition, as inferred from an increase in follistatin mRNA, pituitary follistatin synthesis may increase with the regression of the reproductive system in the old broiler hen (Fig 8.1c). This suggests that
follistatin is also sensitive to ovarian hormone feedback and indicates that the function of the intra-pituitary follistatin/activin loop is altered when plasma FSH is in increased.

8.2.1 Role of GnRH-I mRNA in decreased ovarian function in old broiler breeders

The observation that chGnRH-I did not decrease as ovarian function declined with advancing age (chapter 3) suggests that chGnRH-I gene transcription or chGnRH-I mRNA stability is not actively inhibited when reproductive function decreases with advancing age. This view is supported by supplementary study where a reduction in YYFs in female Japanese quail transferred from long to short days was not associated with a reduction in qGnRH-I mRNA (Chapter 6). In contrast, chGnRH-I mRNA is rapidly reduced after the onset of incubation (chapter 5; Dunn et al., 1996), suggesting that in these birds chGnRH-I gene transcription or chGnRH-I mRNA stability is actively inhibited. The possibility that a similar mechanism may begin to operate when old laying hens stop laying seems unlikely because chGnRH-I mRNA is similar in old laying and out-of-lay hens. It therefore seems that unlike incubating hens, reduced gonadotrophin secretion in old laying hens may be a consequence of reduced stimulation of chGnRH-I release but not of chGnRH-I gene transcription or chGnRH-I mRNA stability.
It is possible that a reduction in processing of pre-proGnRH-I peptide could account for a reduction in gonadotrophin secretion in old laying hens, but this also seems unlikely as in an earlier study there was no difference in hypothalamic chGnRH-I peptide in young and old laying dwarf broiler breeder hens (Sharp et al., 1992). This view is supported by observations in old and young female rats in which no differences are observed in hypothalamic mGnRH-I peptide content, although reproductive function was depressed in older animals (Steger et al., 1979; Rubin et al., 1985). However, it can not be discounted that enzymatic processing and degradation is altered causing a decrease in the amount of stored chGnRH-I or GnIH peptides in their biologically active forms within the median eminence of the old laying hen. Available evidence on the rate of chGnRH-I peptide degradation in the chicken argues against this possibility, because degradation of the chGnRH-I peptide is higher in laying than in out-of-lay hens (Advis et al., 1985).

It is more likely that a reduction of chGnRH-I release in old laying hens accounts for the reduction in gonadotrophin release. In support of this view, in vitro GnRH-I release from old laying female Japanese quail hypothalamic slices is reduced compared to young laying females (Ottinger et al., 2004).
8.2.2 GnRH-I pulse frequency and decreased ovarian function in old broiler breeders

This Thesis provides evidence that pulsatile chGnRH-I presentation is required to stimulate pituitary α-subunit mRNA and to maintain pituitary responsiveness to chGnRH-I in the laying hen (chapter 7), since pulsatile chGnRH-I administered in vitro to pituitary glands stimulated gonadotrophin mRNA while continuous chGnRH-I exposure had no effect on gonadotrophin mRNAs (chapter 5).

A decrease in chGnRH-I release could be manifested in either a reduction in chGnRH-I pulse frequency or a reduction in chGnRH-I pulse amplitude or a combination of both. Changes in LH and FSH release as well as pituitary gonadotrophin subunit biosynthesis are partly dependent on changes in hypothalamic mGnRH-I pulse frequency in mammals (Chapter 1, section 1.x.x; Kasier et al., 1997; Dalkin et al., 1989) and mGnRH-I pulse frequency is reduced in both ageing women (Hall et al., 2000; Rossmanith et al., 1991) and female rats (Rubin and Bridges, 1989). A reduction in chGnRH-I pulse frequency in ageing laying broiler breeder hens explains, at least in part, reduced LH and FSH secretion. An age-related reduction in chGnRH-I pulse frequency in mammals is known to be associated with decreased LH and increased FSH secretion (Wildt et al., 1981; Kaiser et al., 1997; Jayes et al., 1997). It is likely that a similar mechanism operates in ageing broiler breeders. This prediction is consistent with the observation that in old laying broiler breeder hens plasma LH was decreased relative to young laying hens. However,
contrary to what might be predicated from the mammalian literature plasma FSH was decreased rather than increased. The hypothesis that FSH should increase in old broilers as a consequence of decreased GnRH-I pulse frequency is, however, consistent with the increase in plasma FSH seen in the old out-of-lay broiler breeder hen, compared to young or old laying hens. This increase in plasma FSH might be expected as a result of reduced ovarian function and a loss of ovarian feedback signals in old out-of-lay hens which normally inhibits FSH release.

8.2.3 Differential regulation of gonadotrophin secretion

This Thesis provides additional circumstantial evidence that changes in chGnRH-I pulse frequency may play a role in controlling the differential secretion of LH and FSH in broiler breeders. The increase in ovarian follicular growth observed after refeeding food restricted laying broiler breeder hens was associated with an increase in hypothalamic chGnRH-I mRNA (chapter 4) suggesting increased chGnRH-I neuronal activity. If this resulted in an increase in chGnRH-I pulse frequency or amplitude, the finding that plasma LH is higher while plasma FSH is lower in ad libitum than food restricted hens strengthens the view that increased chGnRH-I pulse frequency may play a role in controlling differential gonadotrophin secretion, with a high chGnRH-I pulse frequency preferentially stimulating LH release over FSH secretion. As mentioned above, this conclusion is inconsistent with the low concentrations of plasma FSH observed in old laying broilers, but if chGnRH-I pulse
frequency is decreased in these birds it is suggested that the potential to increase plasma FSH is negated by hormones secreted by yellow yolky follicles to suppress FSH secretion. This is most likely to be oestrogen, since oestrogen has highly inhibitory effects on hen FSH pituitary content and release (Dunn et al., 2003). It is concluded that the regression of the ovary in old out-of-lay broiler breeder hens results in a lifting of inhibitory feedback effects of oestrogen and consequently FSHβ mRNA and FSH release is stimulated, at least in part, by low chGnRH-I pulse frequency.

8.2.4 Role of pituitary follistatin, activin and ovarian inhibin in the regulation of FSH release in broiler breeder hens

A reduction in plasma inhibin in old out-of-lay hens may also account, in part, for the increase in FSHβ mRNA (Ahn et al., 2002) and constitutive FSH release. Evidence that ovarian inhibin secretion is reduced in the out-of-lay hen is suggested by the observation that pituitary follistatin mRNA increases as, in the rat, inhibin suppresses follistatin gene transcription (Prendergast et al., 2004) and a similar mechanism may be operational in the broiler hen. Inhibin is likely to reduce FSH secretion from the pituitary by interfering with activin receptor signaling (see chapter 1, section 1.5). Despite an increase in follistatin mRNA in out-of-lay hens (Fig 8.1c), which may have resulted in an increase in pituitary follistatin protein, free activin B protein would bind more efficiently to pituitary activin receptors as a consequence of
reduced circulating inhibin, resulting in increased FSHβ mRNA and FSH release (Fig 8.2).

Figure 8.2 Proposed intra-pituitary mechanism controlling FSH release in the laying and out-of-lay broiler breeder hen. a). In the laying broiler breeder hen circulating inhibin secreted by the ovary binds to activin receptor II (ActRII) facilitated by inhibin's affinity for betaglycans present on pituitary cells. This interferes with activin signaling and little activin stimulated FSH release occurs. b). In the out-of-lay state there is little, if any, circulating ovarian inhibin. This results in increased follistatin protein that binds activin and neutralises the molecules bioactivity. Remaining free activin that is not bound to follistatin can bind to activin receptor and stimulate second messenger systems that induce constitutive FSH release. See chapter 1, sections 1.5 and 1.6 for further details on this mechanism.

The proposed reduction in chGnRH-I pulse frequency in old broiler hens would be expected to reduce pituitary follistatin and increase pituitary activin (Beseck et al., 1996; chapter 1, section 1.5). This view is supported by the finding in vitro that
follistatin mRNA increase in pituitary gland fragments exposed to pulsatile chGnRH-I while activin βB mRNA was non-significantly lower (chapter 7). The observation that activin βB mRNA was not lowered significantly may be explained by the possibility that there is no direct relationship between activin βB mRNA and activin B protein. In contrast, there is good reason to suppose pituitary follistatin mRNA is directly related to pituitary follistatin content. For example, the increase seen in chGnRH-I mRNA after re-instating ad libitum feeding in broiler breeder hens was associated with increased pituitary follistatin mRNA and decreased plasma FSH while pituitary FSHβ and activin βB mRNA content remained unchanged. Furthermore, follistatin mRNA was increased in pituitary fragments exposed to pulsatile chGnRH-I while FSHβ mRNA remained unchanged. Conversely, in studies in vitro increased FSHβ mRNA observed in broiler hen pituitary gland fragments exposed to pulsatile chGnRH-I was not associated with a change in follistatin mRNA (chapter 7). If the increase in follistatin mRNA is a reflection of increased pituitary follistatin protein then follistatin may play a functional role in suppressing FSH function possibly by neutralising locally released activin, which is not tightly correlated with the pituitary content of activin βB mRNA.

8.2.5 The role of FSH in maintaining ovarian function in ageing broiler breeder hens

In this Thesis it was found that in the old out-of-lay broiler breeders and in the incubating hen there is an increase or no change in plasma FSH associated with the
regression of the ovary and cessation of egg laying. These observations are counterintuitive since increased or maintained plasma FSH might be expected to support or stimulate yellow yolky follicular growth. The fact that the ovary regresses even when plasma FSH is not depressed may be due a change in FSH isoform secretion (Chapter 1, section 1.2.1) and/or a reduction in the responsiveness of the ovary to FSH due to a reduction in ovarian FSH receptor.

Both increased mGnRH-I release and oestrogen stimulate the secretion of less acidic FSH isoforms with increased biological activity (Ulloa-Aguirre et al., 1995). It is suggested that a decrease in chGnRH-I release and plasma oestrogen in the incubating and old out-of-lay hens, may result in a shift in the balance of FSH isoforms with an increase in the secretion of the more acidic forms. These may be less biologically active and consequently may not be as effective in stimulating and maintaining ovarian development in incubating and old out-of-lay hens.

8.2.6 GnRH-I regulation of LHβ and α-subunit gene expression and plasma LH in broiler breeder hens

The reduction in plasma LH in old broiler breeder hens, which is suggested to be causally related to an age-related reduction in chGnRH-I pulse frequency, was correlated with reduced pituitary α-subunit mRNA but not LHβ mRNA. Conversely, increased plasma LH, induced by exposure to 3 long days in female quail (chapter 6)
or by *ad libitum* feeding after food restriction in broiler breeder hens (chapter 4) was associated with increased in hypothalamic GnRH-I gene expression and pituitary α-subunit, but not LHβ mRNAs. These observations highlight a difference in the response of GnRH-I neurone to inhibition and stimulation. While stimulation of GnRH-I release is associated acutely with increased GnRH-I mRNA, the inhibition of GnRH-I release is not associated with an acute decrease in GnRH-I mRNA. It appears that the inhibition and stimulation of the avian GnRH-I neurone is mechanistically distinct.

The data raise the possibility that in birds α-subunit biosynthesis is the rate limiting step for LH release. This conclusion is supported by studies *in vitro* using cockerel pituitary fragments, showing that pulsatile chGnRH-I stimulates LH release and α-subunit but not LHβ mRNAs (chapter 8). This finding is consistent with the findings using cultured turkey (Foster and Foster, 1991) and duck (Hsieh *et al.*, 2001) pituitary glands that chGnRH-I treatment elevated α-subunit mRNA, while, in the chicken, injections of chGnRH-I analogues had no effect on LHβ mRNA (Marsden *et al.*, 1994). Studies in the male Japanese quail also show that plasma LH is correlated with pituitary α-subunit but not LHβ mRNAs (Kobayashi *et al.*, 2002; Kobayashi and Ishii, 2002; Kobayashi *et al.*, 2004). This may reflect differences in the roles of pituitary glycoprotein α and LHβ subunit synthesis as limiting factors for LH secretion in mammals and birds. In mammals, the common α subunit protein is always in excess of the LHβ subunit protein (Shupnik, 1996; Nilson *et al.*, 1983) while in the rat, pituitary concentrations of common α-subunit mRNAs are 3- to 4
fold greater than levels of LHβ (Haisenleder et al., 1987, Papavasiliou et al., 1986).

