Photoperiodic Control of Hypothalamic Gonadotrophin Releasing Hormone mRNA in Japanese Quail

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PUBLICATIONS ARISING

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E Baines, PJ Sharp, IC Dunn, T Boswell, R Talbot (2000) Is Gonadotrophin releasing hormone (GnRH-I) mRNA involved in the development of relative photorefractoriness in Japanese Quail? Abstract no. 1 Biological clocks 2000, The University of Edinburgh, Edinburgh UK.

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ABSTRACT

The research presented in this Thesis investigated the relationship between hypothalamic gonadotrophin releasing hormone-I (GnRH-I) gene expression and the photoperiodic control of reproduction in the Japanese quail (*Coturnix c. japonica*). A cDNA encoding quail GnRH-I was cloned and used to develop a competitive RT-PCR assay for GnRH-I mRNA. The assay was validated and used to quantify levels of GnRH-I mRNA in the hypothalamus in a series of photoperiodic experiments.

The first question addressed was whether a change in GnRH-I mRNA is involved in photoinduced luteinising hormone (LH) secretion, as inferred from an increase in blood plasma LH after transfer from short to long days. An increase in plasma LH was first seen 20h after dawn of the first long day and was found to be associated with an increase in hypothalamic GnRH-I mRNA. Exposure to one photostimulatory day followed by a return to short days, stimulated a surge of LH secretion which persisted for up to 10 days. This photo-induced carry "over effect" for LH secretion was not associated with a "carry over" effect for increased GnRH-I mRNA.

The second question addressed was whether the development of relative photorefractoriness induced by prolonged exposure to long day lengths is associated with a change in hypothalamic GnRH-I mRNA. Relative photorefractoriness was demonstrated by showing that quail maintained on long days (18hr light/day) for a prolonged period went out of breeding condition when transferred to 13 hr light /day, a photoperiod which is photo-stimulatory for fully photosensitive quail. The development of relative photorefractoriness in quail held on 18hr light/day was not associated with a decrease in plasma LH but was associated with a decrease in hypothalamic GnRH-I mRNA. However, this decrease should be treated with caution as it was significant when comparing ANOVA least squares means and two sample T-tests, but not with post hoc Tukey's tests. Since the development of relative photorefractoriness is associated with increased prolactin secretion, a final series of experiments investigated the possibility that prolactin might exert an inhibitory effect on hypothalamic GnRH-I mRNA. Treatment of quail with ovine prolactin for 5 days after photostimulation suppressed the photo-induced increase in GnRH-I mRNA

It is concluded that in the Japanese quail, photoinduced LH secretion is closely linked to an increase in hypothalamic GnRH-I mRNA, but part of the LH release mechanism might operate independently of continued GnRH-I mRNA production. Although the development of relative photorefractoriness is not associated with a decrease in LH secretion, the data suggest that the underlying decrease in photoperiodic "hypothalamic drive" is reflected by a decrease in hypothalamic GnRH-I mRNA. Since prolactin treatment reduced hypothalamic GnRH-I mRNA, the development relative photorefractoriness may be a consequence of increased prolactin secretion.

TABLE OF CONTENTS

1. GENERAL INTRODUCTION

1.1. REPRODUCTIVE PHOTOPERIODISM

In many birds and other animals the annual change in day length is the most important environmental cue for controlling the timing of seasonal breeding. Understanding of how seasonal photoperiodic changes are recognised and measured and how the photoperiodic signal is translated into an appropriate neuroendocrine output and gonadal development is a major scientific challenge.

Photoperiodic mechanisms controlling reproduction in animals share a number of basic components. A ***photoreceptor*** is required to detect the presence of light, a ***clock*** is required to measure photoperiod and a ***neural transducer*** mechanism is needed to convert the neural input from the clock into a ***neuroendocrine output*** (figure 1.1.).

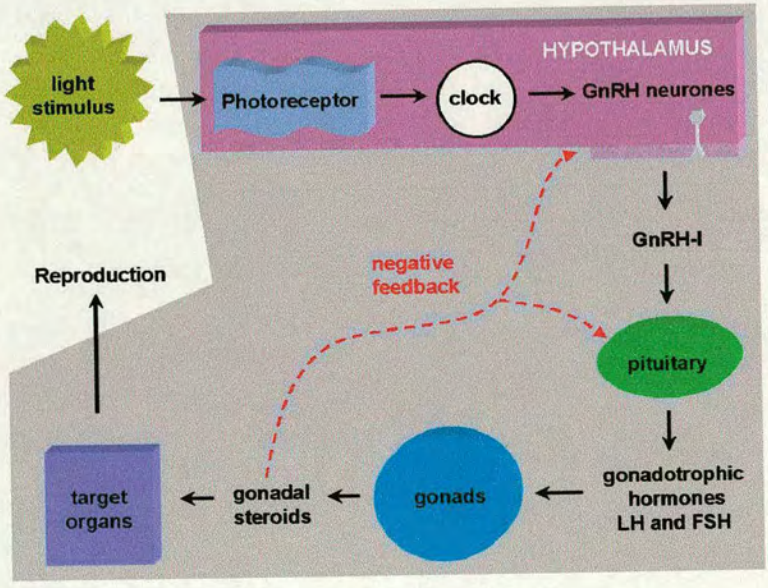


Figure 1.1. Photoperiodic pathway of reproduction: including the basic components: photoreceptor, clock and neural transduction mechanism (shown here as the GnRH neurones).

1.2. THE PHOTORECEPTOR.

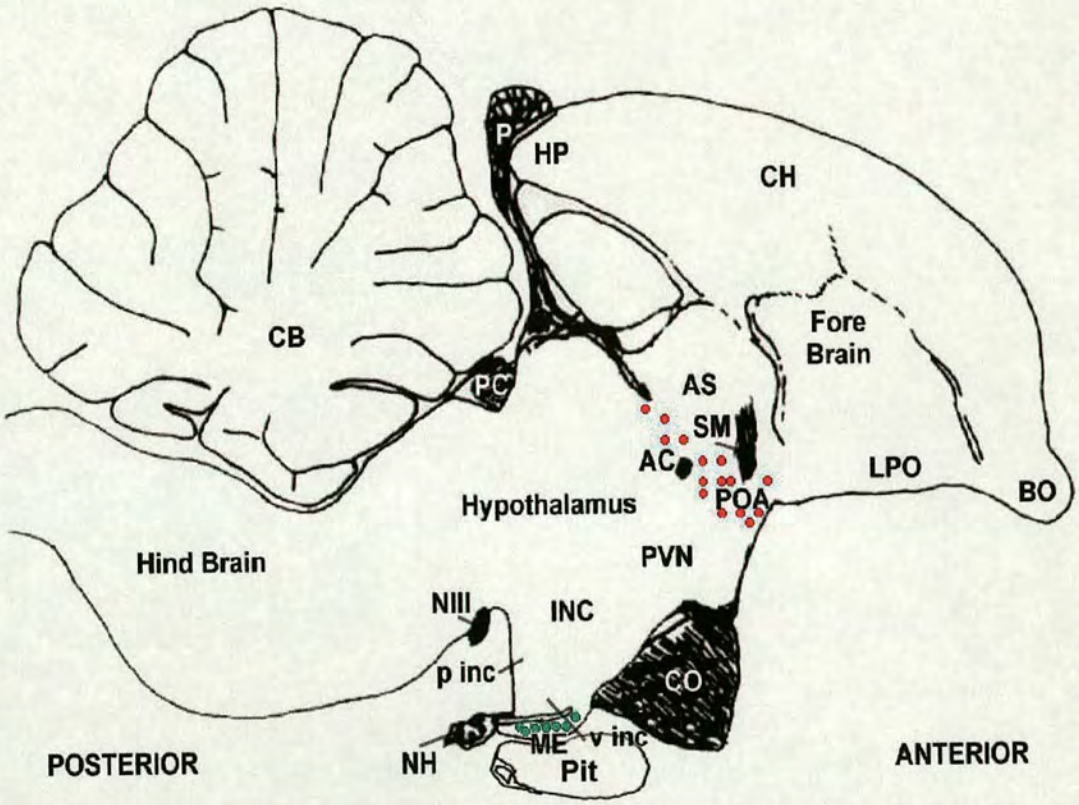
Photoperiodic photoreceptors are extra-retinal

Unlike mammals (Nelson and Zucker, 1981), birds do not need eyes for photoperiodic reproduction. The location of the avian extra-retinal photoreceptor was first hypothesised as being in the testes or feet (Bissonette, 1930) but Benoit in 1935, demonstrated that it was within the avian brain. He found that placing black hoods over the heads of ducks made them unresponsive to the gonado-stimulatory effects of exposure to long days. This response was confirmed in other photoperiodic birds for example, Japanese quail (Sayler and Wolfson, 1968; Homma et al, 1972), house sparrows (Menaker and Keatts, 1968; Menaker, 1970) and chickens (Ookawa 1970a, 1970b). In birds the photoreceptor used for photoperiodic reproduction is therefore 'extra-retinal'. Menaker, Roberts, Elliott and Underwood (1970) demonstrated that light can pass through the avian skull to reach the extra-retinal photoreceptor. They removed the feathers from the heads of a group of house sparrows, while another group had light impenetrable Indian ink painted over their skulls. Both groups were exposed to long days of dim light. After six weeks, photostimulated testicular growth was observed in birds without the Indian ink. While the testes in the Indian ink group remained undeveloped. The eyes were present in both groups and so the difference in testicular size must have been due to the amount of light passing through the skulls. Since then it has been shown that maximal gonadal growth can be induced by direct photostimulation of brain photoreceptors (Follett *et al.,* 1975; Underwood, 1975). However, work by Siopes and Wilson (1980) found that although blinded quail become sexually mature in response to transfer from short to long days, they did not

undergo testicular regression when placed back on short days. This suggests that in quail the eyes may play a regulatory role in the later stages of the photoperiodic response leading to gonadal regression and the recovery of photosensitivity (Siopes and Wilson, 1980; Oliver and Baylé 1982; Homma *et al.,* 1972).

The location of the extra-retinal photoreceptor

The location of extra-retinal photoreceptors within the brain was first suggested to be the hypothalamus by Benoit in the thirties and many experiments have confirmed this (see figure 1.1.). Micro-pellets of radio-luminous paint placed in the posterior tuberal hypothalamus stimulate gonadal growth (Oliver and Baylé, 1976 and, 1982; Saldanha *et al.,* 1994). Whereas, illumination of other brain areas, such as the anterior hypothalamus or pituitary do not stimulate gonadal growth (Oliver and Baylé, 1976; Homma *et al.,* 1980). Lesion experiments in quail also confirm the requirement for the integrity of the infundibular nuclear complex (Sharp and Follett, 1969; Davies and Follett, 1975a, b). Lesions in two areas of the quail tuberal hypothalamus are required for photoinduced gonadotrophin release: the ventral and the posterodorsal infundibular nuclear complex (v inc. and p inc.). Juss (1993) proved that the v inc. lesions did not block photoinduced LH release by destroying the GnRH-I nerve fibres. The reason for the efficacy of these lesions is unknown but could involve the disruption of photoreceptors. The photosensitive pigment opsin has been observed in cells contacting glial cells in the median eminence (Saldanha *et al.,* 1995). These glial cells may be involved in releasing GnRH from the nerve terminal in the median eminence, this is discussed in more detail in chapter 5.



AC	Anterior commissure	NIII	Third nerve, nervous occulomotorius, occulomotor nerve
AS	Septal area, area septalis, nucleus septalis medialis	P	Pineal
BO	Olfactory bulb, bulbus olfactorius	PC	Posterior commissure
CB	Cerebellum	p inc	Posterior infundibular nuclear complex
CH	Cerebral hemisphere	Pit	Pars distalis of the pituitary, adenohypophysis
CO	Optic chiasma, chiasma opticum	POA	Pre-optic area, pre-optic region, nucleus preopticus periventricularis/paraventricularis magnocellularis
HP	Hippocampus	PVN	Paraventricular nucleus
INC	Infundibular nuclear complex, infundibulum, tuberal hypothalamus	SM	Septal tract, tractus septomesencephalicus
LPO	Olfactory lobe, lobus paraolfactorius	TH	Tuberal hypothalamus
ME	Median eminence		
NH	Neural lobe, pars nervosa of the pituitary, neurohypophysis	v inc	Ventral infundibular nuclear complex

Figure 1.1. Schematic drawing of a parasagittal section of a quail brain. The red dots represent the position of GnRH-I cell bodies and the green dots represents the nerve terminals in the median eminence. Adapted from Dunn *et al.,* 1996; Mikami *et al.,* 1988; Foster et al., 1988; Juss, 1993; Perera and Follett, 1992; Follett 1982. See figure 2.3.1. to see areas taken for dissection for the experiments presented in this thesis.

It is also possible that there are photoreceptors outside the infundibular region of the hypothalamus (Oliver *et al.,* 198 ; Sicard *et al.,* 1983). Sicard *et al.* (1983) found that there was an effect of environmental lighting on the firing rates of paraolfactory

neuronal populations in quail. However, in paraolfactory disconnected birds there was no significant difference in firing rates between short and long day photoperiods, suggesting that light may be affecting paraolfactory activity indirectly.

Much of the evidence for the location of the extra retinal photoreceptor in the hypothalamus comes from immunocytochemical studies on pigments involved in photoreception.

Pigments involved in photoreception

There is a large body of evidence to suggest that opsin-like molecules are involved in the non visual deep brain photoreception in vertebrates (for review see Kojima *et al.*, 2000). In quail, green light was found to be most effective at inducing sexual maturation in blinded (Foster *et al.*, 1985) and blinded/pinealectomized birds (Oishi and Ohashi, 1993). This observation and work by Foster and Follett (1985) who found that the action spectrum of the deep brain photoreceptor closely matched the peak sensitivity and standard absorption spectrum of the pigment rhodopsin, suggests that an opsin/11-cis-retinal based photopigment contributes to deep brain photoreception in birds. Opsin-like immunoreactivity has been detected in the cerebrospinal fluid (CSF) contacting neurones in the lateral septum and infundibular region of the duck, quail and pigeon (Silver *et al.*, 1988; Foster *et al.*, 1994; Wada *et al.*, 1998). The ultra structure of these cells revealed a sensory cell-like morphology (Vigh and Vigh-Teichmann, 1973; Grace *et al.*, 1996). Wada *et al.*, (1998) detected the expression of a rhodopsin gene and rhodopsin immunoreactivity in the pigeon lateral septum and infundibular region. The pigeon nucleotide sequence of the deep

brain rhodopsin was the same as retinal rhodopsin. Red sensitive cone opsin-like immunoreactivity has also been detected in the lateral septum and tuberal hypothalamus (see Saldanha *et al.,* 1994). It is possible therefore, that there are several types photoreceptive molecules in the avian brain. The presence of transducin and phosducin like immunoreactivities have also been detected in the CSF contacting neurones of pigeons and juncos respectively in the hypothalamus (Wada *et al.,* 2000, Saldanha *et al.,* 1994). This suggests that the avian extra retinal photoreceptor shares properties with the rod/cone visual photoreceptor cascade and is located in the lateral septum and/or infundibular region.

1.3. PHOTOPERIODIC TIME MEASUREMENT

Biological clocks

All organisms have innate clocks which are used to time a wide variety of physiological and behavioural functions to synchronise them with predictable changes in environmental conditions. These internally driven rhythms may be circadian, circatidal, circalunar or circannual and in optimal conditions will continue to run at a fixed rate even when the organisms are placed in constant conditions. For example, beetles, birds and humans all continue to show biological rhythms with a period of approximately 24 hours when held in total darkness. In this situation the clocks are said to be 'free-running'. However, natural daily environments provide organisms with cues or 'Zeitgebers' that allow them to 'entrain' their clocks so that the period of a circadian rhythm will be precisely 24 hours.

Photoinducible phase

In photoperiodic organisms, including birds, the photoperiodic clock is based on a circadian rhythm of photosensitivity (e.g. Bünning, 1969; Saunders, 1982; Hamner and Enright, 1967; Follett *et al.,* 1974, Follett *et al.,* 1992). For photostimulation to occur a 'window' in the circadian cycle has to be illuminated, this is the photoinducible phase (ϕi)(Follett *et al.,* 1974; Nicholls *et al.,* 1983; Follett *et al.,* 1992, Follett 1973). In quail the photoinducible phase is approximately 12 to 16 hours from dawn (Nicholls *et al.,* 1983). There is species variance in the strength of the ϕi rhythm free running in darkness. For example, starlings and white-crowned sparrows have strong circadian oscillators (Juss *et al.,* 1995; King *et al.,* 1997, Follett *et al.,* 1974) whereas quail have weak rhythms that damp out rapidly in darkness and have oscillators that are reset by every light pulse (Follett *et al.,* 1992, Juss *et al.,* 1995; King *et al.,* 1997). For this reason Saiovici *et al.* (1987) hypothesised that the quail photoperiodic clock is based on an hour glass system and not a circadian oscillator. However, several experiments have shown that photoperiodic induction in quail is dependent on illumination of a particular phase of the circadian cycle. For example, 'resonance' experiments in which quail were exposed to a short light period combined with increasing periods of darkness, e.g. 6L:18D, 6L30D, 6L:42D, 6L:54D showed that gonadal growth only occurred when the light cycles were in multiples of 36h or 60h but not 24h (Follett and Sharp, 1969). Further Follett, Kumar and Juss (1992) were able to demonstrate the circadian nature of the photoinducible phase by gradually training quail to a light schedule of one short day and three days of darkness (SD/DD/DD/DD) over a period of one month. The quail were then treated with a single 6 or 10 hour pulse of light at the times shown in figure 1.2.1. It was

found that the LH secretion was stimulated in a rhythmic nature dependent on the circadian system as had been previously observed in white crowned sparrows (Follett *et al.,* 1974).

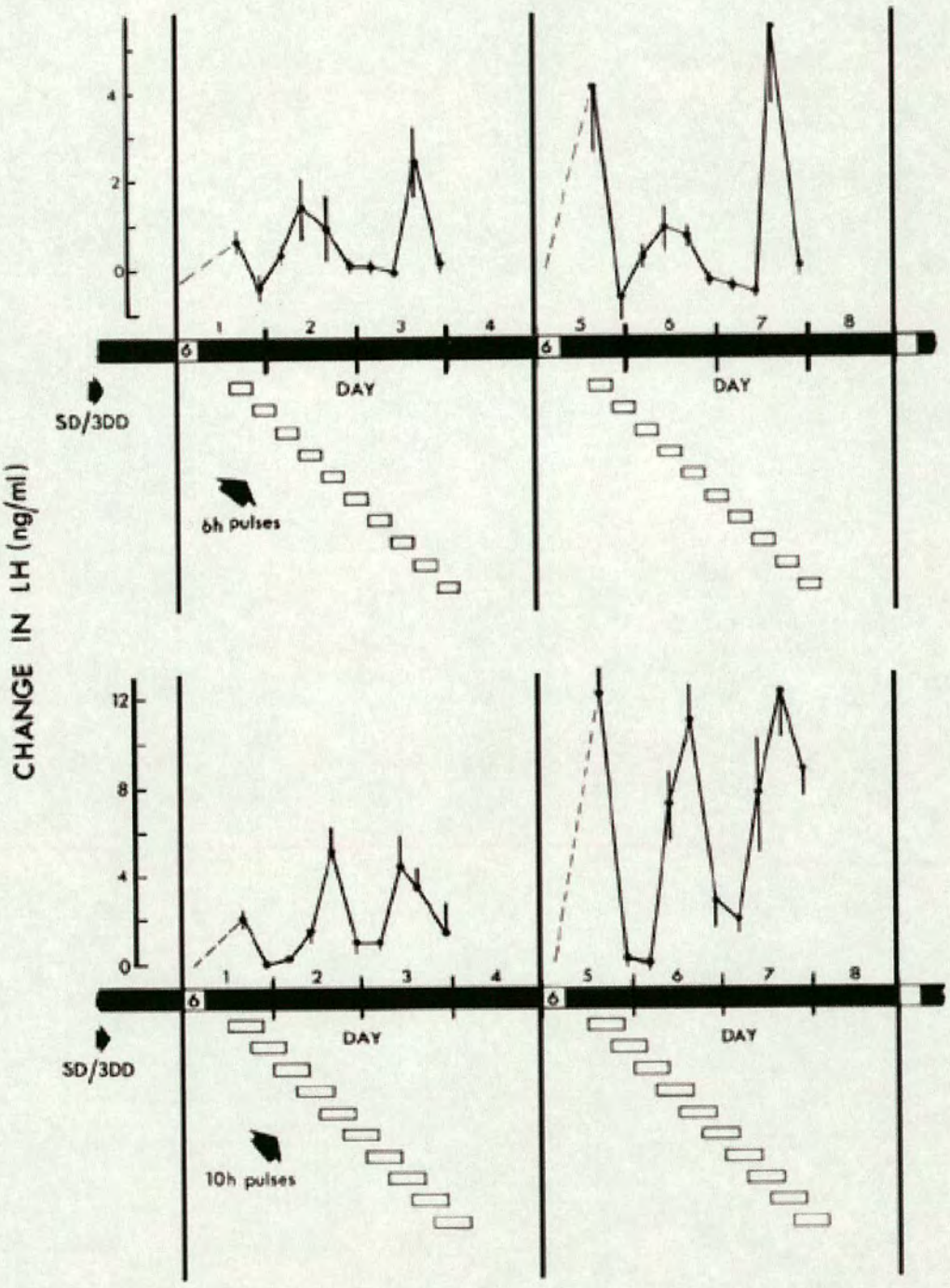


Figure 1.2.1. A resonance experiment demonstrating the circadian nature of the photoinducible phase in Japanese quail. The peak in LH occurs when the 6 hour (top) or 10 hour (bottom) light pulses coincide with the photoinducible phase 12 to 16 hours from dawn. Reproduced from Follett *et al.*, 1992.