The synthesis of gonadotrophin β subunit is therefore seen as the limiting factor for mammalian gonadotrophin synthesis (Gharib et al., 1990). In contrast, in the chicken the pituitary content of α- and β-subunit mRNAs of LH were expressed in equimolar amounts (e.g. Chapter 3, Figure 3.5a: pituitary content, 10^{-14} moles; Chapter 4, Fig 4.3a 10^{-13} moles). It is also possible that common α-subunit synthesis may be a rate limiting step for LH release in other lower vertebrates such as the African catfish (Clarias lazera) where salmon GnRH-I does not stimulate LHβ mRNA (Rebers et al., 2002). However, conclusions on the role α-subunit mRNA production as a rate limiting LH release in birds must be drawn with caution as other anterior pituitary glycoproteins, FSH and TSH, also include the α-subunit.

Furthermore, the mammalian pituitary secretes free α-subunit protein that can be stimulated by mGnRH-I (Weiss et al. 1990a), which is a better indicator of fast mGnRH-I pulse frequency than LH secretion (Hayes et al. 1999). Therefore, it appears, at the very least, that measurements of pituitary α-subunit mRNA are a better indicator than measurements of LHβ mRNA of the chGnRH-I stimulation of plasma LH and ovarian development.
8.2.7 Ovarian control of LHβ biosynthesis in ageing broiler breeder hens

Changes in plasma LH induced by experimental manipulation of reproductive condition in this Thesis were not associated with a change in LHβ mRNA. It is therefore surprising that a decreased plasma LH in old broiler hens was associated with a decrease in LHβ subunit mRNA. Since progesterone receptor is present in LH cells in laying hens (Gasc and Baulieu, 1988) prolonged exposure of the pituitary gland to increased concentrations of plasma progesterone from the largest F1-F3 follicles of the hen ovary during a laying year (Furr, 1973; Williams, 1977) may be responsible for this decrease in LHβ subunit mRNA. In support of this view, studies in sheep show that progesterone decreases the stability of LHβ mRNA by shortening of the poly-A tail (Crawford and McNeilly, 2002). Alternatively, progesterone may decrease LHβ subunit by inhibiting GnRH receptor as demonstrated in sheep (Sakurai et al., 1997). A similar mechanism in the broiler breeder hen, might be responsible for the reduction in gonadotroph sensitivity to chGnRH-I in ageing birds (Sharp et al., 1992). The increase in LHβ mRNA in old out-of-lay hens could be due to the lifting of these inhibitory effects of progesterone and was not associated with an increase in plasma LH. Reduced plasma LH in old out-of-lay birds is associated with decreased α-subunit mRNA (Fig 8.1c), which further supports the view that α-subunit mRNA is the rate limiting step in LH release in the broiler breeder hen.
8.2.8 Roles of GnlH in the loss of ovarian function in ageing broiler 
breeder hens

In addition to the possibility that a reduction chGnRH-I release contributes to the reduction in plasma LH in old laying broiler breeders, the novel RFamide, GnlH may also play a role by suppressing pituitary α-subunit mRNA and constitutive LH release. This suggestion is supported by the observation in vitro that the GnlH inhibition of LH release from cockerel pituitary fragments was associated with reduced α-subunit mRNA but not LHβ mRNA (chapter 7). It is therefore possible that GnlH release increases in old laying broilers causing a reduction in α-subunit mRNA and reduced constitutive LH release. This may explain why, in old laying broiler breeder hens, chGnRH-I injection is less effective in stimulating LH release than in young laying broilers (Sharp et al., 1992). A GnlH induced reduction in α-subunit mRNA in old laying hens may also explain a reduction in plasma FSH. However, if an increase in GnlH neuronal activity does contribute to the age-related loss of reproductive function in broiler breeder hens it is unlikely to involve a direct effect on GnlH gene expression or GnlH mRNA stability since hypothalamic GnlH mRNA concentrations were the same in young laying, old laying and old out-of-lay broiler breeders.
8.2.9 The development of photorefractoriness and age-related loss of ovarian function in broiler breeder hens

The reproductive system is one of the first body systems to show an age-related decline in function, with failing reproductive function in females evident in most species long before the end of the life span (Brann and Mahesh, 2005). There is a growing body of evidence to suggest that a hypothalamic defect underlies reproductive senescence (chapter 1, section 1.7). A defect in hypothalamic function in old laying broiler breeder hens may result in a decrease in chGnRH-I and/or an increase in GnIH release.

The age-related loss of reproductive function in broiler breeders can be ascribed to age per se, the development of photorefractoriness or the duration of the laying period a combination of all three phenomena. A photoperiodic study in broiler breeder hens indicates that age per se is more in addition to the development of photorefractoriness is responsible for reduced egg production at the end of a laying year (Dunn and Sharp, 1992) but a role for the development of photorefractoriness can not be discounted.

Reduced reproductive function in some photoperiodic birds, such as quail and chickens, exposed to long days for a prolonged period is due to the development of a form of photorefractoriness described as “relative” (chapter 1, section 1.10). This is thought to be due to a reduction in chGnRH-I release at the level of the median eminence rather than to a reduction in chGnRH-I biosynthesis (Dawson et al., 2001).
This view is supported by the observations in old laying hens where reduced plasma LH was not associated with reduced hypothalamic chGnRH-I mRNA (chapter 3) or chGnRH-I peptide content (Sharp et al., 1992). In addition to a decrease in chGnRH-I functional activity, an increase in GnIH release could equally be implicated in the development of photorefractoriness in old hens. In support of this view, GnIH-containing neurones are increased in seasonally breeding song sparrows at the termination of the breeding season (Bentley et al., 2003). If this occurs in the photorefractory old laying broiler breeder hen then an increase in GnIH neuronal activity may be mediated at the post-transcriptional level possibly by increasing the processing of pre-proGnIH protein or decreasing the degradation of GnIH, since the amount of hypothalamic GnIH mRNA did not increase in ageing broiler breeders.

The decrease in egg production at the end of a laying year in a broiler breeder flock may not be due to age per se but a consequence of the duration of the laying period, resulting in reproductive “exhaustion”. Prolonged exposure of the hypothalamic-gonadotroph axis to ovarian hormones may mediate this suggested mechanism since, in young female rats, chronic oestrogen exposure reduces mGnRH-I neuronal function and attenuates LH release similar to that observed in aged female rats (Tsai and Legan, 2002). Alternatively, it is possible that the sensitivity of hypothalamus and pituitary gland to the inhibitory effects of ovarian hormones such as progesterone may increase in ageing broiler breeder hens. Yellow yolky ovarian follicle increase in size as laying hens become older (Williams and Sharp, 1978), and since these follicles are the primary source of progesterone in hens progestrone may
contribute to reducing neuroendocrine reproductive function in ageing laying hens.

In support of this view, the progesterone induced pre-ovulatory LH surge is reduced in ageing hens (Williams and Sharp, 1978).

### 8.3 Future perspectives

This Thesis has generated several observations that require further investigation to fully understand their functional implications. To test the hypothesis that chGnRH-I, GnRH-II and GnIH pulse frequency controls gonadotrophin subunit mRNAs and LH and FSH release differentially, additional studies *in vitro* should be carried out using pituitary cells exposed to a wide range of chGnRH-I, GnRH-II and GnIH pulse amplitude and frequencies. Further work is needed to establish whether activin/follistatin are involved in the regulation of FSH secretion in the chicken. It remains to be shown that activin and follistatin protein are produced in the chicken pituitary gland and that activin and follistatin interact to control FSH secretion using cultured chicken pituitary cells. Studies *in vitro* should be extended to the use of RNAi technology to inhibit pituitary follistatin and activin βB gene expression in order to provide an insight into the role these intra-pituitary factors play in the regulation of FSHβ mRNA and FSH secretion. The novel finding that pituitary α-subunit mRNA rather than LHβ mRNA is correlated with LH release in several reproductive states in the chicken should be followed through with further work to establish that pituitary α-subunit protein content correlates with these observed
changes in α-subunit mRNA. A specific chicken antibody raised against pituitary α-
subunit protein, should be developed to facilitate this investigation. Finally, the
possibility that GnIH inhibits constitutive LH and FSH release should be confirmed
by immunising chickens against GnIH.
9. References


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References


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References


References


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Changes in reproductive neuroendocrine mRNAs with decreasing ovarian function in ageing hens

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Abstract

Egg production declines with advancing age in the domestic chicken and this is particularly pronounced in breeding stocks of meat type hens (broiler breeders). The objective of this study was to establish whether declining egg production with reproductive ageing in broiler breeders is correlated with plasma LH and FSH, and with mRNAs encoding hypothalamic gonadotrophin-releasing hormone-I (GnRH-I), gonadotrophin inhibitory hormone (GnIH), and gonadotrophin subunits. Comparisons were made between hens at the peak of egg laying (young: 30 weeks) and at the end of a laying year (old: 60 weeks). Old hens were subdivided into laying and out-of-lay groups. Plasma LH and FSH were lower in old than in young laying hens. Compared with old laying hens, old out-of-lay hens had significantly increased plasma FSH but not plasma LH. There were no differences in total hypothalamic GnRH-I and GnIH mRNAs between young and old hens. In old laying hens, the decrease in plasma LH was correlated with decreased gonadotrophin α-subunit but not LHβ mRNAs. The decrease in plasma FSH was not associated with a change in FSHβ mRNA. In old out-of-lay hens, the increase in plasma FSH was correlated with increased FSHβ mRNA, while unchanged plasma LH was associated with increased LHβ mRNA. A regression analysis of all plasma gonadotrophin and gonadotrophin subunit mRNA data collected from the study demonstrated that plasma LH is correlated with α-subunit but not LHβ mRNAs, while plasma FSH is correlated with FSHβ but not α-subunit mRNAs. It is concluded that the decrease in the rate of lay in ageing broiler breeders is not correlated with decreased GnRH-I mRNA nor with increased GnIH mRNA, but it is related to a decrease in α-subunit mRNA which may account for the associated reduction in plasma LH but not FSH.

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Keywords: GnRH-I; GnIH; Gonadotrophin subunits; LH; FSH; Reproductive ageing

1. Introduction

As the domestic hen becomes older, egg production progressively decreases and this loss of reproductive function is particularly pronounced in broiler breeder hens reared to produce chickens for meat consumption. Egg production is highest in broiler breeders at 5–7 months of age (Robinson et al., 1993; Sharp et al., 1992). The subsequent decrease in egg laying is associated with a reduced recruitment of ovarian follicles into the yellow-yolky follicular hierarchy (Palmer and Bahr, 1992; Waddington et al., 1985), decreased plasma and pituitary LH, and decreased LH responsiveness to GnRH (Sharp et al., 1992). Decreased plasma LH in old laying or out-of-lay hens is not associated with reduced hypothalamic GnRH-I peptide (Sharp et al., 1992). Similarly, decreased plasma LH in incubating hens with regressed ovaries is not associated with decreased GnRH peptide but is associated with decreased hypothalamic GnRH-I mRNA (Dunn et al., 1996), which suggests that the decrease in ovarian function in old laying hens could be mediated by a reduction in GnRH mRNA transcription and/or stability. The discovery of a putative avian gonadotrophin inhibitory hormone (GnIH, Tsutsui et al., 2000) presents a new possibility that reduced...
plasma LH in ageing laying hens could be a consequence of increased GnIH release. This view is strengthened by the observation that increased GnIH mRNA is associated with depressed plasma LH in incubating hens (Ciccone et al., 2004).

Nothing is known in the domestic hen about the relationship between reproductive neuroendocrine gene expression and naturally reduced or terminated egg laying. In Japanese quail, testicular regression induced by food withdrawal or transfer from long to short days is associated with decreased α-subunit mRNA (Kobayashi and Ishii, 2002; Kobayashi et al., 2004) and decreased plasma LH (Kobayashi et al., 2004). Food withdrawal also results in decreased pituitary LHβ and FSHβ subunit mRNAs (Kobayashi and Ishii, 2002). Both the LHβ and α-subunit mRNAs are likely to be regulated by an inhibitory action of oestrogen since in the chicken these mRNAs are increased after ovarioectomy, while ovarioectomy and oestrogen replacement prevent this increase in gonadotrophin mRNAs (Terada et al., 1997).

The objective of this study was to establish whether declining egg production with reproductive ageing in broiler breeders is correlated with plasma LH and FSH, and with mRNAs encoding hypothalamic gonadotrophin-releasing hormone-I (GnRH-I), gonadotrophin inhibitory hormone (GnIH), and gonadotrophin subunits.

2. Materials and methods

2.1. Birds and sample collection

Hens were obtained from a flock of young pedigree broiler breeders (Cobb Vantress) at peak-of-lay at 30 weeks of age and from a flock of old hens of the same breed at 60 weeks of age. The birds were reared and maintained on a commercial restricted feeding programme, as recommended by Cobb Vantress, to maximise egg laying and were held on 16 h light and 8 h dark in floor pens. The hens in both flocks started laying at 22-24 weeks of age.