Internal and external coincidence models

The internal coincidence model and external coincidence models explain how circadian rhythms might be involved in photoperiodic time measurement. In the external coincidence model photostimulation occurs when there is a simple coincidence between the photoinducible phase and light (Pittendrigh and Minnis, 1964). Whereas in the internal coincidence model photoperiodic induction occurs because the stimulatory day length causes a coincidence between phases of two (or more) internal circadian oscillators. In this model light received at a particular time causes the rhythms to entrain differently and the phases to coincide (Pittendrigh and Daan, 1976; Gwinner, 1973, 1974). It is very difficult to distinguish between the internal and external hypotheses experimentally. It is not until the relevant oscillators involved with photoperiodic time measurement have been identified that we are likely to be able to determine which model fits the quail.

Differences between photoperiodism in birds and mammals

Although the circadian nature of the photoinducible phase is the same in birds as it is in mammals there are differences in the mechanisms of the reproductive photoperiodic response. The mammalian 'master' biological clock is located in the suprachiasmatic nucleus (SCN) and this controls both the circadian and photoperiodic responses (Stephan and Zucker, 1972; Moore and Eichler, 1972; Ralph *et al.,* 1990). The mammalian SCN receives input about the day length from the retina and uses the information to create a circadian rhythm of melatonin secretion from the pineal gland. This circadian rhythmic secretion of melatonin drives circadian function, including photoperiodic reproduction (see Goldman, 2001 for a

review of the mammalian photoperiodic system). However, in birds it has been found that although the pineal and its rhythmic melatonin secretion is important in the generation of some circadian rhythms (Gwinner *et al.,* 1987), the pineal gland plays no major role in the avian photoperiodic response: pinealectomy in blinded or intact birds does not affect photoperiodic induction (Siopes and Wilson, 1974; Simpson *et al.,* 1983) and melatonin injections do not block photoinduced testicular growth (Follett *et al.,* 1985; Kumar, 1996).

The location of the avian neural structures equivalent to the mammalian SCN is uncertain. Retinohypothalamic projections revealed two potential sites for the avian hypothalamic pacemaker, the medial SCN (mSCN) and visual SCN (vSCN) (Hartwig, 1974; Shimizu *et al.,* 1984; Norgren and Silver, 1989a, b). Circadian activity rhythms are disrupted by lesions to both the mSCN and vSCN (Takehashi and Menaker, 1982; Cassone *et al.,* 1990) and clock genes are expressed in both locations (Yoshimura *et al.,* 2000; Brandstatter *et al.,* 2001).

1.4. NEURAL TRANSDUCTION MECHANISM

The GnRH neurones

The photoreceptor and photoperiodic clock transduce neural information into hormone output through GnRH neurones. The cell bodies of the GnRH-I neurones span from the preoptic area (POA) sweeping caudally and dorsally into the septal region of the hypothalamus (Dunn and Millam, 1998)(see figure 1.1). Long axons

extend from the cell bodies to the median eminence, where they are surrounded by glial (non-neuronal) cells. The GnRH neuronal terminals open onto a network of capillaries to release GnRH which then drains into the pituitary gland to stimulate the release of gonadotrophins. The activation of GnRH neurones after exposure to a long photoperiod may occur at the level of cell bodies or at the terminals in the median eminence. The precise mechanism controlling photoperiodic GnRH peptide release from the GnRH neurones is not known but is likely to involve a combination of separate synthesis and release mechanisms (Meddle and Follett, 1997; Dawson, 2000). To investigate the possibility of a change in peptide synthesis being involved in the induction of photoperiodic reproduction a semi-quantitative reverse transcription PCR assay (Q RT-PCR) was developed to measure GnRH-I mRNA (chapter 3) and used as a measure of gene transcription during photostimulation (chapter 5).

GnRH peptide

The structure of GnRH was first discovered in mammals (Matsuo *et al.,* 1971; Schally *et al.,* 1971; Burgus *et al.,* 1972). The decapeptide amino acid sequence of mammalian GnRH (mGnRH) was found to be highly conserved in pigs, sheep, humans, mice and rats (Seeburg and Adelman, 1984; Mason *et al.,* 1986; Adelman *et al.,* 1986). The first described avian, and non-mammalian, amino acid sequence for GnRH was in chicken (King and Millar, 1982a; Miyamoto *et al.,* 1982).

Glu^{1}-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly^{10}

Chicken GnRH-I (cGnRH) differs from mGnRH at position 8, where arginine is substituted for glutamine.

The chicken genomic GnRH-I sequence is 6.3 kilo bases in length and the intron exon structure is similar to that found in fish and mammals (Dunn et al., 1993; Dunn and Millam, 1998) and is 3002 bases from exon I to exon IV. The 4 exons encode a 5' -untranslated region, the signal peptide for GnRH, the enzymatic amidation and precursor processing site, followed by a 56 amino acid GnRH-associated peptide (GAP) which is followed by the 3' -untranslated part of the mRNA (figure 1.3.1.). The fully mature mRNA is 390 bases long. The chicken precursor or prepropeptide GnRH transitional start site is in exon II, as in mammals (Dunn *et al.,* in 1993).

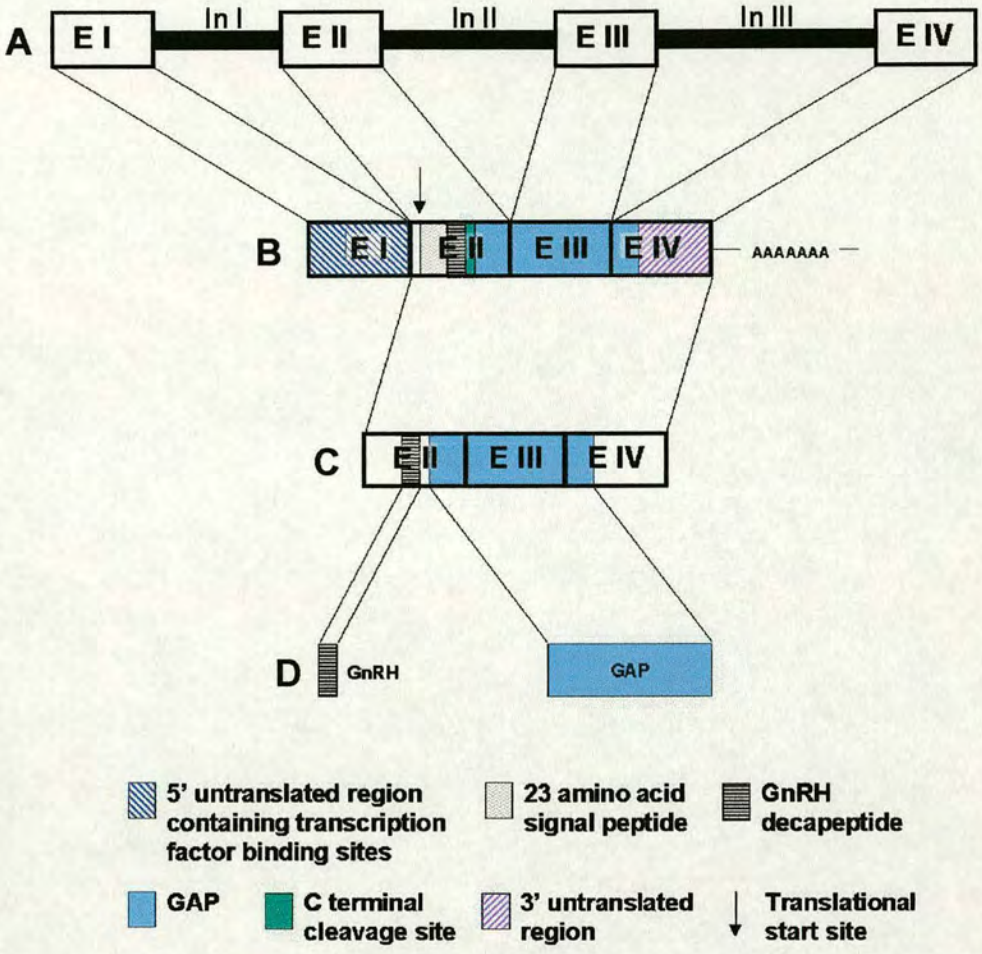


Figure 1.3.1. A diagram of the chicken GnRH-I gene showing **A)** the intron (in I, II and III) exon (E I, II, III and IV) genomic structure, **B)** GnRH-I mRNA with poly-A tail (one spliced form shown), **C)** GnRH-I prepropeptide and **D)** GnRH and GAP peptides (adapted from Dunn *et al.,* 1993 and Sun *et al.,* 2001).

Alternative forms of cGnRH-I mature RNA transcripts (and also the GnRH receptor) occur in all levels of the avian hypothalamic-pituitary-gonadal axis and in some other tissues (Sun *et al.,* 2001). The function of alternative transcripts is unknown, though the phenomenon is well documented (Dong *et al.,* 1996; Botté *et al.,* 1998; Seeburg and Adelman, 1984; Von Schalburg and Sherwood, 1999; Seong *et al.,* 1999). It is thought that they may play a role in the regulation of mRNA stability or translation.

Multiple Forms of GnRH

There are multiple GnRH genes in many vertebrates (for review see: Dunn and Millam, 1998). The roles of chicken gonadotrophin releasing hormone -I (cGnRH-I) and -II (cGnRH-II) in the physiological regulation of gonadotrophin release have been studied most extensively. cGnRH-II is also a decapeptide but it differs from cGnRH-I by three amino acids (King and Millar, 1982b, Miyamoto *et al.,* 1984).

$$Glu^{1}\text{-}His\text{-}Trp\text{-}Ser\text{-}His\text{-}Gly\text{-}Trp\text{-}Tyr\text{-}Pro\text{-}Gly^{10}$$

Both cGnRH-I and cGnRH-II stimulate the release of LH and FSH from the pituitary in vitro and in vivo (Hattori *et al.,* 1986; Sharp *et al.,* 1987; Connolly and Callard, 1987). However, there is no evidence that cGnRH-II exerts any effect on gonadotrophin release under physiological conditions. Measurements of the hypothalamic content of GnRH-I show significant changes according to different physiological conditions. For example hypothalamic cGnRH-I increases at the onset of puberty in chickens (Knight, 1983a; Knight *et al.,* 1985), during the ovulatory cycle in hens (Advis *et al.,* 1985; Sharp *et al.,* 1990), after castration (Knight *et al.,* 1983b; Sharp *et al.,* 1990, Lal *et al.,* 1990) and GnRH-I release increases from quail hypothalamic explants taken during photostimulation (Perera and Follett, 1992). Similar studies on GnRH-II have found that although hypothalamic content increases in turkeys aat the onset of lay, it does not do so in chickens (Sharp *et al.,* 1990; Millam *et al.,* 1989). In the domestic hen there are changes in GnRH-II correlated with the progesterone induced preovulatory LH surge and diurnal rhythms in plasma

1.5. THE NEUROENDOCRINE OUTPUT

Gonadotrophin Release

Until recently most information about the photoperiodic control of GnRH was inferred from changes in plasma LH. An increase in gonadotrophin secretion initiates gonadal development, FSH acts by stimulating gonadal growth, while LH stimulates the synthesis and release of gonadal steroids. In males LH stimulates the Leydig cells to release testosterone which triggers sexual behaviour and the development of secondary sexual characters. In females LH stimulates the granulosa cells of mature follicles to release progesterone and oestrogen secretion (Maung and Follett, 1977; Marrone and Hertelendy, 1983). Gonadal steroids exert inhibitory actions on gonadotrophin secretion and this creates homeostasis between gonadotrophins and gonadal steroid secretion. Gonadectomy disrupts this homeostasis and causes gonadotrophin secretion to increase (Nicholls *et al.,* 1983), and this effect can be reversed by administering gonadal steroids. The inhibitory effects of gonadal steroids are mediated at both levels of the hypothalamus-pituitary axis. They decrease the response of the pituitary gland to GnRH by reducing the expression of the GnRH receptor (GnRH-R) (Sealfon *et al.,* 1990; Wu *et al.,* 1994; Quinones-Jenab *et al.,* 1996; Cowley *et al.,* 1998; Sun et al, 2001), by directly inhibiting GnRH release (Sharp and Gow, 1983) and by changing the expression of the GnRH gene (von Schalburg and Sherwood, 1999; Spratt and Herbison, 1997; Scott *et al.,* 1997; Sun et al, 2001).

Photostimulation

Most temperate and high latitude birds are long day breeders and so under short daily photoperiods most photoperiodic birds remain sexually inactive with regressed gonads. The Japanese quail (*Coturnix coturnix japonica*) is a highly photoperiodic temperate bird and like the closely related domestic chicken, starts breeding in early spring and finishes breeding in the late summer or early autumn (Phillips et al, 1985).

Critical, marginal and saturation day lengths

In spring under natural circumstances, breeding in quail is initiated when day lengths reach about 12 hours. Sexual maturity is reached within the next 30 - 40 days in early May when day lengths are about 16.75 hours (Follett and Maung, 1978). The plasma levels of FSH rise gradually to maximal levels by late July whereas plasma LH increases more rapidly, reaching maximal levels in early May (figure 1.4.1.).

Under artificial lighting quail can be stimulated into breeding condition if transferred from short days to photoperiods of more than 11.7 hours (Follett and Maung, 1978). The minimum photoperiod required to initiate photostimulated breeding is termed the *critical day length* and differs between species. For example in the Bewick swan it is 16 hours and in the rook it is 10.4 hours. The species variance in critical day length occurs because it plays a role in the timing of the breeding season, and different birds initiate breeding at different times in spring and summer. In general the higher the latitude of the breeding site the longer the critical day length. For example, Bewick swans which breed at 65^0 N have a critical day length of 16 hours

whereas black swans breeding at 28^0 S and have a critical day length of about 11 hours (Murton and Westwood, 1977).

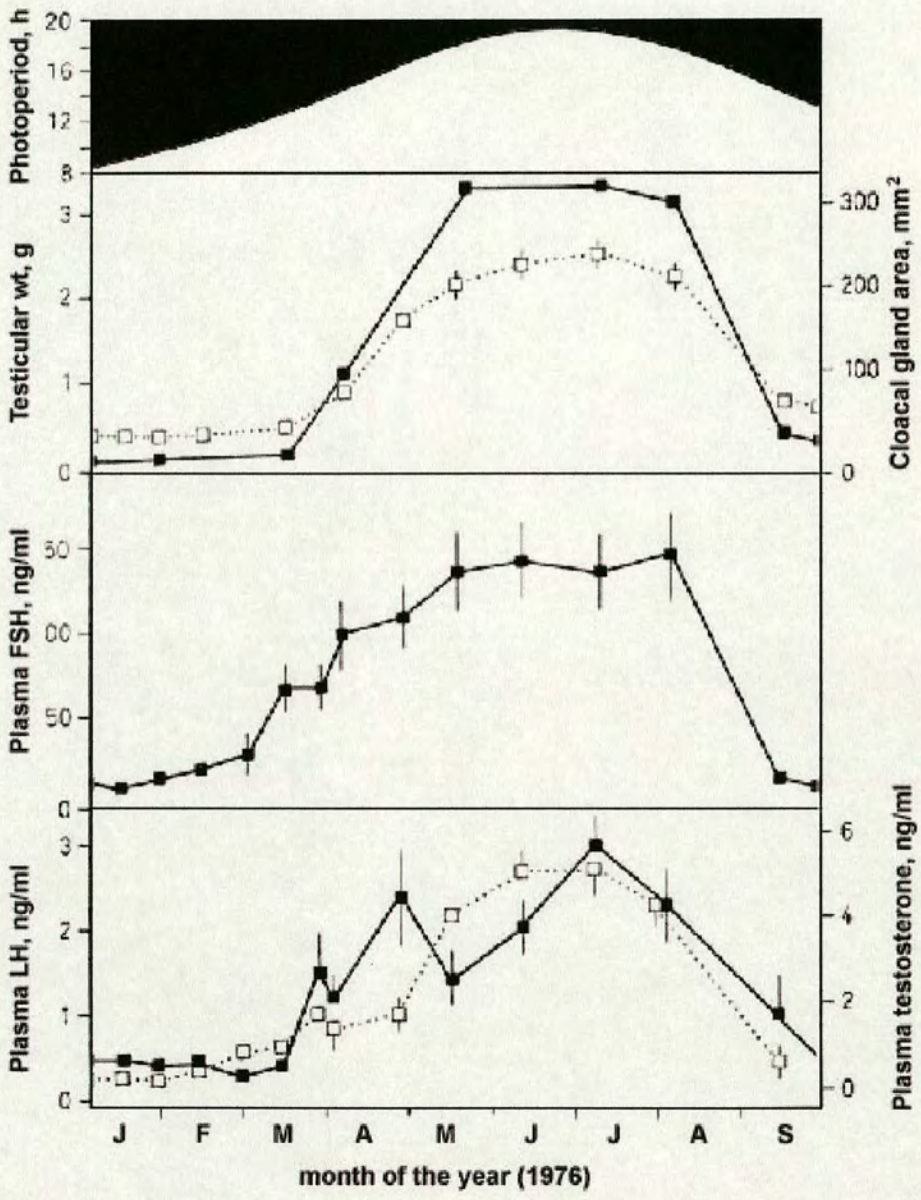


Figure 1.4.1. The seasonal changes in male quail testicular size (black circle), cloacal gland size (black circle), gonadotrophin secretion FSH and LH (solid lines) and testosterone (broken line). The top panel shows the day length. From Follett and Maung (1978).

Maximal testicular growth rates are achieved on 14L:10D, 16L:8D and 20L:4D (Follett and Maung, 1978). Japanese quail placed on long photoperiods of 16-20

hours become sexually mature within 3 weeks (Follett, 1976; Gledhill and Follett, 1976; Follett, Davies and Gledhill, 1977). This is reflected in LH levels which reach a maximum values on 14.7 to 16 hours of light, any further increase above this *saturation day length* has no additional stimulatory effect on LH secretion (Follett and Sharp, 1969; Follett *et al.,* 1977). Day lengths longer than the critical day length but shorter than the saturation day length are called *marginal day lengths*. There is a direct relationship between the duration of marginal day lengths and the magnitude of the neuroendocrine response (Urbanski and Follett, 1982). There is species variation in the range of marginally stimulatory day lengths and it is possible that this has adaptive significance in regulating the time taken to reach sexual maturation (Phillips *et al.,* 1985).

In quail moved from 8 hours to 20 hours of light a day, FSH levels increase 12 fold after 9 days of photostimulation while LH is increased 5 fold (Follett, 1976). The photoperiodic response for plasma LH is far greater in castrated than in intact quail. In castrates plasma LH increases ten-fold during the first week of photostimulation and then remains relatively stable (Follett and Maung 1978).

First day release model

The response to an increase in day length in Japanese quail is rapid resulting in a significant rise in circulating LH levels 18-20 hours after dawn of the first long day. This photoperiodic response is the basis for the 'first day release' model for photoinduced gonadotrophin secretion (Follett *et al.,* 1977). The quail is not the only bird to exhibit this first day release, the chaffinch (Follett and Robinson, 1980) and white-crowned sparrow (Follett *et al.,* 1975) also show a significant rise in gonadotrophin secretion before the end of the first long day.

The quail first day release model is useful for experimental purposes for a number of reasons. It largely eliminates entrainment problems associated with repeat light-dark cycles. In castrates the first day release response is sufficiently robust to demonstrate the position of the photoinducible phase, hours 12 to 16 from dawn (Nicholls *et al.,* 1983). And it has also made possible the determination of the timing of key events in photoperiodic induction including the presence of a lag in the system. Follett *et al.,* (1977) transferred short day quail for 8 hours of light to various longer day lengths and found that for Japanese quail to show a first day release response the photoperiod must be 14.7 hours or more. All of the necessary photoperiodic information must therefore, have been received by about 15 hours of photostimulation, yet the first rise in LH is not seen until hours 18-20 of that first long day. There is then, a lag in the endocrine system between the recognition of the long day and the first evidence of photoinduced LH release at hour 18-20 (Follett *et al.,* 1977). This lag in the system also occurs in White crowned sparrows (Follett *et al.,* 1975) and black-headed buntings (Kumar *et al.,* 1996).

The 'Carry-over' effect

If short day quail are exposed to one long day and returned to short days they exhibit increased LH and FSH induction that lasts for more than a week (Nicholls *et al.,* 1983, Follet and Nicholls, 1985b). This extended response is termed the 'carry-over' effect. The half life of gonadotrophins in the circulation of quail is approximately 20 minutes (Davies *et al.,* 1976) and so the hypothalamo-hypophysial system must continue to be active for a long period before gradually returning to its state prior to stimulation. Pituitary responsiveness to GnRH does not change when quail are photostimulated (Follett *et al.,* 1977). Therefore, the 'carry-over' effect must be due to a change in the system upstream of the pituitary. The possibility of the 'carry-over' effect being due to continued transcription of the GnRH-I gene is explored in chapter 6.

Photorefractoriness

In many photoperiodic mid- to high- latitude birds the breeding season is terminated by the development of photorefractoriness. This is a process by which photoperiods which were once photostimulatory become non-photostimulatory (Bissonette and Wadlund, 1932; Hamner, 1968; Follett and Maung, 1978). Photorefractoriness is initiated by prolonged exposure to long photoperiods (Nicholls *et al.,* 1988; Dawson *et al.,* 2001; Dawson, 2001). The development of photorefractoriness allows the termination of breeding to occur ahead of deteriorating environmental conditions associated with seasonal decreasing day lengths. This mechanism ensures no attempt is made to rear offspring in unfavourable conditions (Follett and Nicholls 1985b;

Nicholls *et al.,* 1988) and results in asymmetric breeding seasons with respect to the annual cycle of day length. For example quail are stimulated into breeding in the spring, after day lengths are about 12 hours, whereas photorefractoriness develops in autumn when day lengths fall below about 14 hours (Wada *et al.,* 1992; Nicholls and Follett, 1984; Boswell *et al.,* 1993). Mechanisms that are homologous to long day photorefractoriness also exist in seasonally breeding lemmings, which exhibit spontaneous testicular regression (Gower *et al.,* 1998; Dawson *et al.,* 2001 and for a review of mammal photoperiodism see Goldman, 2001). However the use of the term photorefractoriness in mammalian literature often refers to the loss of responsiveness to the inhibitory effects of short days (see Nicholls *et al.,* 1988).