To determine the effect of reproductive ageing on the distribution of yellow-yolky follicles (YYFs) greater than 8 mm in the ovary and the proportion of hens out-of-lay, 30 hens were selected at random from the old flock and 15 hens from the young flock.

Observations on the relationship between plasma gonadotrophins and reproductive neuroendocrine mRNAs were made on the 15 hens taken from the young flock for the analysis of the distribution of YYFs, and on 12 old laying and 15 out-of-lay hens selected from the old flock by palpation of the pelvic bones which are softer and more widely spread in laying than in out-of-lay hens. Reproductive condition was assessed by dissection; out-of-lay hens were characterised by the absence of hierarchical YYFs larger than 8 mm diameter and regressed oviducts in out-of-lay hens (n = 15), while laying hens were characterised by the presence of 5-7 YYFs in the ovaries and fully developed oviducts (n = 12). All 15 young hens randomly sampled had 5-9 YYFs in the ovaries. Oviduct and residual ovarian weights were recorded after removal of follicles larger than 8 mm. Pituitary glands and whole hypothalami were dissected into 1 ml of “RNA later” (Ambion, Huntingdon, Cambridgeshire, UK) before transport to the laboratory where they were stored at —80°C until RNA extraction. The hypothalamus was dissected as described by Lal et al. (1990), and contained the GnRH-I (Dunn et al., 1996) and GnIH cell bodies (Tsutsui et al., 2000) and their terminals in the median eminence.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from neuroendocrine tissues using Matrix D lysing matrix (Q-biogene-Alexis, Bingham, Nottingham, UK) in a FastPrep FP120 homogeniser (Q-biogene-Alexis, Bingham, Nottingham, UK). Pituitary glands were extracted in 600 μl Trizol (Invitrogen Life Technologies, Paisley, UK) and the hypothalamus in 1 ml Trizol. Final precipitation of pituitary RNA was facilitated by addition of 2 μl glycogen solution (20 mg/ml, Roche Diagnostics, East Sussex, UK). The total RNA pellet was briefly dried under vacuum and was reconstituted in 100–150 μl dH₂O. The yield of RNA was quantified by measuring the optical density of a sample diluted to 1:50 at 260 and 280 nm, and its quality was checked by running a sample on a formaldehyde gel.

Samples of total RNA (4 μl) were reverse transcribed using a First Strand synthesis kit (Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, UK) following the manufacturer’s instructions.

2.3. Quantitative competitive QC RT-PCR assays for quantification of neuroendocrine mRNA genes

Chicken hypothalamic GnRH-I mRNA was measured by quantitative competitive RT-PCR (QC RT-PCR) as described by Dunn et al. (1996). GnIH and pituitary gonadotrophin α, FSHβ, and LHβ subunit mRNAs were also measured using QC RT-PCR as described by Ciccone et al. (2004).

2.4. Gonadotrophin radioimmunoassays

Blood samples for gonadotrophin radioimmunoassay were taken from a brachial vein using a heparinised syringe. Plasma fractions were stored at —20°C.

Plasma FSH was measured as described by Krishnan et al. (1993) and plasma LH as described by Sharp et al. (1987). Plasma samples were measured in single assays.
The intra-assay coefficients of variation for the FSH and LH assays were 8.0 and 5.2%, respectively.

2.5. Statistical analysis

All statistical analyses were carried out using Genstat 6th edition (VSN International, Oxford) unless otherwise indicated. Differences between experimental groups were considered significant at $p<0.05$ or as otherwise indicated. One-way ANOVA was performed followed by least significance difference or unpaired $t$-tests if appropriate to test for significance between means. Analyses of tissue weights were performed using body weight as a co-variante in a one-way ANOVA. Plasma LH and FSH, and neuroendocrine mRNA data were log-transformed to normalise variance. The relationships between all plasma gonadotrophins and gonadotrophin subunit mRNAs measurements were analysed using least-square regression analysis (Minitab 14 (www.minitab.com)) with the proportion of variance explained by the model and the significance values of the fit ($r^2$ and $p$ values).

3. Results

3.1. Survey of reproductive status in young and ageing flocks

An analysis of the numbers of YYFs more than 8 mm in diameter in 30 old hens selected at random within the sample flock showed that, compared with the young flock, there is a shift in the distribution of the numbers of YYFs greater than 8 mm to a lower median, from 5 to 7 YYFs (Fig. 1). Of the 30 hens selected at random from the old flock, 4 (13%) were out-of-lay as indicated by the absence of YYFs greater than 8 mm and regressed oviducts. No young hens selected at random were out-of-lay (Fig. 1).

3.2. Comparison of body weight and ovarian morphology in young and ageing hens

Body and pituitary weights were higher in old than in young laying hens, but were lower in old out-of-lay than in old laying hens selected for neuroendocrine mRNAs and plasma gonadotrophin measurements (Table 1). The mean number YYFs greater than 8 mm in diameter in the ovary of young laying hens was significantly higher than in old hens (Table 1).

Residual ovary weight was the same in young and old laying hens but was greater than out-of-lay hens, and the oviducts of young laying hens were heavier than old laying hens (Table 1).

3.3. Comparison of plasma gonadotrophins in young and ageing hens

Plasma LH was higher in young than in old birds, while there was no difference in plasma LH between old laying and out-of-lay hens (Fig. 2). Plasma FSH was
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Young laying (n=15)</th>
<th>Old laying (n=12)</th>
<th>Old out-of-lay (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>3.57 ± 0.05a</td>
<td>5.11 ± 0.19b</td>
<td>4.35 ± 0.14c</td>
</tr>
<tr>
<td>Pituitary weight (mg)</td>
<td>9.70 ± 0.25d</td>
<td>11.87 ± 0.61e</td>
<td>9.67 ± 0.39d</td>
</tr>
<tr>
<td>YYFs (&gt;8 mm)</td>
<td>6.76 ± 0.62a</td>
<td>5.92 ± 0.23c</td>
<td>9.90</td>
</tr>
<tr>
<td>Residual ovary (g)</td>
<td>10.05 ± 0.62a</td>
<td>9.53 ± 0.74a</td>
<td>4.60 ± 0.40b</td>
</tr>
<tr>
<td>Oviduct (g)</td>
<td>65.40 ± 3.15a</td>
<td>41.42 ± 1.61b</td>
<td>1.79 ± 0.31c</td>
</tr>
</tbody>
</table>

Means within row followed by different superscripts are significantly different, ab/p < 0.001, cd/p < 0.05, by analysis of variance and least-significant differences using body weight as a co-variant. Significantly different, p < 0.05 using unpaired t-test. YYFs, number of yellow-yolkY follicles in the ovary.

Fig. 2. Comparison of plasma LH and FSH in young laying (n=15), old laying (n=12), and old out-of-lay (n=15) hens. Means ± SEM. For each hormone: a vs. b; p < 0.001, a vs. c; p < 0.01, b vs. d; p < 0.05. Statistical significance by unpaired t-test if one-way ANOVA revealed significant differences between means on log transformed data.

3.4. Comparison of reproductive neuroendocrine mRNAs in young and ageing hens

There were no differences in hypothalamic GnRH-I nor GnIH mRNAs between young and ageing hens irrespective of whether they were laying or out-of-lay (Fig. 3A). To account for differences in pituitary weight between groups gonadotrophin subunit mRNAs were expressed as a concentration (mol/mg pituitary gland). The concentration of \( \alpha \)-subunit mRNA was higher in young than in laying or out-of-lay old hens and was not different between old, laying, and out-of-lay, hens (Fig. 3B). The concentration of pituitary LH\( \beta \) mRNA was higher in young than in old laying hens (Fig. 3B) and was higher in old out-of-lay than in young or old laying hens (Fig. 3B). Pituitary FSH\( \beta \) subunit mRNA concentration did not differ between young and old laying hens but was higher in old out-of-lay than in young or old laying hens (Fig. 3B).

3.5. Correlation of plasma gonadotrophin and pituitary mRNA concentration

Plasma FSH was correlated with pituitary FSH\( \beta \) mRNA (Fig. 4A) but not with \( \alpha \)-subunit mRNA concentration (Fig. 4B), while plasma LH was correlated with pituitary \( \alpha \)-subunit mRNA (Fig. 4D) but not with LH\( \beta \) mRNA concentration (Fig. 4C). Regression analysis showed that 52% of the variation in plasma LH was explained by the \( \alpha \)-subunit mRNA concentration.

4. Discussion

This study shows that reduced egg production in laying ageing broiler breeder hens is associated with a reduction in YYFs over 8 mm diameter, and in reduced plasma LH and FSH. The reduction in numbers of YYFs and in plasma LH agrees with a previous study in ageing dwarf broiler breeders (Sharp et al., 1992), but a reduction in plasma FSH in ageing laying hens has not been previously reported. It is therefore likely that reduced ovarian function in ageing hens is caused by a reduction in gonadotrophin secretion. The finding that there were no differences in GnRH-I and GnIH mRNAs between young and old laying hens suggests that changes in the synthesis of GnRH-I and GnIH are not the immediate cause of decreased gonadotrophin secretion in old laying hens.

The absence of a decrease in hypothalamic GnRH-I mRNA in old laying hens is consistent with an earlier finding that hypothalamic GnRH-I peptide content does not decrease with decreased egg laying in ageing dwarf broilers (Sharp et al., 1992). However, the absence of a decrease in GnRH-I mRNA in ageing broiler hens does not imply that changes in GnRH-I release are not responsible for decreased gonadotrophin secretion. This is supported by an observation on a mammalian GnRH cell line (GT1), where steady state levels of GnRH mRNA do not correlate with GnRH release (Pitts et al., 2001).

In ageing female rats and women, GnRH pulse frequency is reduced (Rossmanith et al., 1991; Rubin and Bridges, 1989), and a similar decrease in GnRH pulse frequency may explain the changes in plasma FSH and LH in old out-of-lay hens. The observation that plasma FSH, but not LH increases in old out-of-lay hens is similar to the finding that ovarian atrophy induced in
hens by feed deprivation is also associated with increased plasma FSH but not LH (Lovell et al., 2000; Vanmontfort et al., 1994). Changes in GnRH pulse frequency with advancing age may also explain why ovarian regression was associated with increased plasma FSH but not LH (Fig. 2). In mammals, a fast GnRH pulse frequency preferentially stimulates LH release whereas a slow GnRH pulse frequency stimulates FSH release (Dalkin et al., 1989; Kaiser et al., 1997). In post-menopausal women reduced GnRH pulse frequency (Rossmanith et al., 1991) may explain why serum FSH levels are elevated (Rubin, 2000) while LH levels decrease (Matt et al., 1998). The same mechanism may apply to ageing out-of-lay hens. This leaves the question of why plasma FSH is not elevated in ageing laying hens. It is suggested that this may be a consequence of high circulating concentrations of plasma oestrogen, relative to out-of-lay hens (Tanabe et al., 1981), which exert a greater inhibitory effect on FSH than LH pituitary content (Dunn et al., 2003) resulting in FSH secretion being more sensitive to the inhibitory action of oestrogen than LH secretion.

The present study shows that pituitary FSHβ subunit mRNA concentrations are higher in out-of-lay than in-lay hens and this may also be a consequence of reduced GnRH pulse frequency and plasma oestrogen.

The inhibitory effects of oestrogen on gonadotrophin secretion could be either at the level of the anterior pituitary or the hypothalamus. There is evidence in birds that the hypothalamus is a site of the inhibitory action of oestrogen on gonadotrophin secretion, as demonstrated in adult male Japanese quail, where oestrogen treatment reduces basal release of GnRH-I from the hypothalamus in vitro (Li et al., 1994). Further, in the cockerel, hypothalamic GnRH-I peptide is increased by treatment with the anti-oestrogen, tamoxifen (Rozenboim et al., 1993), while GnRH-I peptide (Wilson et al., 1990) and GnRH-I mRNA (Dunn and Sharp, 1999) are suppressed in juvenile birds after treatment with oestrogen. At the level of the anterior pituitary in mammals, FSHβ subunit gene transcription is highly sensitive to the inhibitory effect of oestrogen (Miller and Miller, 1996; Phillips et al., 1988) and this may be the case in the chicken since in the juvenile hen, pituitary FSH content is more responsive to the depressive action of oestrogen than LH content (Dunn et al., 2003). The observation that LHβ mRNA was increased in out-of-lay compared to laying hens may also be ascribed to reduced circulating oestrogen, since oestrogen depresses avian LHβ mRNA (Terada et al., 1997). In out-of-lay birds, a reduction in oestrogen may remove an inhibitory effect on LHβ mRNA.

The increase in pituitary FSHβ mRNA and plasma FSH in old out-of-lay hens may not only be due to decreasing oestrogen and GnRH pulse frequency but also to a fall in circulating ovarian inhibin derived from the hierarchical YYFs (Johnson, 1993). Evidence for this comes from the inverse relationship between the presence of YYFs and, therefore, inhibin and circulating FSH (Johnson et al., 1993; Lovell et al., 2000; Vanmontfort et al., 1994).
This study shows that pituitary FSHβ mRNA but not α-subunit mRNA concentration is positively correlated with plasma FSH (Fig. 4A) and supports the view that avian FSH (Hattori et al., 1986), like mammalian FSH (Farnworth, 1995) is in large part, constitutively released. Hattori et al. (1986) showed in quail that pituitary glands treated with hypothalamic extracts released more FSH than was initially present. Further, FSH but not LH secretion continued after the glands were collected and incubated in vitro. Similar observations have been reported in mammals (Kartun and Schwartz, 1987). These studies demonstrate that LH and FSH are differentially controlled and that LH secretion is more immediately dependent on the stimulatory action of GnRH than is FSH. This raises the possibility that an unidentified hypothalamic factor differentially controls FSH and LH secretion. The recently discovered avian hypothalamic RFamide gonadotrophin inhibitory hormone, GnIH (Tsutsui et al., 2000), is a potential candidate for this undiscovered factor in birds.

In the chicken, GnIH suppresses pituitary FSHβ and α-subunit mRNAs, and the secretion of LH and FSH in vitro (Ciccone et al., 2004), while increased GnIH mRNA (Ciccone et al., 2004) and GnIH peptide (Tsutsui, Ciccone et al. unpublished) are associated with ovarian regression in incubating hens. An increase in GnIH mRNA in ageing laying hens might, therefore, explain the decrease in plasma LH and FSH. However, since there was no change in GnIH mRNA between young and old or laying and out-of-lay hens this seems unlikely. However, it is possible that an increase in GnIH secretion may occur in ageing hens independent of a change in GnIH mRNA.

Of particular interest was the observation that concentrations of plasma LH were correlated with concentrations of α-subunit but not LHβ subunit mRNA. A direct relationship between α-subunit mRNA and plasma LH has been observed previously in quail deprived of food (Kobayashi and Ishii, 2002) or after transfer from long to short days (Kobayashi et al., 2004). These findings suggest that in birds unlike mammals, α-subunit synthesis may be the rate limiting step for LH release. In mammals, α-subunit mRNA is always in excess of LHβ mRNA (Nilson et al., 1993; Shupnik, 1996) implying that LHβ mRNA production is the rate limiting step for LH release. However, this does not apply to the hen where α-subunit mRNA and LHβ subunit mRNA are present in the pituitary in equimolar amounts (pituitary content, $10^{-15}$ mol, Fig. 3B).

Fig. 4. Linear regression analysis of the relationship between plasma gonadotrophins and pituitary gonadotrophin subunit mRNA concentrations for all hens in the study. (A) Plasma FSH versus FSHβ mRNA, (B) plasma FSH versus α mRNA, (C) plasma LH versus LHβ mRNA, and (D) plasma LH plasma versus α mRNA. Data were log transformed; values for the coefficient of determination (the proportion of the variation explained by the fit) $r^2$ and probability values associated with the $F$ test of the relationship between the fitted values for each regression analysis are shown.
In conclusion, this study demonstrates that the decline in reproductive performance in ageing laying broiler breeders is correlated with a decrease in both LH and FSH secretion but not with decreased GnRH-I mRNA or with increased GnIH mRNA. Observations on the relationship between plasma FSH and concentration of FSHβ subunit mRNA support the view that FSH secretion is largely constitutive, as in mammals, but unlike mammals, the expression of α-subunit mRNA, rather than LHβ mRNA may be the rate limiting step in avian LH secretion.

Acknowledgments

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References


Light intensity can influence plasma FSH and age at sexual maturity in domestic pullets

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Abstract

1. Shaver White and ISA Brown pullets were reared to 140 d in cage groups of 8 on a 10-h photoperiod of incandescent light and maintained at an illuminance of 3 or 25 lux, or transferred from 3 to 25 lux or from 25 to 3 lux at 63 or 112 d of age.

2. Plasma follicle stimulating hormone (FSH) concentration at 63 and 112 d was higher in both breeds for pullets maintained at an illuminance of 25 lux compared with 3 lux. After 2-4 d, and relative to constant-illuminance controls, plasma FSH increased significantly for ISA Brown transferred from 3 to 25 lux at 63 d and for Shaver White transferred at 112 d. Irrespective of genotype, plasma FSH for pullets given a decrease in illuminance at 63 or 112 d showed a tendency for less change than did constant-illuminance controls.

3. There was no significant difference in sexual maturity for ISA Brown maintained on 3 or 25 lux, but Shaver White pullets exposed to constant 3 lux matured later than those maintained on 25 lux. Shaver White matured later following an increase from 3 to 25 lux at 63 or 112 d, and earlier subsequent to a decrease from 25 to 3 lux at 112 d. ISA Brown pullets were not significantly affected by a change in illuminance at 63 or 112 d, though their responses were in the same direction as Shaver White.

4. Changes in plasma FSH in the 2- to 4-d period following a change in illuminance at 63 or 112 d were not significantly correlated with sexual maturity.

INTRODUCTION

Age at sexual maturity is similar for ISA Brown but later for Shaver White pullets maintained on 10-h photoperiods and exposed to an illuminance of 3 lux compared with 25 lux (Lewis et al., 2004). These findings agree with earlier studies which revealed a threshold of 2 lux for ISA Brown pullets to detect the presence of light necessary to fully modify sexual development (Lewis et al., 1999a), and indicate a threshold of more than 3 lux for Shaver White. Sexual maturity is delayed in Shaver White pullets by a transfer from 3 to 25 lux at 63 or 112 d, and is advanced by a change from 25 to 3 lux at 112 d (Lewis et al., 2004). Sexual maturity in ISA Brown pullets is not significantly affected by either an increase or a decrease in illuminance (Lewis et al., 2004). Plasma luteinising hormone (LH) concentration, in both breeds, is lower in birds maintained at 3 lux than at 25 lux, is depressed by a transfer from 25 to 3 lux at 63 and 112 d, and is increased by a transfer from 3 to 25 lux, but only at 63 d. However, changes in plasma LH that follow changes in illuminance are not significantly correlated with changes in sexual maturity. This paper reports the effects that the various illuminance treatments had on plasma follicle stimulating hormone (FSH) concentration.

MATERIALS AND METHODS

A total of 800 Shaver White and 800 ISA Brown pullets were placed in the top tier of 8-bird cages in 4 light-proof rooms at one day of age, with each room containing 200 birds of each breed. All birds received a 23L:1D lighting regime for the first 5 d, 18L:6D between d 6 and 10, and 10L:14D from d 11 to 140. Mean illuminance at the feed trough was 63.0 ± 1.40 lux for the first 7 d, 370 ± 0.40 lux from 8 to 14 d, and 25.2 ± 0.25 lux (nominally 25 lux) in two rooms and 2.8 ± 0.03 lux (nominally 3 lux) in the other two rooms from 14 d onwards. At 63 and 112 d, reductions in illuminance were achieved by...
transferring 40 birds of each breed from the two 25-lux rooms into the two 3-lux rooms. Similarly, increases in illuminance were achieved by transferring 40 birds of each breed from the two 3-lux rooms into the two 25-lux rooms. Simultaneously, another 40 birds of each breed were moved between rooms of the same illuminance to provide constant-illumiance (but moved) controls. Each treatment was represented in two rooms so that a between-room error could be used to determine the significance of any differences in sexual maturity when conducting an ANOVA.

A 1 ml blood sample was taken during the first 2 h of the photoperiod from a brachial vein of 8 birds (one cage) of each breed at 63, 65 and 67 d for the early changes in intensity, and from different birds at 112, 114 and 116 d for the later changes (0, +2 and +4 d of each change in illuminance, or of each move for constant 3- and 25-lux controls). Samples were identified to bird, centrifuged at 500 g for 15 min, and the sera stored frozen. Subsequently, FSH was measured by homologous radioimmunoassay (Krishnan et al., 1993) in a single assay that had an intra-assay coefficient of variation of 8-0%.

A regression of the standard errors of the mean (SEM) for plasma FSH concentrations on the corresponding means for each breed x sampling time indicated that the SEM increased in direct proportion to the mean. This heterogeneity of variance was removed by transforming FSH concentrations to log10 values before conducting an ANOVA on the 0-d values using a model from Genstat 6th Edition (Lawes Agricultural Trust 2002). Untransformed percentage changes in FSH for individual birds were also analysed by ANOVA. It was not possible to test for room effect because samples for each breed x lighting x age treatment were taken from one room only, and, as a result, the error term used for tests of significance was measured within-room. Consequently, caution is required when comparing the means for treatments not in the same room. However, no significant room effect was identified for the egg production, feed intake or body weight data reported in Lewis et al. (2004), so it is a reasonable assumption that none existed for the FSH responses. Significant differences between means were identified using a t-test.

RESULTS

‘Plasma FSH concentration’ has been abbreviated to FSH, and the results are presented separately for each breed because there were significant treatment x breed interactions for the initial FSH values and for the percentage change in FSH.

Shaver White

The initial (0 d) mean FSH for the 4 groups maintained on 25 lux or given 25 lux prior to a change to 3 lux was higher than the equivalent four 3-lux groups, although the difference was only significant at 112 d (Table 1). At both light intensities, FSH was higher at 112 d than at 63 d, though the difference was significant only for the 25 lux birds. There was no significant difference between the 3 to 25 lux and 25 to 3 lux groups for percentage change in FSH at 63 d, but at 112 d, the 51% increase for the birds transferred from 3 to 25 lux was significantly different from the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shaver White</th>
<th>ISA Brown</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-3 lux at 63 d</td>
<td>1.40 ± 0.03 (0.074)b</td>
<td>1.28 ± 0.03 (0.091)b</td>
</tr>
<tr>
<td>25 lux moved at 63 d</td>
<td>0.66 ± 0.01 (0.269)c</td>
<td>0.90 ± 0.01 (0.013)bc</td>
</tr>
<tr>
<td>25-3 lux at 112 d</td>
<td>1.98 ± 0.04 (0.257)c</td>
<td>1.32 ± 0.03 (0.070)b</td>
</tr>
<tr>
<td>25 lux moved at 112 d</td>
<td>2.34 ± 0.06 (0.332)c</td>
<td>1.52 ± 0.02 (0.096)c</td>
</tr>
<tr>
<td>5 lux initial mean</td>
<td>1.57 ± 0.03</td>
<td>1.20 ± 0.09</td>
</tr>
<tr>
<td>3 to 5 lux at 63 d</td>
<td>1.28 ± 0.01 (0.091)b</td>
<td>1.58 ± 0.03 (0.188)bc</td>
</tr>
<tr>
<td>3 lux moved at 63 d</td>
<td>0.90 ± 0.07 (0.053)bc</td>
<td>1.32 ± 0.03 (0.070)b</td>
</tr>
<tr>
<td>3-25 lux at 112 d</td>
<td>1.32 ± 0.03 (0.070)b</td>
<td>1.52 ± 0.01 (0.096)c</td>
</tr>
<tr>
<td>3 lux moved at 112 d</td>
<td>1.52 ± 0.01 (0.096)c</td>
<td>1.20 ± 0.09</td>
</tr>
</tbody>
</table>

SED and significance levels for 0 d (log10 data):
Light intensity = 0.06120 d, P < 0.001; Breed = 0.0306, P = 0.474; Treatment x Breed = 0.0865, P = 0.021.

SED and significance levels for percentage change:
Light intensity = 21.04, P < 0.001; Breed = 10.52, P = 0.468; Treatment x Breed = 29.75, P < 0.001.
Within a column, means with the same superscript are not significantly different at P > 0.05.
1 Calculated from individual percentage change data.
18% fall in FSH for the birds transferred from 25 to 3 lux.

**ISA Brown**

The initial (0d) mean FSH for the 4 groups maintained on 25 lux or given 25 lux prior to a change to 3 lux was significantly higher at 63 and 112 d than those maintained on 3 lux (Table 1). FSH was significantly higher at 112 d than at 63 d in both light intensity groups. The 141% increase in FSH for birds transferred from 3 to 25 lux at 63 d was significantly higher than the 5% rise in the birds changed from 25 to 3 lux, but, at 112 d, the 6% fall for birds transferred from 3 to 25 lux was not significantly different from the 15% fall for those changed from 25 to 3 lux.