Absolute and Relative Photorefractoriness

There are two main types of photorefractoriness in birds, absolute and relative. In 'absolute' photorefractory birds such as starlings (Burger, 1947), willow ptarmigan (Stokkan *et al.,* 1982), mallards (Haase, 1983), white crowned sparrows (Lam and Farner, 1976), canaries (Story and Nicholls, 1976), Svalbard ptarmigan (Stokkan *et al.,* 1988), LH becomes unresponsive to photoperiodic stimulation and can not be re-stimulated by longer photoperiods until the photorefractory state has been dissipated. Decreases in LH and prolactin secretion and subsequent gonadal regression will occur spontaneously in these birds if held on fixed long days. Absolute photorefractoriness develops for prolactin but not LH in castrated domestic chickens (Sreekumar and Sharp, 1998; Sharp *et al.,* 1992).

Photorefractoriness may be 'relative' as it is in quail (Follett and Maung, 1978). If quail are held on fixed long days gonadotrophin secretion and full gonadal function is maintained indefinitely (Robinson and Follett, 1982). However, a decrease in day length results in the photostimulatory input being overridden, which causes a decrease in plasma LH and prolactin. Relatively photorefractory quail placed on short days to initiate gonadal regression can be brought back into reproductive condition without the need for dissipation of the refractory state by increasing the day lengths. Under fixed day lengths the critical day length that induces regression depends on the duration that quail have been on long days (Robinson and Follett, 1982). Therefore, in order to demonstrate the development of relative photorefractoriness in quail, the birds must be exposed to a day length that is stimulatory for photosensitive birds but non-stimulatory for photorefractory birds. For quail that have become photorefractory while exposed to 18-20 hours of light, 13 hours is such a photoperiod.

Dissipation of photorefractoriness

The photorefractory state for PRL and LH is dissipated by exposure to short days (Nicholls *et al.,* 1988; Sreekumar and Sharp, 1998). Absolutely photorefractory birds transferred to short days have low concentrations of plasma LH which increase once photorefractoriness has dissipated (e.g., Wilson, 1990; Dawson, 2001). This is also the case for prolactin the great tit (Silverin and Goldsmith, 1997) and bantam (Sreekumar and Sharp, 1998). Observations of white-crowned sparrows and house finches show that the longer birds are maintained on short days the greater the gonadal growth in response to long days (Hamner, 1968; Wilson, 1990). In 1990,

Follett and Pearce-Kelly in quail demonstrated that the dissipation of relative photorefractoriness by exposure to short days is progressive. They moved relatively photorefractory quail from long days to short days and photostimulated them at various times after the change in photoperiod. They found that photoinduced LH secretion was directly proportional to the time the birds had been on short days and that the photorefractory state is completely dissipated after 5 weeks of exposure to short days.

It is concluded that both absolute and relative photorefractoriness are characterised by similar seasonal patterns in LH and prolactin secretion and are dissipated progressively by exposure to short days. However, it remains to be established whether the difference between relative and absolute photorefractoriness are due to different mechanisms controlling the GnRH neurones or whether they merely represent quantitative differences (Hahn and Ball, 1995; Dawson *et al.,* 2001). In absolute photorefractory birds there is a decrease in hypothalamic content of GnRH whereas there is no decrease in relatively photorefractory Japanese quail (Foster *et al.,* 1988; Dawson *et al.,* 2001; Bluhm *et al.,* 1991). The possibility that development of relative photorefractoriness is correlated with a change in GnRH-I gene transcription in photorefractory quail, as inferred from changes in hypothalamic GnRH-I mRNA levels, is explored in chapter 7.

Prolactin and photoperiodism

The development of photorefractoriness for LH secretion is initiated before that for prolactin in both absolute and relative photorefractory birds (Stokkan *et al.,* 1988;

Boswell *et al.,* 1993; Boswell *et al.,* 1995) and as LH begins to decrease there is a seasonal peak in prolactin concentration (Dawson and Goldsmith, 1982, 1983; Ebling *et al.,* 1982; Sharp and Sreekumar, 2001). The association between this peak and the onset of photorefractoriness strongly implies that increased prolactin secretion is involved in the development of reproductive photorefractoriness. In birds the hypothalamic factor which induces the release of prolactin from the pituitary is vasoactive intestinal polypeptide (VIP) and active immunisation against VIP inhibits prolactin secretion (Macnamee *et al.,* 1986; El Halawani *et al.,* 1996; Sharp *et al.,* 1989). Active immunisation against VIP in European starlings (Dawson and Sharp, 1998) did not prevent the development of reproductive photorefractoriness but did significantly delay its development. This suggests that the role of prolactin is to adjust the timing of the end of the breeding season. Also consistent with this hypothesis is the lack of a seasonal peak in plasma prolactin in the ring dove which breeds all year round, except for during the moult (Lea *et al.,* 1986). However, it remains to be established, how and why plasma prolactin alters the timing of seasonal gonadal regression (Sharp and Sreekumar, 2001). It is suggested that seasonally increased prolactin could act on the GnRH neurones to depress GnRH-release by depressing GnRH-I gene expression. This was investigated by measuring GnRH-I mRNA in prolactin injected and vehicle injected quail and this is discussed in chapter 8.

1.6. SUMMARY

In Japanese quail the precise location and nature of the extra-retinal photoreceptors involved in photoperiodic reproduction are beginning to be elucidated by studies on opsins and the cells containing them. The location and mechanisms governing the biological clock/s measuring photoperiodic time are uncertain. Studies on clock genes and their expression are providing new means with which to investigate the clock. The neural mechanism that transforms the neural information into endocrine output is also not fully understood. The research presented in this thesis is centred upon this latter part of the photoperiodic reproductive system and investigates the effect of photoperiodic induction and refractoriness on the expression of the GnRH-I gene as inferred from changes in GnRH-I mRNA.

1.7. SUMMARY OF OBJECTIVES AND HYPOTHESES

The overall objective of this thesis was to investigate the possibility that photoperiodically induced changes in reproductive function in Japanese quail involve changes in GnRH-I gene transcription.

The specific aims were:

1. to clone the quail GnRH-I gene and use the knowledge of its DNA sequence to develop and validate a competitive reverse transcription PCR assay for GnRH-I mRNA. This research is presented in chapter 3.

2. to test the hypothesis that the GnRH-I mRNA transcripts exist in many non-reproductive tissues in quail but in extremely small numbers. The result of this work is presented in chapter 4.

3. to investigate the hypothesis that photoperiodically induced GnRH-I peptide release associated with a change in GnRH-I mRNA at the time of photoinduction. The experiments investigating this hypothesis are presented in chapter 5.

4. to establish whether the carry over effect of photoinduced LH is due to continued GnRH-I gene expression. This is addressed in chapter 6 by measuring changes in GnRH-I mRNA in quail that have been exposed to a single long day and compared to the time course of photoinduced LH release.

5. To test the hypothesis that relative photorefractoriness is a consequence of reduced GnRH-I gene transcription. To address this GnRH-I mRNA was measured in photorefractory quail and compared to photosensitive controls. The results of this experiment are described in chapter 7.

6. To determine whether increased plasma prolactin is responsible for the development of relative photorefractoriness by depressing GnRH-I gene transcription. GnRH-I mRNA was measured long and short day quail injected with either prolactin or vehicle. The results of this experiment are reported in chapter 8.

2. MATERIALS AND METHODS

2.1. ANIMALS AND HUSBANDRY CONDITIONS

The birds used in these experiments were a highly photoperiodic strain of Japanese quail (*Coturnix coturnix japonica*) hatched in the Roslin Institute. This strain has been selectively bred to remain non-reproductive when held on short days for a prolonged amount of time. The majority of these quail exhibit a rise in LH on the first day of photostimulation, and have been described as a 'first day release model' (Follett *et al.,* 1977). The quail were reared in brooder cages for one to three weeks on long days and then transferred to individual cages. All the birds used in these experiments were kept at an ambient temperature of 18 ± 5^0C. The light intensity in the middle of the rooms was 250 - 350 lux, measured using a Megatron DL 3 light meter. Birds were ringed with numbered and coloured split plastic rings (A. C. Hughes Ltd, Middx, UK) for identification purposes.

2.2. COLLECTION OF PLASMA SAMPLES

Blood samples were taken from live quail by wing venipuncture. For this procedure the brachial vein was pricked with a sterile needle and the escaping blood was collected with heparinised capillary tubes (Hawksley and Sons Ltd, Sussex, UK) and emptied into 1.5 ml microcentrifuge tubes (Greiner, UK). In cases where only one sample was taken from an individual quail, a volume of 500µl of blood or less was collected. When serial bleeds were necessary no more than 200 µl of blood was removed and on not more than three occasions in one week. This is in compliance with home office regulations. Blood samples taken from birds at the time of

dissection were taken from the neck stump after decapitation and collected into 15 ml plastic tubes (Greiner) containing 20 µl of PUMP-HEP® (Leo Laboratories Ltd., Bucks, UK), a heparin sodium solution of approximately 20 units per µl. The blood samples were kept on ice until spun at 1000 x g for 10 minutes in a bench top centrifuge. The plasma fractions were then removed and stored at -20^0C until assayed.

2.3. DISSECTION OF QUAIL AND COLLECTION OF TISSUES

Birds were killed by decapitation and the specific tissue samples were dissected, weighed, snap frozen in liquid nitrogen and then stored in a -80 ^{0}C freezer. This process was done as quickly as possible to minimise RNA degradation in the tissues prior to mRNA quantification. One disposable scalpel blade was used per quail brain to minimise cross contamination between animals.

2.3.1. Dissection of brain tissues

The brain tissues dissected for experiments were: the pituitary, the cerebellum, the cortex, the pre-optic area (POA) and the basal hypothalamus (BH). In most of the experiments just the POA and BH were used (figure 2.3.1.). The POA tissue samples weighed approximately 60 mg and included all of the GnRH-I neurone cell bodies. The basal hypothalamus weighed approximately 20 mg and included the median eminence with the GnRH-I neurone terminals (Lal e*t al.*, 1990). The dissection of the POA and BH were as outlined for the chicken brain (Dunn *et al.*, 1996). The pituitaries were taken whole and because of their small size were processed as male and female pools.