**DISCUSSION**

In neither breed, nor at either age, was there a significant correlation between the change in FSH that occurred in the 4 d following transfer to a new light intensity and age at sexual maturity, whether in actual terms (Table 1), or relative to birds maintained at the final illuminance (Table 2). This agrees with the previously reported findings for changes in LH (Lewis et al., 2004 and Table 2). Changes in FSH have been reported to be significantly correlated with age at sexual maturity following a change in photoperiod (Lewis et al., 1998, 1999b); however, blood samples in these two trials were taken at 7 and 14 d, respectively, after the changes in illuminance. In the current trial, blood samples were only taken 2 and 4 d (periods previously found to be satisfactory for detecting a change in LH) after the changes in illuminance, and the opportunity to conduct an FSH assay only presented itself after the trial and LH assay had been completed. It is possible that 4 d is too soon after a change in illuminance to detect any effect on FSH; Lewis et al. (1999b) found a 0.359±0.066 log10 ng/ml mean increase in FSH 14 d after a transfer from an 8- to a 13- or 16-h photoperiod at 63 or 105 d, and this is a 10-fold increase over the 0.035±0.046 log10 ng/ml increase recorded in the current trial. Furthermore, the ranges in sexual maturity in the earlier investigations (54 d in Lewis et al., 1998, and 39 d in Lewis et al., 1999b) were much wider than the 8-d spread in this trial, and so the lack of a significant correlation between change in FSH and sexual maturity in the current trial might not necessarily indicate a difference between a response to a change in photoperiod and one to a change in illuminance.

In both breeds, there was a tendency (P=0.060) for the changes in FSH that occurred between 63 and 67 d and between 112 and 116 d, whether they were for constant-illuminance controls or following a decrease or an increase in illuminance, to be positively correlated with the changes in LH reported by Lewis et al. (2004). This suggests a common influence on the hypothalamopituitary axis, even though the changes failed to lead to predictable changes in sexual maturity.

It can be concluded that illuminance per se has a positive effect on FSH, particularly at 112 d, and that, relative to constant-illuminance controls, changes in light intensity modify FSH over the 4 d that follow a change in light intensity, whether to brighter or dimmer, but that the size and direction of the response vary with age and genotype, and give no indication of age at sexual maturity (Table 2).

**ACKNOWLEDGEMENTS**

The work was supported by the Ontario Ministry of Agriculture and Food, Guelph, Ontario (S.L.), a BBSRC/CORB CASE studentship (N.C.) and a BBSRC Core Strategic Grant (P.J.S.).

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Lighting regimens and plasma LH and FSH in broiler breeders

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Animal and Poultry Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa and Roslin Institute, Roslin, Midlothian, Scotland

Abstract 1. Egg production by meat-type fowl is markedly inferior to that from commercial laying hens, and so, to assess the degree to which photorefractoriness might be a contributing factor, male- and female-line broiler breeders were maintained on 8-, 11- or 16-h photoperiods. In addition, to determine the age-related rate of change in response to an increment in photoperiod, other birds were transferred from 8- to 16-h photoperiods at 67 or 124 d.

2. Blood samples were taken from all groups, except those on constant 11-h photoperiods, in both genotypes at 67, 69, 124 and 126 d, and from all lighting groups in the female line at 58 weeks (end of trial), and the plasma was assayed for plasma luteinising hormone (LH) and follicle stimulating hormone (FSH) concentration to investigate possible correlations with rate of sexual maturity, total egg numbers and terminal rates of lay.

3. Prepubertal LH was consistently higher for the female line than for the male line, and higher for 16-h birds than for 8-h birds. At 69 and 126 d, LH values were not significantly different from those 2 d earlier for 8-h birds, but significantly reduced for 16-h birds. There was an increase in LH following photostimulation at 67 d, but no significant change after the 124-d light increase.

4. There were no significant differences in FSH between the two genetic lines, nor any effect of photostimulation at 67 or 124 d. There was a tendency for FSH in 8-h birds to be higher than for 16-h birds, and this difference became significant for male-line birds at 67 d.

5. At 58 weeks, LH was higher for constant 11- and 16-h birds and for birds photostimulated at 67 d than for constant 8-h controls or birds transferred from 8 to 16 h at 124 d.

6. Neither baseline nor photoinduced prepubertal changes in plasma LH nor FSH were found to be of value for predicting age at sexual maturity or subsequent rates of egg production. At 58 weeks, LH was not generally correlated with sexual maturity, total eggs or terminal rates of lay, however, there was a negative correlation with age at first egg in birds photostimulated at 124 d.

7. It must be concluded that plasma LH and FSH concentrations are of minimal value to the broiler breeder industry for predicting the degree of photorefractoriness, the age at sexual maturity, or subsequent egg production.

INTRODUCTION

Changes in plasma luteinising hormone (LH) and follicle stimulating hormone (FSH) concentrations in egg-type domestic fowl, both with age and following a change in photoperiod, are well documented (Sharp, 1975; Williams and Sharp, 1977; Vannmontfort et al., 1995; Lewis et al., 1998). The effects of controlled feeding on plasma LH concentrations in dwarf broiler breeders maintained on 8 h, or transferred to a range of photoperiods between 10-5 and 20 h (Dunn and Sharp, 1990), and at various ages between 3 and 19 weeks (Dunn et al., 1990), have also been reported, but there appears to be a dearth of information on the effects of changes in photoperiod, and of constant long days on plasma LH and FSH concentrations in control-fed normal-size broiler breeders.

It has been suggested that plasma LH concentration during the rearing period might be useful as a predictor of sexual maturity and rate of egg production in egg-type hybrids, however, the evidence is equivocal. Whereas data reported by Wilson (1978) were supportive of the proposal, Lewis et al. (1994, 1998) reported that prepubertal LH was a poor predictor of sexual maturity, and Sharp et al. (1981) concluded that it gave no indication of subsequent
rate of lay. However, the hypothesis has not been tested in meat-type hybrids.

Accordingly, this paper reports plasma LH and FSH data for a male and a female line of broiler breeder maintained on 8- or 16-h photoperiods, or transferred from 8-, 11- to 16-h photoperiods at 67 or 124 d, and correlates these with sexual maturity, total and terminal egg production data. In addition, it reports plasma LH data for the female-line birds at 58 weeks of age and correlates these with the above performance traits.

MATERIALS AND METHODS

Male- and female-line Ross broiler breeder pullets were reared in light-proof litter-floor rooms from one day until 15 weeks and then transferred to individual cages in different light-proof rooms until depletion at 58 weeks. They were maintained on 8-, 11- or 16-h photoperiods or transferred from 8- to 16-h photoperiods at 67 or 124 d of age, and fed according to the primary breeding company’s recommendations to achieve a 2.1 kg body weight at about 20 weeks.

Within each line and lighting treatment, a 1 to 2 ml blood sample was taken from a brachial vein of 8 birds at the same time each day (1 to 3 h into the photoperiod), excluding constant 11-h photoperiods, at 67, 69, 124 and 126 d, and centrifuged at 500 g for 15 min. Unpublished data from an earlier investigation of the photoperiodic response in normal-size broiler breeders had shown that a 2-d interval was sufficient to detect a photoinduced increase in LH. Plasma LH and FSH concentrations were measured using an LH radioimmunoassay (Sharp et al., 1987) and an FSH radioimmunoassay (Krishnan et al., 1993) each in a single assay. The intra-assay CV was 6.9% for LH and 8.0% for FSH (suggesting that pulsatility was not a concern for either hormone). A further 10 blood samples were taken, at random, from each of the three constant-photoperiod and two photostimulated groups at 58 weeks, and assayed for plasma LH concentration.

Regressions of the standard errors of the mean (SEM) for plasma LH and FSH concentrations on the corresponding means for each line × lighting treatment indicated that the SEM increased in direct proportion to the mean, and so this heterogeneity of variance was removed by transforming concentrations to log_{10} values prior to statistical analysis. Initial values and the percentage change in actual plasma hormone concentration between d 0 and d 2 were subjected to an ANOVA using a model from Genstat 6th Edition (Lawes Agricultural Trust, 2002).

RESULTS

‘Plasma LH concentration’ and ‘plasma FSH concentration’ will be abbreviated to LH and FSH, respectively, and ‘age at first egg’ to AFE.

LH

Although the female-line birds had significantly higher LH than the male-line birds at all ages and for all lighting treatments, the responses of the two genotypes for each of the lighting treatments were similar (Table 1). Birds on 8-h photoperiods had significantly lower LH than 16-h birds at

Table 1. Plasma LH and FSH concentrations (ng/ml), and mean individual percentage change over 2 d for male- and female-line broiler breeder females maintained on 8- or 16-h photoperiods or transferred from 8- to 16-h photoperiods at 67 or 124 d

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 d</th>
<th>+2 d</th>
<th>% Change</th>
<th>0 d</th>
<th>+2 d</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant 8 h at 67 d</td>
<td>1.75 ± 0.33bc</td>
<td>1.30 ± 0.13</td>
<td>-13 ± 16a</td>
<td>0.98 ± 0.36a</td>
<td>1.10 ± 0.26</td>
<td>84 ± 49bc</td>
</tr>
<tr>
<td>Constant 16 h at 67 d</td>
<td>2.11 ± 0.61c</td>
<td>1.37 ± 0.14</td>
<td>-29 ± 13a</td>
<td>1.31 ± 0.29c</td>
<td>0.81 ± 0.09</td>
<td>-28 ± 8a</td>
</tr>
<tr>
<td>8 to 16 h at 67 d</td>
<td>1.52 ± 0.19abc</td>
<td>2.26 ± 0.27</td>
<td>69 ± 29ab</td>
<td>0.99 ± 0.14ab</td>
<td>1.89 ± 0.47</td>
<td>163 ± 116c</td>
</tr>
<tr>
<td>Constant 8 h at 124 d</td>
<td>1.10 ± 0.19a</td>
<td>1.09 ± 0.22</td>
<td>6 ± 24ab</td>
<td>1.42 ± 0.32bc</td>
<td>1.30 ± 0.23</td>
<td>10 ± 22ab</td>
</tr>
<tr>
<td>Constant 16 h at 124 d</td>
<td>1.69 ± 0.29ab</td>
<td>1.06 ± 0.21</td>
<td>-29 ± 12a</td>
<td>1.80 ± 0.36c</td>
<td>0.81 ± 0.14</td>
<td>-47 ± 11a</td>
</tr>
<tr>
<td>8 to 16 h at 124 d</td>
<td>1.29 ± 0.22ab</td>
<td>1.50 ± 0.27</td>
<td>20 ± 19ab</td>
<td>1.00 ± 0.20ab</td>
<td>0.93 ± 0.20</td>
<td>11 ± 25ab</td>
</tr>
<tr>
<td>Residual DF</td>
<td>83</td>
<td>82</td>
<td></td>
<td>83</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td><strong>FSH data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant 8 h at 67 d</td>
<td>1.05 ± 0.06ab</td>
<td>1.00 ± 0.11</td>
<td>1 ± 15ac</td>
<td>1.59 ± 0.37c</td>
<td>1.52 ± 0.42</td>
<td>22 ± 30a</td>
</tr>
<tr>
<td>Constant 16 h at 67 d</td>
<td>1.02 ± 0.14a</td>
<td>1.08 ± 0.10</td>
<td>27 ± 29ab</td>
<td>0.75 ± 0.05a</td>
<td>0.78 ± 0.11</td>
<td>-6 ± 14a</td>
</tr>
<tr>
<td>8 to 16 h at 67 d</td>
<td>0.88 ± 0.15a</td>
<td>0.76 ± 0.03</td>
<td>-25 ± 8ab</td>
<td>1.11 ± 0.13b</td>
<td>0.97 ± 0.19</td>
<td>8 ± 5a</td>
</tr>
<tr>
<td>Constant 8 h at 124 d</td>
<td>1.12 ± 0.08b</td>
<td>0.89 ± 0.13</td>
<td>-17 ± 15ab</td>
<td>1.03 ± 0.18b</td>
<td>0.95 ± 0.09</td>
<td>5</td>
</tr>
<tr>
<td>Constant 16 h at 124 d</td>
<td>1.25 ± 0.21b</td>
<td>1.09 ± 0.32</td>
<td>-49 ± 15a</td>
<td>0.96 ± 0.06b</td>
<td>0.79 ± 0.10</td>
<td>-81</td>
</tr>
<tr>
<td>8 to 16 h at 124 d</td>
<td>1.27 ± 0.15b</td>
<td>0.621</td>
<td>-481</td>
<td>0.87 ± 0.07ab</td>
<td>0.98 ± 0.28</td>
<td>23 ± 57a</td>
</tr>
<tr>
<td>Residual DF</td>
<td>68</td>
<td>39</td>
<td></td>
<td>68</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

n = 1. Within a column, and for each hormone, means with the same superscript letter are not significantly different at P > 0.05.
both 67 and 124 d, but, within a photoperiod group, initial LH did not change with age. LH for birds maintained on 8 h did not significantly change between 67 and 69 d or between 124 and 126 d, however, there were significant reductions in LH for birds maintained on 16 h (Figure 1). In the female line, the transfer from 8- to 16-h photoperiods at 67 d produced a significant 69% increase in LH (+2 d), compared with a 13% fall for constant 8-h controls, but a non-significant 20% increase after the increase in photoperiod at 124 d, compared with a modest 6% increase for controls. In the male line, photostimulation had similar effects on LH, increasing by 163% between 67 and 69 d compared with 84% for 8 h controls (though $P > 0.05$), but the same 10 to 11% change as controls after the 124-d transfer (Table 1).

At 58 weeks, birds maintained on 11- and 16-h photoperiods, or photostimulated at 67 d, had significantly higher LH than constant 8-h controls or birds transferred to 16 h at 124 d (Table 2). When all individual LH data were regressed on individual AFE, there was no significant correlation. However, when separate regressions were conducted for each lighting treatment, there was a significant negative relationship between LH at 58 weeks and AFE for birds photostimulated at 124 d, with LH reducing by 0.05 ng/ml for each one-day delay in maturity ($r^2 = 0.723$, $P = 0.002$, slope SE = 0.011), while none of the regressions for the other treatments approached significance (Figure 2).

**FSH**

At neither age, nor for any lighting treatment, was there a significant difference in FSH between the two lines (Table 1). FSH at 67 d was not significantly different from 124 d, and was also unaffected by either of the transfers from 8 to 16 h. FSH in 8-h birds was generally not significantly different from that in 16-h birds, although in male-line birds, FSH for birds maintained on 8-h photoperiods was significantly higher than for those maintained on 16-h photoperiods at 67 d.

**DISCUSSION**

The higher LH for the female-line compared with the male-line birds concurs with evidence from Dunn and Sharp (1990) that there are genotypic differences in baseline LH. Data for brown-egg hybrids (Dunn and Sharp, 1990; Lewis et al., 1994, 1998), dwarf broiler breeders (Dunn and Sharp, 1990; Dunn et al., 1990), and the two lines of normal-size broiler breeders in the current trial suggest that LH baselines are inversely correlated with genotypic body weight. However, the baselines for broiler breeders would have been even lower had they been fed *ad libitum*, because plasma LH concentrations

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**Table 1.** Mean plasma LH (solid symbols) and FSH (open symbols) concentrations (ng/ml) on d 0 and d 2 for male- and female-line broiler breeder pullets maintained on 8- (○, ●) or 16-h (■, □) photoperiods, or transferred from 8- to 16-h photoperiods (▲, △) at 67 or 124 d.

![Figure 1](image1.png)

**Figure 1.** Mean plasma LH (solid symbols) and FSH (open symbols) concentrations (ng/ml) on d 0 and d 2 for male- and female-line broiler breeder pullets maintained on 8- (○, ●) or 16-h (■, □) photoperiods, or transferred from 8- to 16-h photoperiods (▲, △) at 67 or 124 d.

**Table 2.** Plasma LH concentrations (ng/ml) at 58 weeks of age for female-line broiler breeder females maintained on 8-, 11- or 16-h photoperiods or transferred from 8- to 16-h photoperiods at 67 or 124 d.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma LH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant 8 h</td>
<td>2.02 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Constant 11 h</td>
<td>2.51 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Constant 16 h</td>
<td>2.33 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 to 16 h at 67 d</td>
<td>2.30 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 to 16 h at 124 d</td>
<td>1.85 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means with the same superscript letter are not significantly different at $P > 0.05$. 

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At 58 weeks, birds maintained on 11- and 16-h photoperiods, or photostimulated at 67 d, had significantly higher LH than constant 8-h controls or birds transferred to 16 h at 124 d (Table 2). When all individual LH data were regressed on individual AFE, there was no significant correlation. However, when separate regressions were conducted for each lighting treatment, there was a significant negative relationship between LH at 58 weeks and AFE for birds photostimulated at 124 d, with LH reducing by 0.05 ng/ml for each one-day delay in maturity ($r^2 = 0.723$, $P = 0.002$, slope SE = 0.011), while none of the regressions for the other treatments approached significance (Figure 2).

**FSH**

At neither age, nor for any lighting treatment, was there a significant difference in FSH between the two lines (Table 1). FSH at 67 d was not significantly different from 124 d, and was also unaffected by either of the transfers from 8 to 16 h. FSH in 8-h birds was generally not significantly different from that in 16-h birds, although in male-line birds, FSH for birds maintained on 8-h photoperiods was significantly higher than for those maintained on 16-h photoperiods at 67 d. 

**DISCUSSION**

The higher LH for the female-line compared with the male-line birds concurs with evidence from Dunn and Sharp (1990) that there are genotypic differences in baseline LH. Data for brown-egg hybrids (Dunn and Sharp, 1990; Lewis et al., 1994, 1998), dwarf broiler breeders (Dunn and Sharp, 1990; Dunn et al., 1990), and the two lines of normal-size broiler breeders in the current trial suggest that LH baselines are inversely correlated with genotypic body weight. However, the baselines for broiler breeders would have been even lower had they been fed *ad libitum*, because plasma LH concentrations...
for control-fed birds are higher than for full-fed (Dunn and Sharp, 1990; Dunn et al., 1990).

The higher prepubertal LH for birds on 16-h compared with 8-h photoperiods (Table 1), irrespective of age or genotype, concurs with data for egg-type hybrids maintained on 8- or 14-h day-lengths (Lewis et al., 1998).

The most likely explanation for the reduction in LH that occurred at each d-2 sampling for birds maintained on 16-h photoperiods (Table 1, Figure 1) is that it was a response to handling during the earlier sampling (Wilson and Sharp, 1975). A stress response to handling is more evident when initial LH values are high, and this explains the difference between the 8- and 16-h birds. An additional reason why the photostimulated birds did not show the depression was that the stress response to handling was counteracted by the acute stimulatory effect of the increase in photoperiod, resulting in an increase in LH at 69 d and a plateau at 126 d.

The significant increase in LH in both genotypes following the transfer from 8 to 16 h at 67 d, but non-significant increase in LH following an increase at 124 d, reflects the findings for ad-libitum-fed egg-type hybrids given the same increment in photoperiod at 63 and 126 d (Lewis et al., 1994). However, the effects on sexual maturity for the two types of bird were contradictory, with the 9-week photostimulation delaying maturity by about 2 weeks in the broiler breeder (Lewis et al., 2003) but advancing it by more than 3 weeks in the commercial layer (Lewis et al., 1997), whilst at 18 weeks, it advanced sexual maturity by 5 weeks in the broiler breeder but by only 10 d in the layer. Much of the difference in sexual maturity between the broiler breeder and egg-type hybrid can be explained in terms of photorefractoriness and feed allocation, but the LH responses appear to be independent of these factors, and this is further evidence that changes in LH are not correlated with sexual development.

The lack of correlation between prepubertal baseline and photoinduced changes in LH with either sexual maturity or subsequent egg production concurs with the findings of Lewis et al. (1994, 1998) and Sharp et al. (1981) for egg-type pullets, but contrasts with those of Wilson (1978). LH at 58 weeks was not significantly related to either total egg production or egg production between 54 and 58 weeks, however, it correlated well with mean prepubertal LH values \(r^2 = 0.947, P = 0.027\), suggesting that the rate at which LH changes during the laying period is independent of any concurrent photoperiodic influence. Although there was a significant negative correlation of LH at 58 weeks with AFE for birds photostimulated at 124 d, there were no links with AFE for any of the other lighting groups (Figure 2). Notwithstanding that the response might have been spurious, it could just indicate that, whereas LH is unaffected by spontaneous maturation, it is enduringly modified by a photosexual response that is strong enough to advance maturity by 5 weeks. It is difficult, however, to understand why the earlier maturing birds, which would have been in lay longer, should have had higher LH values at 58 weeks than the later maturing birds. The lower values for the constant 8-h controls and those photostimulated at 124 d, compared with the constant 11- and 16-h birds and those photostimulated at 67 d, may suggest that there is some AFE-independent interaction with the rate at which juvenile photorefractoriness is dissipated,
with faster dissipation leading to lower LH at 58 weeks.

The tendency for FSH to be higher in 8- than in 16-h birds concurs with the findings of Lewis et al. (1998) for egg-type pullets. However, the similarity of FSH values at 67 and 124 d contrasts with an age-related increase in FSH in laying birds. This could be a response to restricted feeding, which has been reported to suppress ovarian and oviducal development, and, as a likely corollary, FSH release in dwarf broiler breeders (Dunn and Sharp, 1990). The lack of any significant change in FSH following the 8-h increments in photoperiod, especially at 124 d, could be due to the second sample being taken too soon (+2 d) after the change, because FSH must have increased at some stage following the light increase at 124 d to have advanced AFE by 5 weeks. Lewis et al. (1999) reported that the correlation in egg-type hybrids was between sexual development and change in FSH 14 d after photostimulation, and so this is also a likely explanation for the lack of correlation in 16-h birds. Additionally, it is evident that LH is depressed by handling though not at 124d, and has no effect on FSH.

It is also evident that LH is depressed by handling in 16-h birds, but not in birds maintained on 8-h or transferred from 8- to 16-h photoperiods. Furthermore, there is no correlation of prepubertal LH, FSH or LH at 58 weeks with either sexual maturity or subsequent rate of lay, and baseline LH in domestic fowl appears to be negatively correlated with genotypic body weight potential.

ACKNOWLEDGEMENTS

The authors wish to thank Ross Poultry (South Africa) for the generous supply of 1-d-old chicks. Nick Ciccone and Peter Sharp were supported by a BBSRC/CASE award and a BBSRC Core Strategic Grant, respectively.

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Gonadotrophin Inhibitory Hormone Depresses Gonadotrophin α and Follicle-Stimulating Hormone β Subunit Expression in the Pituitary of the Domestic Chicken

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Key words: GnIH, hypothalamus, reproduction, gonadotrophin mRNAs, chicken.

Abstract

Studies performed in vitro suggest that a novel 12 amino acid RF amide peptide, isolated from the quail hypothalamus, is a gonadotrophin inhibitory hormone (GnIH). The aim of the present study was to investigate this hypothesis in the domestic chicken. Injections of GnIH into nest-deprived incubating hens failed to depress the concentration of plasma luteinizing hormone (LH). Addition of GnIH to short-term (120 min) cultures of diced pituitary glands from adult cockerels depressed follicle-stimulating hormone (FSH) and LH release and depressed common α and FSHβ gonadotrophin subunit mRNAs, with no effect on LHβ subunit mRNA. Hypothalamic GnIH mRNA was higher in incubating (out-of-lay) than in laying hens, but there was no significant difference in the amount of hypothalamic GnIH mRNA in out-of-lay and laying broiler breeder hens at the end of a laying year. It is concluded that avian GnIH may play a role in controlling gonadotrophin synthesis and associated constitutive release in the domestic chicken.

A novel RF amide, SIKPSAYLPLRF-NH₂, isolated from the quail hypothalamus, inhibits luteinizing hormone (LH) but not prolactin secretion from the male quail pituitary in vitro. Accordingly, the peptide was named gonadotrophin inhibitory hormone (GnIH) (1). This peptide has a transiently depressive effect on plasma LH in the white crowned sparrow in vivo (2). Immunocytochemical studies in quail (1, 3) and song sparrow (4) show that GnIH is located in cells in the paraventricular nucleus of the hypothalamus, with terminals in the median eminence, with a potential to control anterior pituitary function. The cDNA sequence encoding GnIH has been cloned in the Japanese quail (5), domestic chicken (NCBI accession number AB120325) and white crowned sparrow (2). The predicted amino acid sequence for chicken GnIH (SIRPSAYLPLRF-NH₂) differs from quail GnIH at position 3 where arginine conservatively substitutes lysine. It is unknown whether the expression of the gene encoding GnIH in the avian hypothalamus changes with reproductive state consistent with an inhibitory action on gonadotrophin secretion. Furthermore, it is not known whether, in addition to inhibiting LH release, GnIH also inhibits gonadotrophin synthesis. This appears to be possible because, by analogy, gonadotrophin-releasing hormone-I (GnRH-I) stimulates both the release and synthesis of chicken (6, 7) and mammalian gonadotrophins (8, 9). The present study investigated the role of GnIH in chicken reproductive function by determining whether GnIH decreases plasma LH concentrations in vivo, whether GnIH depresses gonadotrophin subunit mRNAs in vitro and whether there is an increase in hypothalamic GnIH mRNA associated with the atrophy of the ovary in incubating hens (10, 11) and in broiler breeders at the end of a laying period (12).