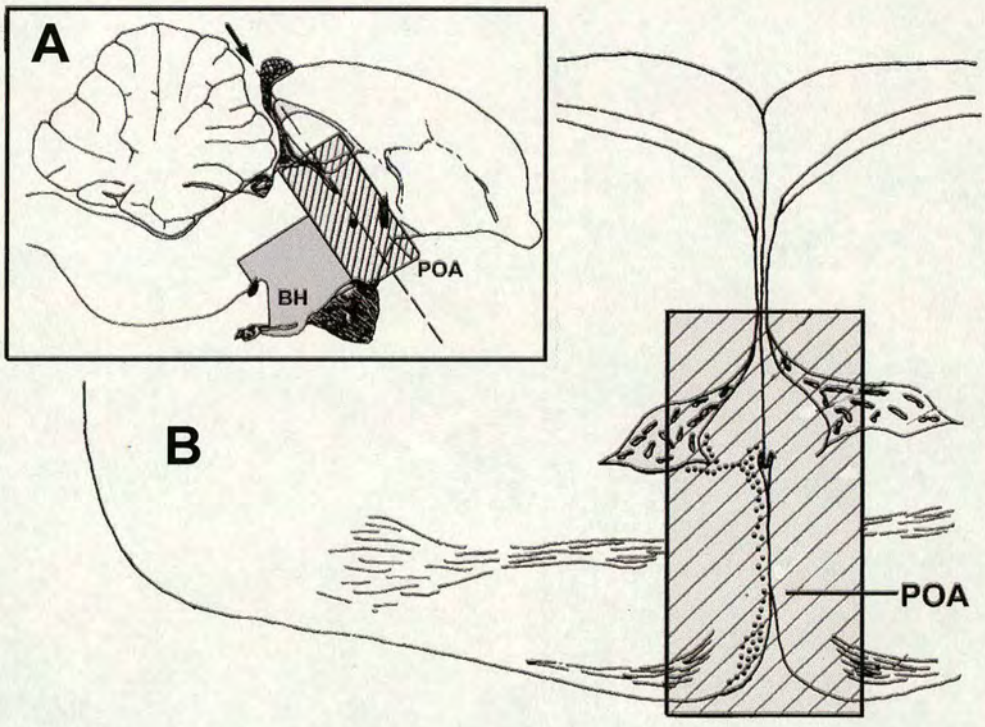


Figure 2.3.1. A diagram showing the POA and BH samples taken for GnRH-I mRNA analysis in the Q RT-PCR. The dashed line in diagram **A**, marked by the arrow, corresponds to the plane of the transverse section in diagram **B**. The striped area in **A** and **B** represents the tissue taken which includes the pre-optic area (POA) and the grey area in **A** represents the tissue taken which includes the basal hypothalamus (BH). Picture taken from Dunn *et al.,* (1996) with slight modification.

2.3.2. Dissection of other organs

For experiments investigating the distribution and quantification of GnRH-I mRNA, tissue samples were taken from, testes, ovary, theca and granulosa cell layers from mature ovarian follicles, spleen, liver, kidney and small intestine. The testes tissue samples were approximately 80 mg blocks taken from the dorsal side of the largest testes. The sample referred to here as the ovary included the cortex, medulla and stroma, but not yolky follicles and weighed approximately 60 mg. The theca and granulosa cell layers were taken from the largest pre-ovulatory follicle (F1) and weighed approximately 100 mg and 20 mg respectively. The spleen was taken whole

and varied from 30 to 90 mg. The samples of small intestine were taken from the jejunum and were approximately 2-3 mm in length and weighed approximately 80 mg.

2.4. HORMONE MEASUREMENTS

LH and PRL measurements were made using radioimmunoassay of plasma samples. All radioimmunoassays used a radioimmunoassay diluent.

Radioimmunoassay diluent

160 ml 0.5 M phosphate buffer, pH 7.5

17.5 g NaCl

5.84 g EDTA (disodium salt)

2 g sodium azide (BDH)

40 ml horse serum (GIBCO)

H_2O MilliQ to make a final volume of 2 litres.

pH adjusted to 7.5 using 1 M NaOH

2.4.1. Radioimmunoassay of quail LH

The quail radioimmunoassay procedure was a micro-modification of the chicken radioimmunoassay method first described by Sharp *et al.* in 1987. The LH used for the standards and iodinations was a preparation of the native chicken LH (code RI-LH-1) which was also first described by Sharp *et al.* (1987). The assay sensitivity was 10 pg as measured by the ED80 and the inter- and intra- assay coefficients of variation were 16.5 % and 18.1 % respectively.

2.4.1.1. Preparation of radiolabelled LH

All reagents used in the preparation of the radiolabelled LH were dissolved in 50mM sodium phosphate buffer, pH 7.5. Before the start of the iodination a PD-10 Sephadex G-25 column (LKB-Pharmacia) was equilibrated with Tris/tween/gelatin buffer.

Tris/tween/gelatin buffer

0.1M Tris/HCl pH7

0.9% NaCl

0.1% tween 20 (company, address)

0.1% Na Azide (NaN_3)

0.2% gelatin

2.5ug of LH (LH-RI-1) in a 1.5 ml microcentrifuge tube was set up in a lead shielded area and clamped over a magnetic stirrer. A mini stirrer bar was added to the tube and kept spinning throughout the reaction. Magnetic stirrer bars were pieces of paperclip approximately 2mm in size. Next, 20µl of phosphate buffer and 37 Mbq of [^{125}I]-NaI (IMS-30, APBiotech) in 10µl were added to the LH. The radiolabelling reaction was started by the addition of 10µl of fresh 3.55mM chloramine T sodium salt (Fisons). This was mixed for exactly 45 seconds before the reaction was stopped by adding 100µl 5.26mM Sodium metabisulphate (NaS_2O_5, Fisons) and 100µl of 0.6M potassium iodide (KI, Fisons, Loughborough, UK). The contents of the reaction tube were then added to the equilibrated PD-10 column. The reaction tube

was then washed with 250µl of column buffer, which was also added to the PD-10 column. Once the contents of the reaction tube had entered the column, the column reservoir was topped up with column buffer. The drips from the PD-10 column were then collected into LP4 plastic tubes (Denley-Luckham Ltd, UK), approximately 15 tubes were collected and each tube contained 13 drips (approximately 0.5 ml). The column was refilled during this process with column buffer when necessary. The amount of gamma radioactivity in each tube was then counted using a Mini-Assay type 6-20 counter (Mini-Instruments Ltd, Essex, UK). The first peak of radioactivity in these tubes contained the labelled LH and these fractions were pooled and diluted in radioimmunoassay diluent at approximately 400 000 counts/minute/10 µl and stored for up to 4 weeks at 4°C.

2.4.1.2. Preparation of standards

Stock LH was stored lyophilised in 192ug aliquots and was reconstituted into a 192ug/ml solution with 50 mM phosphate buffer (1 ml) for use in the assay. Further dilutions of this stock were made using radioimmunoassay diluent as shown in the table below (Table 2.4.1.2).

Table 2.4.1.2 Dilutions of LH standards for RIA.

	LH	Diluent	µg/ml	pg/tube
1	26 µl of stock	974 µl diluent	5	
2	100 µl of #1	900 µl diluent	500	
3	400 µl of #2	3.6 ml diluent	50	1000
4	1.6 ml of #3	0.4 ml diluent	40	800
5	1.0 ml of #3	1 ml diluent	25	500
6	1.0 ml of #4	1 ml diluent	20	400
7	1.0 ml of #6	1 ml diluent	10	200
8	1.0 ml of #7	1 ml diluent	5	100
9	1.0 ml of #8	1 ml diluent	2.5	50
10	1.0 ml of #9	1 ml diluent	1.25	25
11	1.0 ml of #10	1 ml diluent	0.625	12.5
12	1.0 ml of #11	1 ml diluent	0.3125	6.25

2.4.1.3. Radioimmunoassay procedure

The radioimmunoassay procedure took four days to run. On the first day the LH, either as plasma samples or standards (20 µl each), and anti-chicken LH primary antibody (20 µl, code: α-LH118 diluted 1:19000 in radioimmunoassay diluent) were added to plastic LP2 tubes (Denley-Luckham, UK), vortexed and then incubated overnight at 4°C. On the second day the radiolabelled [125I]-LH (20 µl) was added to all of the tubes to give an approximate count of 12000 cpm per tube and again the tubes were vortexed and incubated at 4°C overnight. On the third day, the second antibody (20 µl, donkey anti-rabbit serum (DARS), 1:20 dilution, Scottish Antibody Production Unit) and serum (20 µl, normal rabbit serum (NRS), 1:200 dilution, Scottish Antibody Production Unit) were added, vortexed and incubated overnight at 4°C. On the fourth day all of the tubes except for the total counts were centrifuged (Sorvall RC-3B, Dupont (UK) Ltd) at 2000 x g for 30 minutes at 4°C. To the spun tubes a 6% starch solution (50 µl) was added and the tubes were then centrifuged again for 20 min. The supernatant was then aspirated and the tubes were counted for

60 sec on a gamma counter (1277 Gamma Master, LKB-Pharmacia). The count data was analysed using the programme AssayZap™ (AssayZap Universal Assay Calculator, Biosoft). All reagents were dispensed with a Hamilton Microlab-500 automated dispenser (Howe and Co Ltd.) except for the starch, which was dispensed with a 125 µl multi-dispensing pipette (Eppendorf, Cambridge, UK).

Table 2.4.1.3. Radioimmunoassay procedure.

Tubes	Day 1	Day 2	Day 3	Day 4
Total Counts (T)	_	Labelled Ag	AB^2 + NRS	No spin or starch. Just step 5. Below.
Non-specific Binding (NsB)	40µl of diluent	Labelled Ag	AB^2 + NRS	1. Spin for 30 minutes at 2000g at 4^0C. 2. Add 50µl of 6% starch solution. 3. Spin for 20 minutes at 2000g at 4^0C. 4. Aspirate supernatant. 5. Count on a gamma counter.
Total Binding (Bo)	AB^1 + diluent	Labelled Ag	AB^2 + NRS	
Standards (1-12)	AB^1 + Ag	Labelled Ag	AB^2 + NRS	
Quality controls (QC)	AB^1 + Ag	Labelled Ag	AB^2 + NRS	
Unknown samples	AB^1 + Ag	Labelled Ag	AB^2 + NRS	

20µl of each reagent in the table was added, unless stated otherwise. The final volume in all tubes, except for the total counts, was 100µl.

Abbreviations: AB^1; native chicken LH antibody raised in rabbit – code αLH118, AB^2; Donkey anti-rabbit serum (DARS), NRS; normal rabbit serum, Ag; LH standard or plasma sample as appropriate.

2.4.2. Radioimmunoassay of quail PRL

The procedure for measuring PRL in quail plasma was a micro-modification from Talbot and Sharp (1994). The recombinant-derived chicken prolactin used for the standards and radio-iodination was as described by Talbot and Sharp (1994). The sensitivity of the assay as measured by ED80 was 30 pg/ml. The inter- and intra-assay coefficients of variation were 16.5 % and 19.6 % respectively.

2.4.1.1. Preparation of radiolabelled quail PRL

The following reagents were mixed :

5 µg chicken prolactin	15 µl
0.25 M Na phosphate buffer	100 µl
37 MBq [125-I]- NaI	5 µl
25 u/ml Lactoperoxidase	10 µl
0.002 % hydrogen peroxide	10 µl

Then 15 µ of 1.2 mg/10 ml Cysteine was added. This was mixed for 20 seconds before applying to PD-10 column (APBiotech) equilibrated with elution buffer 2.