Materials and methods

Animals

All experimental procedures were carried out under UK Home Office regulations. Incubating and laying domestic hens required for studies performed in vivo were obtained from the Roslin Institute’s breeding flocks. The hens were a hybrid between White Leghorn and Silkie breeds. Pituitary glands for studies performed in vitro were from adult ISA Brown cockerels (ISA Poultry Services Ltd, Peterborough, UK) purchased at 1 day old. All birds obtained from Roslin Institute were held under a LD 16:8 h light/dark cycle with free access to food and water.

Broiler breeder hens (Cobb Vantress Inc., East Hanningfield, UK) were obtained from a flock of pedigree broiler breeders at the end of a commercial laying period at 58 weeks of age. The birds were fed a commercial restricted feeding programme to maximize egg laying as recommended by Cobb Vantress and were held under a LD 16:8 h light/dark cycle.
The objective was to investigate the effect of GnIH on LH release in nest-deprived incubating hens. Nest removal results in an increase in plasma LH (10) and could be a consequence of reduced GnIH release, which was predicted to be reversed by exogenous administration of GnIH. Quail GnIH was synthesized in the laboratory of K. Tsutsui (1), and dissolved in physiological saline and injected (100 μl) into a brachial vein of a nest deprived incubating hen. Incubating hens were given three injections of 50 μg quail GnIH per kg body weight or saline at 5.5, 6.5 and 7.5 h after nest deprivation. The dose of GnIH was chosen based on analogous experiments in the incubating hens using GnRH analogues, which suggested that injections of reproductive neuroendocrine peptides of 50 μg/kg are likely to affect gonadotrophin secretion (11). This is similar to the dose of GnIH shown to be effective in depressing LH in vivo in the white crowned sparrow (12). Three injections of GnIH were given at hourly intervals to cover the possibility that an inhibitory effect of the peptide on LH release may be secondary to a longer term inhibitory effect on synthesis. Blood samples (approximately 1 ml) were taken from a brachial vein before nest deprivation at 0 h, and at 5.5, 6.5, 7.5 and 8.5 h thereafter for LH assay. GnIH or saline injections were given immediately after withdrawing blood samples at 5.5, 6.5 and 7.5 h after nest deprivation.

Experiment 5

The objective was to test the prediction that hypothalamic GnIH mRNA is higher in out-of-lay than in laying hens, and that this is inversely related to pituitary gonadotrophin subunit mRNAs and plasma LH. Hypothalamic GnRH-I mRNA was also measured to test the alternative prediction that ovarian regression in out-of-lay hens is a consequence of reduced GnRH-I mRNA. This experiment took advantage of the natural presence of laying and out-of-lay hens in a flock of broiler breeder hens at the end of a commercial laying period at 58 weeks old. Neuroendocrine tissues were collected at a commercial broiler breeding unit. Out-of-lay hens were identified by the absence of hierarchical yellow yolky ovarian follicles and regressed oviducts, and laying hens by the presence of fully developed oviducts and six or seven yellow yolky follicles in the ovary. Whole hypothalami, containing the GnIH and GnRH-1 neurones, and pituitary glands were dissected into 1 ml of "RNA Later" (Ambion, Huntingdon UK). The neuroendocrine tissues were stored at -20 °C before RIA extraction. Blood samples for LH RIA were taken from a brachial vein. Collected blood samples were centrifuged and blood plasma was stored at -20 °C before RIA.

RNA extraction

Total RNA was extracted from neuroendocrine tissues in Matrix D tubes (Q-biogene-Alexis Ltd, Bingham, Nottingham, UK) containing 600 μl Trizol (Invitrogen Life Technologies) for pituitary fragments or 1 ml Trizol for hypothalamus. The tissues were disrupted using a FastPrep FP120 homogeniser (Q-biogene-Alexis Ltd). Final precipitation of pituitary RNA was facilitated by addition of 2 μl glycerol solution (20 mg/ml, Roche Diagnostics Ltd, East Sussex, UK). The total RNA pellet was briefly dried under vacuum and reconstituted in 100-150 μl of dH2O. The yield of RNA was quantified by measuring the optical density of a sample diluted to 1:50 at 260 nm and 280 nm, and its quality was confirmed by running a sample out on a formamide gel.

Reverse transcription of total RNA

A 4 μl sample of total RNA was reverse transcribed using a First Strand synthesis kit (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK). Reverse transcribed samples were diluted to 40 μl of dH2O.

Quantitative competitive RT-PCR assays for pituitary gonadotrophin subunits, common alpha, follicle-stimulating hormone (FSH) beta, LH beta, and for GnIH and GnRH-I

Chicken GnRH-I mRNA was measured by quantitative competitive reverse transcription-polymerase chain reaction (QC RT-PCR) (14), and the same methodology was used to develop and validate assays for gonadotrophin common α, FSHβ and LHβ subunit mRNAs, and for GnIH mRNA. Oligonucleotide primers for the amplification of FSHβ, LHβ, common α gonadotrophin subunits and GnIH were designed using the 'primer' computer package (11) (Whitehead Institute for Biomedical Research Cambridge, MA, USA) with sequences of published cDNA transcripts (Table 1). Sequence information for all these genes was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez). All primers were designed to span at least one exon-intron boundary, to allow detection of any contaminating gDNA.

All plasmids used as standards in the QC RT-PCR assays were constructed by cloning the product of RT-PCR amplification into the pBSK II + (Stratagene Europe, Amsterdam, The Netherlands) cloning vector using chicken pituitary (for common α, FSHβ, LHβ subunits) or hypothalamic (for GnIH) cDNAs as templates. Cloned fragments were transformed into XL1 Blue competent Escherichia coli cells (Stratagene Europe). Plasmid DNA was purified using a Qiagen Qiaprep Spin Midiprep kit (Qiagen Ltd, Crawley, West Sussex, UK) and sequenced to check gene identity.

Construction of competitor plasmids

Competitor plasmids were made by shortening (common α and FSHβ subunits), or by inserting a fragment of foreign DNA (LHβ subunit, GnIH) into the cloned standard cDNAs. Primer specific sequences were retained at the 5' and 3' ends to allow PCR amplification.

The common α subunit competitor was constructed using a BplI (New England Biolabs, Hitchin, UK) and AflII (New England Biolabs) double digestion of a plasmid containing the common α subunit cDNA.
enzyme digest of the cloned alpha standard. Both endonucleases cut once within the standard sequence to produce a competitor fragment of 335 bp after blunt ending and religation. The FSHβ competitor was constructed by digestion with PmlI (Roche Diagnostics) and BglI (Roche Diagnostics Ltd) followed by blunt ending and religation. Both LHβ subunit and GnIH competitors were constructed by insertion of a 200 bp fragment of a PBSK II+ vector backbone produced by a HaellI digestion (Roche Diagnostics Ltd). The LHβ standard was cut by PflM I (New England Biolabs) and GnIH standard was cut using BglI (Roche Diagnostics Ltd). Both restriction enzymes cut once within each respective gene sequence in preparation for the subcloning of the foreign DNA insert. The standard and competitor bp sizes, respectively, for each gene were: α subunit: 521 and 335; FSHβ subunit: 341 and 115; LHβ subunit: 239 and 439; GnIH: 552 and 752, and GnRH-I: 358 and 463.

**QC RT-PCR assays**

The assays were carried out in tubes containing plasmid DNA standard and competitor for standard curves, or experimental sample cDNA and the competitor.

Standard curves were made up of eight, two-fold dilutions of the respective standard plasmid. The concentration of standard plasmids ranged between $2.60 \times 10^{-14}$ and $1.79 \times 10^{-12}$ mol, depending on the assay, in a volume of 5 µl. The competitor plasmids were diluted to a concentration that fell in the middle of the standard range. A fresh standard curve and competitor dilutions were made on the day of each assay. Each tube for all assays contained standard or cDNA neuroendocrine sample (5 µl) and competitor (5 µl) made up to a PCR mix of 20 µl. The PCR assays were carried out in PCR buffer (x 10) with 1.5 mm MgCl2 (Roche Diagnostics Ltd), 2 mm dNTP (Advanced Biotechologies Ltd (Abgene), Epsom, Surrey, UK), 0.025 U/µl Taq DNA polymerase [Advanced Biotechologies Ltd (Abgene)] and 0.5 µm of assay specific forward and reverse primers (Sigma-Genosys, Cambridge, UK). The PCR amplification was carried out in a Thermo-Fast® low profile 96-well plate [Advanced Biotechologies Ltd (Abgene)] on a Hybaid MBS 0.2G programmable heating block (Hybaid Ltd, Ashford, Middlesex, UK). The PCR conditions were 30 cycles (94 °C, 20 s; 60 °C, 20 s; 72 °C, 20 s) for FSHβ, LHβ, common α subunits and GnRH-I, and 30 cycles (94 °C, 20 s; 60 °C, 20 s; 72 °C, 20 s) for GnIH. After the PCR amplification was completed, a final incubation step of 80 °C for 20 min was added to ensure heterodimers of amplified competitor and standard or cDNA were eliminated.

The product (10 µl) was loaded with 3 µl of gel loading dye [30% glycerol, 70% 1 x Tris-Acetate/EDTA (TAE), 0.4% Orange G] on a 3% agarose gel (BDH Laboratory Supplies, Poole, UK) containing ethidium bromide (0.2 µg/ml) and electrophoresed (6 V/cm) in 1 x TAE running buffer until band separation was visible. The relative amounts of cDNA in the bands were measured as a function of ethidium bromide fluorescence produced by UV illumination via a gel transilluminator at 312 nm (UVI, Cambridge, UK.), and captured using a video camera linked to a personal computer running the Multi-Analyst program (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplified cDNA in each lane was quantified using gel plotting macros in the Scion Image computer package (Scion Corporation, Frederick, MD, USA). To obtain a linear standard curve, the ratio of the two bands, standard and competitor, was calculated, log-transformed and plotted against the number of moles of standard plasmid present in each sample.

### Chicken gonadotrophin radioimmunoassays

FSH and LH were measured by homologous radioimmunoassays (15, 16). The intrassay coefficients of variation were 8 and 5.4%, respectively. Culture medium samples were diluted serially in assay diluent. The sensitivity of the FSH assay was too low to measure FSH reliably in the plasma of adult hens.

### Statistical analysis

All analyses were carried out using Genstat 6 edition (VSN International Ltd, Oxford, UK). Differences between experimental groups were considered significant at $P < 0.05$. Data from experiments 1, 2, 4 and 5, log-transformed where necessary to normalize variance, were analysed using analysis of variance (ANOVA). In Experiment 1, treatment and time were used as variables and bird as a block in the analyses. In Experiment 2, the treatment and location of pituitary fragments within a tissue culture plate were used as variables for ANOVA, followed by Student’s t-test, if appropriate, to test for significance between means. For Experiment 3, degradation of gonadotrophin subunit mRNA was calculated by subtraction of the control values from the actinomycin D-values to give the change in the concentration of mRNA due to degradation. The value for change in the concentration of mRNA was log-transformed and plotted against time. Half-lives were calculated by taking the reciprocal of the slope of the regression between the change in the concentration of mRNA and time, because a change of 1 in log-transformed data equals a decrease in concentration of a half.

### Results

**Effect of an intravenous injection of GnIH on plasma LH in nest deprived incubating hens**

Three intravenous injections of 50 µg GnIH per kg given at hourly intervals after nest deprivation of incubating hens for...
5.5 h failed to attenuate or block the increase in plasma LH observed (Fig. 1).

**Effect of GnIH on gonadotrophin subunit mRNAs and gonadotrophin secretion in vitro**

Incubation of cockerel pituitary fragments for 120 min with GnIH at doses of $1 \times 10^{-7}$ and $1 \times 10^{-6}$ M but not at $1 \times 10^{-5}$ M depressed the concentration of common α subunit mRNA (Fig. 2A). GnIH had a similar depressive effect on FSHβ mRNA, although this effect was seen with doses of $1 \times 10^{-7}$ and $1 \times 10^{-5}$ M but not $1 \times 10^{-6}$ M (Fig. 2B). By contrast, GnIH did not depress LHβ subunit mRNA (Fig. 2C). GnIH inhibited both FSH and LH release into the incubation medium at the lowest but not the highest doses (Fig. 2D,E). The experiment was repeated, and the combined results are shown.

**Estimation of the half-lives of gonadotrophin subunit mRNAs**

The half-lives of the gonadotrophin α, FSHβ and LHβ subunit mRNAs were calculated to be 8.6, 4.1 and 13 h, respectively, although the error estimates are relatively large (Fig. 3A–C).

**Comparison of hypothalamic GnIH, GnRH-I and pituitary gonadotrophin subunit mRNAs and plasma LH in incubating and laying hens**

Concentrations of common α and LHβ subunit mRNAs were lower in incubating than in laying hens (Fig. 4A) and were directly correlated with depressed plasma LH (Fig. 4B). No significant difference in the amount of FSHβ mRNA was observed between incubating and laying hens (Fig. 4A). Hypothalamic GnRH-I mRNA was lower, whereas GnIH...
Fig. 3. Estimates of the half-lives for chicken gonadotrophin subunit mRNAs. Cockerel anterior pituitary fragments were incubated in the presence or absence of 8 μM actinomycin D for 1–9 h and subunit mRNAs were measured during this period. The half-lives were calculated by taking the reciprocal of the slope of the fitted line. Regression analyses are shown for (A) the common α subunit, (b) follicle-stimulating hormone (FSH) β subunit and (c) luteinizing hormone (LH) β subunit mRNAs. Values are mean ± SEM (n = 3).

(A) α subunit  
$\frac{1}{t_{1/2}} = 8.6 ± 10.2$ h

(B) FSHβ  
$\frac{1}{t_{1/2}} = 4.1 ± 11.5$ h

(C) LHβ  
$\frac{1}{t_{1/2}} = 13 ± 7$ h

Fig. 4. Comparison of hypothalamic gonadotrophin inhibitory hormone (GnIH), gonadotrophin-releasing hormone-I (GnRH-I) and gonadotrophin subunit mRNAs and plasma luteinizing hormone (LH) in incubating and laying hens. (A) Comparison of concentrations of gonadotrophin subunit mRNAs and (B) of GnIH and GnRH-I mRNAs and plasma LH. Values are mean ± SEM (n = 5). ANOVA was performed on log-transformed data. *P < 0.05, **P < 0.01, ***P < 0.001 compared to laying hens.

(A) LHβ (x 10^{-16} M)  
FSHβ (x 10^{-17} M)  
Common α (x 10^{-17} M)

(B) Plasma LH  
GnRH-I mRNA  
GnIH mRNA

mRNA was higher in incubating than in laying hens (Fig. 4b).

Comparison of hypothalamic GnIH, GnRH-I and pituitary gonadotrophin subunit mRNAs and plasma LH in laying and out-of-lay broiler breeder hens

The concentration of LHβ mRNA was lower, whereas that of FSHβ mRNA was higher, in out-of-lay than in laying hens (Fig. 5A). The concentrations of the common α subunit mRNA (Fig. 5A) and plasma LH (Fig. 5B) was not significantly different between out-of-lay and laying hens (Fig. 5A). The concentrations of hypothalamic GnRH-I and GnIH mRNAs were not significantly different between out-of-lay and laying hens (Fig. 5A).

Discussion

This study confirms, in the cockerel, the observation in male quail (1), that GnIH inhibits LH secretion in vitro. However repeated injections of GnIH failed to suppress the increase in plasma LH that occurs in incubating hens after nest deprivation (Fig. 1). These observations are in contrast to a study in the white crowned sparrow where an intravenous injection of GnIH transiently suppressed plasma LH after 2 min but not 10 min, and an injection of 1000 ng GnIH antagonized the stimulatory effect of 10 ng GnRH on LH release (2). The sampling procedure used in the present study would not have detected a transient depression in plasma LH after 2 min. The failure to demonstrate a long-term inhibitory effect of GnIH in vivo in nest deprived incubating hens (Fig. 1) may be explained by an inappropriate dose or timing of GnIH administration. Alternatively, it is possible that the quail GnIH used in the study, which differs from the predicted sequence of chicken GnIH by a conservative substitution of lysine by arginine at position three, may be inactive in the chicken in vivo. This is unlikely because quail and white crowned sparrow GnIH are equally effective in depressing plasma LH in the white crowned sparrow (2) and the predicted sequence of white crowned sparrow GnIH...
However, in the cockerel pituitary, the concentrations were four-fold greater than levels of LHL3 in the rat (31, 32).

In agreement with the finding in the quail (1), GnIH inhibited LH release in vitro in the cockerel (Fig. 2e) and further also inhibited FSH release (Fig. 2d). This depressive effect of GnIH on LH and FSH release correlated with a depression in the concentrations of common α and FSHβ subunit, but not LHβ subunit mRNAs. It remains to be demonstrated whether this depressive effect of GnIH is a consequence of an inhibition of common α and FSHβ subunit gene transcription, or of decreased stability of the gonadotrophin subunit mRNAs. The possibility that GnIH also depresses LHβ mRNA cannot be excluded because the half-life of chicken LHβ mRNA was calculated to be 13 h (Fig. 3c) which is much longer than the 120 min experiment performed in vitro. Mammalian LHβ mRNA has a long half-life, 44 h in the rat (17), whereas the shorter, 4-8 h half-lives of common α and FSHβ subunit mRNAs calculated for the chicken (Fig. 3a,b) are similar to those reported for the corresponding mammalian mRNAs (17-21).

It is possible that the depressive action of GnIH on LH release is mediated by more than one mechanism: the first appears to be a short-acting effect, as suggested by the observations of Osugi et al. (2), whereas a second, long-acting mechanism is suggested by the observations in vitro reported in the present study: GnIH may inhibit LH release as a consequence of decreased LH synthesis associated with a decrease in constitutive LH release. The possibility that LH is released without concurrent GnRH stimulation has been reported in mammals (22, 23). Gonadotrophin subunit mRNA levels are maintained for at least 30 h after GnRH deprivation (24). In birds, this is supported by the observation that, in the male turkey, increased baseline plasma LH during sexual maturation is not associated with a change in GnRH pulse frequency or amplitude (25). It is relevant to note that baseline concentrations of LH in laying hens are not pulsatile, providing further evidence for a constitutive release of LH in the chicken (26). If GnIH inhibited LH release in vitro as a consequence of inhibition of synthesis, LH synthesis would have been depressed within 120 min of the pituitary fragment incubation experiment. There is no information on the rate of LH synthesis in birds but, in mammals, synthesis to processing and packaging of a mature LH dimer takes 1.5 h (27). This was within the time-frame of the incubation experiment and supports the view that GnIH may suppress LH release secondarily to a reduction in synthesis.

The possibility that the GnIH-induced depression in the common α subunit mRNA is responsible for the reduction in LH synthesis and release would be unlikely in mammals where the common α subunit protein is always in excess of the LHβ subunit protein (28, 29). The synthesis of gonadotrophin β subunit is therefore seen as the limiting factor for mammalian gonadotrophin synthesis (30), because pituitary concentrations of common α subunit mRNAs are three- to four-fold greater than levels of LHβ in the rat (31, 32). However, in the cockerel pituitary, the concentrations of common α subunit mRNAs and the two gonadotrophin β subunit mRNAs are similar in concentration, ranging between $1 \times 10^{-18}$ and $1 \times 10^{-19}$ M in vitro (Fig. 2a-c) and this does not take into account the concentration of TSHβ subunit mRNA, for which no information is available. The view that the common α rather than the LHβ mRNA levels is predictive of LH secretion in birds is supported by observations in Japanese quail where changes in plasma LH induced by feed restriction and refeeding were correlated with common α, but not LHβ subunit mRNAs (33, 34). This is in contrast to mammals where neither the common α or LHβ subunit mRNAs are predictive of plasma LH concentrations (35).

LH release into the incubation medium and gonadotrophin α subunit mRNA were inhibited in the presence of $1 \times 10^{-7}$ M and $1 \times 10^{-6}$ M but not of $1 \times 10^{-5}$ M GnIH (Fig. 2a,e). This correlation further strengthens the view that GnIH inhibits LH release secondarily to a depression in common α subunit mRNA. The lack of an effect of a high dose of GnIH on LH release can be ascribed to a desensitization of the gonadotrophs. By analogy, the phenomenon of desensitization has been demonstrated for the response of the chicken (11, 36) and mammalian (24, 37-39) gonadotroph to continuous GnRH-I exposure.

GnIH also inhibited FSH release in vitro (Fig. 2d), which confirmed a similar, but non-significant inhibitory effect on FSH release in vitro in the quail (1). However, it was not possible to demonstrate whether GnIH also inhibits FSH in vivo in adult hens because the chicken FSH assay was not sufficiently sensitive to measure it. The inhibitory effect of GnIH on FSH release in vitro correlated with a depression in common α and FSHβ subunits. The highest doses of GnIH did not depress FSH release, again suggesting, as for LH release, a desensitization of gonadotroph function to GnIH. The inhibitory effect of GnIH on FSH release is suggested to be a consequence of reduced common α or FSHβ mRNAs, resulting in reduced synthesis. In the chicken, FSH release is partially constitutive (40), as it is in mammals (41-43), and a reduction in FSH synthesis is predicted to result in a rapid decrease in release, as observed in the present study (Fig. 2d).

The physiological relevance of the depressive effect of GnIH in gonadotrophin secretion in vitro was evaluated by determining whether ovarian regression in the hen in two physiological conditions is correlated with changes in GnIH and gonadotrophin subunit mRNAs. In the first of two physiological conditions chosen for study (i.e. the incubating hen), ovarian regression was associated with increased GnIH mRNA, decreased common α and LHβ mRNA, and decreased plasma LH (Fig. 4a,n). This cascade of reduced neuroendocrine gene expression is consistent with the predicted increase in GnIH release into the hypophysial portal vasculature, resulting in a depression in LH synthesis and constitutive release. This is also consistent with the depressive effect of GnIH on common α mRNA and LH release in vitro (Fig. 2a,e). However, ovarian regression in the incubating hen, in confirmation of an earlier study (14), is also associated with a decrease in hypothalamic GnRH-I mRNA (Fig. 4b), which could also be responsible for decreased GnRH-I release and, consequently, decreased gonadotrophin subunit mRNA synthesis and LH secretion. In support of this view, it has been demonstrated in the chicken that GnRH-I stimulates...
common α subunit mRNA (6; N. A. Ciccone, unpublished observation). There was no significant difference in concentration of FSHβ mRNA between laying and incubating hens, although it was not possible to determine whether this was related to plasma FSH concentrations because FSH cannot be reliably measured in laying hens.

By contrast to incubating hens, ovarian regression in broiler hens at the end of a laying year was not associated with a change in plasma LH, nor in hypothalamic GnIH and GnRH-I mRNAs (Fig. 5a). No information was available for plasma FSH. Ovarian regression in these hens therefore appears to be downstream of a change in GnIH or GnRH-I gene transcription. However, it cannot be ruled out that ovarian regression is not due to altered patterns of GnRH-I or GnIH release controlled independently of steady-state concentrations of GnRH or GnIH mRNAs. However, it appears to be unlikely that ovarian regression at the end of laying year is a result of increased GnIH release and associated depressed plasma FSH release because, in contrast to the studies performed in vitro (Fig. 2), FSHβ subunit mRNA in vivo was increased with no change in common α subunit mRNA (Fig. 5a). The increase in FSHβ mRNA in the out-of-lay hens can be most readily ascribed to the removal of the inhibitory effect of circulating ovarian steroids. In support of this view, FSHβ subunit gene transcription is highly sensitive to the inhibitory effect of oestrogen and progesterone in mammals (44, 45) and, in the juvenile female chicken, pituitary FSH content is more responsive to the depressive action of oestrogen than is LH content (46). The depression in LHβ mRNA in out-of-lay birds (Fig. 5a) could be due either to removal of the trophic influence of GnRH-I or to an increase in inhibitory effect of GnIH, although there is no evidence that chicken LHβ mRNA is controlled by either neuropeptide.

In conclusion the results of this study provide sufficient evidence to suggest that GnIH may play a role in the neuroendocrine control of reproductive function. It remains to be established whether GnIH plays a pivotal role in avian reproduction. GnIH may have a modulatory function and be particularly important in the control of the onset of puberty, as well as the regulation of the timing of seasonal breeding.

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