0.5ml samples were collected into tubes containing 50µl of glycerol. Tubes are counted in single well Geiger tube to confirm iodination. The radioactive peak eluted between 2 and 4 ml is mixed with the glycerol in the tubes and applied directly to a 30 x 1.6 cm column of Sephacryl HR200 (APBiotech). The column is equilibrated and eluted using elution buffer 1. The flow rate is 1ml/min and 1ml fractions are

collected. The elution is monitored by counting the radioactivity i the fractions using the single tube Geiger counter. Fractions eluted between 37 and 41 minutes are taken for use in the assay.

Elution buffer 1	Elution buffer 2
0.1M Tris/HCl pH7.5	Elution buffer 1 containing 0.2% gelatin
0.15M Sodium chloride	
0.1% Tween 20	
0.1% Sodium azide	

2.4.1.2. Preparation of standards

PRL for use in the assay was made within two to three weeks of each assay by Richard Talbot and was stored in 50 mM phosphate buffer at 4 ^{0}C. The PRL was diluted to 5ug/ml for the top standard and was serially diluted by a factor of 2 for the subsequent 13 standards using radioimmunoassay diluent.

2.4.1.3. Radioimmunoassay procedure

For PRL radioimmunoassay procedure see table 2.4.1.3 but substitute abbreviations AB^{1} for native chicken PRL antibody raised in rabbit – code 31/1, and Ag for PRL standard as appropriate. The PRL AB1 31/1 was used at a dilution of 1:4000 and was diluted with radioimmunoassay diluent.

2.5. EXTRACTION OF RNA FROM QUAIL TISSUE

Total RNA was prepared using a commercial modification of the method of Chomczynski and Sacchi (1987). Individual tissue samples were homogenised in a volume of 900 µl of RNAzol B (AMS Biotechnology UK Ltd., Witney, Oxon, UK) using a polytron (Kinematica GmbH, Kriens, Luzern, Switzerland). Sample size in each case was approximately 60 mg. Once the tissue was homogenised, a further 900 µl of RNAzol B was added to the tubes. The tubes were then thoroughly vortexed, left on ice for 15 minutes and vortexed again before adding 180 µl of chloroform ($CHCl_3$). Then the tubes were vortexed, left on ice for 15 minutes and vortexed again before spinning in a bench top centrifuge for 15 min at 100 x g (13000 rpm) and 700 µl of the top phase was removed into a 1.5 ml microfuge tube. To these tubes 700 µl of isopropanol alcohol (IPA) was added and they were then frozen at –20 ^{0}C for a minimum of 15 minutes, to solidify the RNA precipitate. The tubes were then centrifuged for 15 min at 100 x g (13000 rpm) to pellet the RNA. The supernatant was then poured off and 1 ml of 75 % alcohol added to wash the RNA pellet. The tubes were then centrifuged again and the wash repeated with 75 % ethanol until the RNA pellet had been washed three times. After the last wash had been tipped off the pellets were dried in a vacuum desiccator for approximately 30 min. The total RNA isolated was dissolved in 120 µl of milliQ H_2O (Ultra Pure Water System, Milli Pore, Herts, UK).Once the mRNA was extracted a quality check was carried out with gel electrophoresis and optical density readings to insure that the mRNA had not been degraded by RNAses and was of a good and pure quality. The optical density

readings also provided a quantification of the total mRNA in μg/μl and were used in the validation of competitive assays and quantification studies (see chapters 4 and 5).

2.5.1.1. mRNA Formaldehyde Gel Electrophoresis

To 90 ml of heated dH_2O; 1.5 g agarose (1%), 60 ml gel-running buffer and 54 ml formaldehyde were added, swirled and poured into a large gel tray.

3.5 μl of RNA (approximately 3.5 μg) of each sample was placed in a 96 well plate (Advanced Biotech, California, USA) or PCR Microstrip (Eight 0.2 ml tubes with caps, Advanced Biotech) and the following were added:

2 μl gel-running buffer

3.5 μl formaldehyde

10 μl formamide

1 μl ethidium bromide

The samples were then heated at 65 ^{0}C for 15 min in a PCR machine and loaded into the gel immediately. The tank buffer was 1 part gel-running buffer to 4 parts dH_2O. The gel-running buffer used for the formaldehyde gel was made as follows:

Gel-running buffer

20.6 g 3,N-Morpholimopropanesulfuric acid sodium salt (MOPS)

13.3 ml 3 M sodium acetate pH 7

10 ml 0.5 M EDTA pH 8

All solutions were autoclaved and made up to 1 litre with dH_20 and filtered.

2.5.1.2. mRNA Non-Formaldehyde Gel Electrophoresis

For the non-formaldehyde gel all reagents and bench areas were kept free of RNAse with alcohol and Rnase AWAY® (Molecular Bioproducts Inc., address) or RNAseZap® (Ambion, Cambridge, UK). The gels ranged between 1 and 2% agarose and were made in 10mM phosphate buffer (pH 6.8). 2 µl of ethidium bromide solution (5 mg/ml) was added per 100 ml of gel mixture prior to pouring. The electrophoresis buffer was a 10 mM phosphate buffer (pH 6.8) with 2 µl of ethidium bromide solution (5 mg/ml) per 100 ml and was re-circulated throughout the electrophoresis. Electrophoresis was carried out at 3-7 V/cm. To 5 µl of RNA sample (approximately 5 µg) 1 µl of gel loading buffer (see below) was added and the samples were then heated at 75 ^{0}C for 5 minutes and loaded into the gel immediately.

RNA Gel Loading Buffer

0.25% bromophenol blue

0.25% xylene cyanol

30 % glycerol

1.2% SDS

60 mM sodium phosphate pH 6.8

2.5.1.2. mRNA Optical Density Measurements

Optical density readings at 260 and 280 nm were made on freshly extracted mRNA diluted to 1/50. The ratio between each pair of readings was determined and compared to pure RNA, which has a ratio of 2 (OD_{260}/OD_{280}). In this project the ratios ranged between 1:8 and 2. For a 60 mg piece of tissue the quantity of total mRNA ranged between 0.6 and 1.0 μg/μl. The quantity of total mRNA in the sample was determined by multiplying the 260nm reading by 40 because an OD reading of 1 corresponds to approximately 40 μg/ml and by the dilution factor (50). This figure is then divided by 1000 to give the total in μm/μl.

$$(OD_{260} \times 40 \times 50) \ / \ 1000 = \text{total mRNA } \mu m/\mu l$$

2.6. REVERSE TRANSCRIPTION PCR OF mRNA

Reverse transcription polymerase chain reaction (rt-PCR) was performed using a first strand synthesis kit (Pharmacia Biotech Ltd., Buckinghamshire, UK). The primer used for reverse transcription of mRNA for use in the competitive PCR assays was the not I-d$(T)_{18}$ bifunctional primer supplied in the kit:

5' -d[AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}] -3'

This primer and the Frohmann adapter primer (Frohmann et al., 1988 & 1989) were used in attempts to sub-clone mRNA from within genes to the polyA+ tail. This is possible because both the not I-d$(T)_{18}$ and Frohmann primers hybridise to the polyA+ tail of all mRNA and add a unique sequence onto the ends of the cDNA. This unique sequence of the resulting cDNA can then be used as a template for one half of a primer pair in subsequent PCR reactions. The reverse-transcribed sample was diluted by a factor of 5 before PCR.

2.6.1. Test of Reverse Transcribed RNA for QC-PCR

It was necessary to prove that negative PCR in tissue distribution analyses were truly negative and not just due to unsuccessful reverse transcription (chapter 4). Therefore, the success of the reverse transcription was confirmed by PCR for prolactin receptor (PRL-R) (figure 2.6.1.). PRL-R is known to be present in the all tissues examined in chickens (Ohkubo *et al.*, 1998). This was not necessary for experiments in subsequent chapters where only the POA or BH were used because GnRH mRNA was detected in every sample. The PRL-R primers used were:

PRL57F 5'-[dCCCAATTCCTGCTACTTTGA]-3'

PRL334R 5'-[dCTCTCCTTCTTCAGGCTTCA]-3'

2.7. SEQUENCING OF GENES FOR USE IN COMPETITIVE PCR ASSAYS

Primers were designed to chicken sequences from the labs previously obtained sequence data. If the resulting products proved to be the expected size or similar (allowing for differences between the species), the products were sub-cloned and sequenced.

2.7.1. Sub-cloning cDNA

2.7.1.1. Ligation Reactions

DNA generated by PCR was blunt-ended by incubation with 2 units of Klenow fragment of DNA polymerase (Amersham Pharmacia Biotech UK Ltd (APBiotech), Bucks, UK.). This was then inserted into the blunt ends of a linearised pBluescript® II SK+ (Stratagene, Amsterdam, Netherlands) plasmid which had been digested with the restriction enzyme *EcoRV* (Amersham Pharmacia Biotech UK Ltd (APBiotech), Bucks, UK.). Most of the PCR products used were in their original solution containing PCR buffer (Mg++ 1.5 mM) and free dNTPs and so it was not necessary to add these. The mix was heated at 37 ^{0}C for 10 min and used immediately or stored at -20 ^{0}C.

The blunt-ended PCR products were then separated by gel electrophoresis and the appropriate bands cut out and purified with a GENECLEAN® KIT (Anachem, Luton, UK) if in standard grade agarose. Occasionally NuSieve® GTG® agarose (FMC BioProducts, Denmark) was used and excised bands could be used directly in ligations after melteding at 37 ^{0}C for 10 min.

The plasmid used for ligations was pBluescript® II SK+:

To Cut Plasmid

0.2 µl pBluescript® II SK+

0.5 µl *Eco RV* (3 units)

1 µl buffer B

8.2 µl dH_2O

Incubate at 37 ^{0}C for 30 min

Add 20 µl dH_2O

Ligations were carried out using a Rapid DNA Ligation Kit (Roche Diagnostics Ltd., East Sussex, UK). Microfuge tubes for each sample and three controls were made up as according to table 2.7.1.1.

Table 2.7.1.1. Rapid DNA Ligation at room temperature. n = any number of insert samples; C 1 to 3 = controls

	Sample µl	H_2O µl	cut vector µl	DNA dilution buffer µl	Flick, pulse spin, flick	ligase buffer µl	T4 ligase µl	Flick, pulse spin, flick
1	7		1	2		10	1	
2	7		1	2		10	1	
n	7		1	2		10	1	
C1		7	1	2		10	1	
C2		7	1 uncut	2		10	1	
C3		7	1	2		10	-	

To each sample, but not controls, 5 units (0.5 µl) of *EcoRV* was added after ligation and the samples were incubated at 37 ^{0}C for 20 min. This step reduced the numbers of plasmids without inserts in the *EcoRV* restriction site as addition of an insert removes this enzyme restriction site. The plasmids were used to transform competent cells immediately or frozen at -20 ^{0}C. Control 1 contained no inserts so the plasmids should re-circularise and form many more ampicillin resistant blue colonies than colonies with inserts. Control 2 was uncut plasmids so many blue colonies were expected and again more than for the colonies with inserts. Control 3 had no T4 ligase and so the number of colonies should be greatly reduced as the plasmids should remain linear and unable to replicate in the cells.

2.7.1.2. Competent Cells

The Hannahan method of transformation were used to generate competent *Escherichia coli* (*E. coli*) cells.

50 ml of SOB with 1 ml of 1 M MgSO4 and 250 µl of 2 M MgCl2 added was placed in a sterile flask with 1 ml of an overnight growth of XL1-Blue *E. coli* (Stratagene). This was then placed in a shaking incubator at 37 ^{0}C for approximately 3 hours. After this time an OD reading at 600 nm was taken and the culture was ready for use if the OD was between 0.5 and 0.8. The flask was then put on ice for 10 min. The contents of the flask were then transferred to a 50 ml Greiner polypropylene tube and centrifuged at 2500 rpm (700 x g) for 10 min at 4 ^{0}C in a Beckman centrifuge. The supernatant was poured off and the remaining pellet was re-suspended in 16 ml of TFB (approximately 1/3 dilution).

TFB

800 ml dH_2O

10 ml 1M MES pH 6.3 (N-morpholino]ethanosulfonic acid)

8.91 g $MnCl_2.4H_2O$

1.47 g $CaCl_2.2H_2O$

7.46 g KCl

0.8 g Hexamminecobalt chloride

Made up to one litre with dH2O then filtered through a disposable filter. Stored at 4 °C

The *E. coli* was then placed on ice again for 10 min and centrifuged again as before. The supernatant was poured off for a second time and the pellet re-suspended in 3 ml TFB (approximately 1/12.5 dilution). The cells were then put on ice for 15 min. 140 µl of DND was added and swirled and then on ice again for 15 min. The cells could then be used for transforming a ligation or stored frozen in liquid nitrogen for later use.

DND

1.53 g dithiothreitol

9 ml DMSO

100 ml 1 M potassium acetate (pH 7.5)

make up to 10 ml with dH_2O

2.7.1.3. Transformation

To each chilled ligation tube (including controls) 150 µl of competent XL1-Blue *E. coli* cell mix was added. They were then left on ice for 30 min, heat pulsed at 42 ^{0}C for 90 sec and then returned to the ice for a further 1 min. To each tube 700 µl of SOC was added.

SOC

25 ml of SOB without $MgCl_2$ or $MgSO_4$

Add 500µl of 20% glucose and 125 µl of $MgCl_2$

The plasmid-bacteria mixtures were then placed in a shaking incubator at 37 ^{0}C for 45 min and plated out onto selective Agar plates and grown over night in a 37 ^{0}C incubator.

2.7.1.4. Selection for bacterial colonies with recombinant plasmids

The plasmid pBluescript® II SK+ confers ampicillin resistance. XL1-Blue *E. coli* host cells that contain the pBluescript® II SK+ plasmids can be selected by adding ampicillin to the growth medium.

The XL1-Blue *E. coli* cells contain only the carboxy-terminal portion of β-galactosidase gene (lacZ), whereas the pBluescript® II SK+ plasmid contains only the operator-proximal segment part of the lacZ gene. When the plasmid is introduced into XL1-Blue, the cells can then make the enzymatically active protein in a type of partnership called α-complementation. The resulting Lac^+ bacteria form blue colonies in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). When an insert is ligated into pBluescript® II SK+ in the multiple-cloning site the LacZ gene operator-proximal segment is interrupted and the bacteria are no longer able to make the active galactosidase enzyme. Colonies containing plasmids with inserts can be distinguished from those without, as they are white when grown on X-gal containing medium and not blue.

Selective Agar Plates

500 ml of Agar melted and cooled to approximately 47 ^{0}C

Add 640 µl of 20 mg/ml solution of X-gal in dimethylformamide

Add 320 µl of 0.1 M isopropylthio-β-D-galactoside (IPTG)

Add 400 µl of 1 g/100ml solution of Ampicillin Sodium salt

Pour into sterile petri dishes

Once set, dry the agar at 55 ^{0}C for 30 min until wrinkly.

2.7.1.5. Screening Colonies for recombinants

White colonies on the AIX plates were picked with sterile pipette tips and recorded on a grid matrix agar plate. The colonies were then tested for inserts in one of two methods. In the first and longer method, overnight cultures of the colonies were grown in 30 ml L-Broth with ampicillin (120 µl of 1 g/100ml solution of Ampicillin Sodium salt to 200 ml L-Broth). The plasmids were then extracted from the bacteria using a QIAprep miniprep kit (QIAGEN®, West Sussex, UK) using the protocol provided. The structure of these plasmids was verified by restriction digests of the minipreps by gel electrophoresis. Minipreps of colonies that contained the appropriate size of insert were then used for sequencing.

For the second method the sterile tips used to pick the colonies were vigorously mixed in 10 µl L-Broth plus ampicillin (140 µl per 100ml L-Broth). This L-broth and colony mixture was then used directly in a pre-sequencing PCR reaction without any need for further growth of the bacteria. The primers used for this were designed to the pBluescript® II SK+ plasmid flank the Eco RV cloning site and were therefore, universal for all inserts.

Primer BSKPF CAATTTCACACAGGAAACAG

Primer BSKPR CGATTAAGTTGGGTAACGC

Pre-sequencing PCR Mix and Conditions

10 x Buffer (Mg^{++} 1.5 mM)	2 µl per well
2 mM dNTPs	2 µl per well
100 µM BSKPF	0.3 µl per well
100 µM BSKPR	0.3 µl per well
Expand Hi-Fi Taq	0.1 µl per well
5 M Betaine	4 µl per well
dH_2O	10.75 µl per well

Stage 1: [95 ^{0}C for 5 min] x 1 cycle

Stage 2: [95 ^{0}C for 15 sec; 56 ^{0}C for 20 sec; 72 ^{0}C for 2 min] x 30 cycles

This PCR reaction mixture was then used directly in the sequencing reaction for those samples with the correctly sized inserts, as determined by gel electrophoresis. This latter method of checking for inserts proved quicker but was more likely to give false positives.

2.7.2. Sequencing quail GnRH-I mRNA

2.7.2..1 Sequencing PCR

A 96 well PCR plate was marked in a portrait direction with rows A, C, G, T and R (figure 2.7.2.1.). The reagents for the PCR (strictly speaking this is not a PCR as there is not an exponential build up of product) were taken from the Thermo Sequenase™ DYEnamic™ Direct Cycle Sequencing Kit (APBiotech) with IR labelled dNTPs.

Sequencing PCR Mix and Conditions

1.5 µl of primer R (or U) to each well marked R (or U)

16.1 µl dH2O to each R (or U) well

2 µl of DNA sample to each R (or U) well

1.4 µl DMSO to R (or U) wells

1.5 µl of A mix to all A wells, and likewise for mixes C, G and T.

4.5 µl of mix from R (or U) well to wells A, C, G and T.

a drop of chill out wax or cover with plastic strip lids.

Stage 1: [95 ^{0}C for 30 sec] x 1 cycle

Stage 2: [95 0C 10 sec; 54 0C for 30 sec; 65 0C for 30 sec] x 20 cycles

Stage3: [95 0C for 10 sec; 70 0C for 30 sec] x 15 cycles

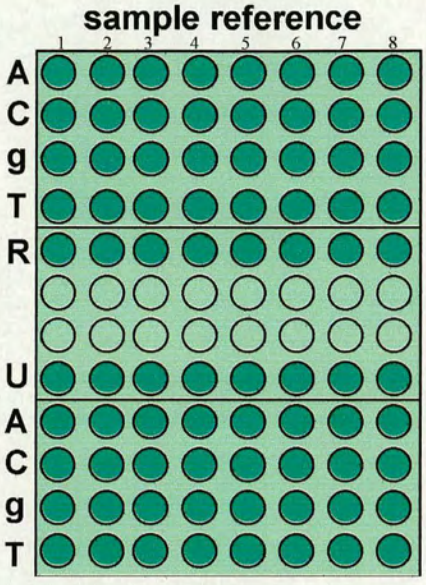


Figure 2.7.2.1. Sequencing PCR layout on a 96 well plate. The rows marked R and U contain the DNA sample, primers R or U, H20 and DMSO. The rows A, C, g and T contain the appropriate PCR mixes supplied with the kit.

2.7.2.2. Li-Cor Automated DNA Sequencer (MWG-Biotech, Milton Keynes, UK).

The standard Li-Cor protocol was used for sequencing gel electrophoresis and data collection.

Li-Cor sequencing gel (for 41 cm sequencing plates)

21 g urea

28 ml dH_20

5 ml 10 x TBE long run buffer

7.5 ml rapid gel excel sol 40%

Stir without heat for 1 - 2 hours then de-gas with tap suction pump for 10 min.

Clean 41 cm plates and clamp together then add the following to the mix:

350 µl 0.1M ammonium persulphate

500 µl DMSO

50 µl TEMED

Pour gel immediately using a syringe

Add well former and casting plate and allow to set for at least 2 hr

TBE long run buffer	Li-Cor gel-electrophoresis running buffer
162 g tris base	100 ml TBE long run buffer
27.5 g boric acid	900 ml dH_2O
9.3 EDTA	
make up to 1 litre with dH_2O	

Prior to loading the samples, the gel was placed in the Li-Cor and run for 30 min at 45 ^{0}C, then any urea that had accumulated in the wells was washed away with a Pasteur pipette. 4 µl of Loading dye was then added to each sample well (A, C , G and T) and heated at 65 ^{0}C for 10 min. The samples were then loaded onto the gel using micro syringes. The gel was run for approximately five hours depending on the length of the inserts with the following conditions: 1500 V, 35 mA, 40 W, 45 ^{0}C.

2.7.2.3. Analysis programme

The LiCor software BaseImage1R V4.0 was used for analysing the digital picture of the sequencing gel. The single lane automatic reader was used for all sample quartets and was double-checked and corrected by eye. The resulting sequences were then analysed using GCG Wisconsin and the NCBI web site.

2.8.1. Oligonucleotides used in sequencing quail GnRH-I mRNA

With the exception of sequencing primers the oligonucleotides used in this study were obtained from Cruachem, Strathclyde, UK.

The specific oligonucleotides used in isolation of the quail sequence were based on the sequence of chicken GnRH-I (Dunn et al. 1993);

564r, 5'-[ATTTTCCAGCGGGAAGAGTTG]-3';

771t, 5'-[TCCTGGTTGCAGGCCATAAGACCAGTCTTG]-3';

z706, 5'-[GCAGCCTCTCTCAGCAAACAG]-3';

772t, 5'-[CAAGACTGGTCTTATGGCCTGCAACCAGGA]-3';

2.9. mRNA QUANTIFICATION

2.9.1. Competitive reverse transcribed PCR of GnRH-I mRNA

The Q RT-PCR assay amplified a 286 base pair region of GnRH-I mRNA from exon I to exon IV. Oligonucleotide primers for the amplification were designed to the quail sequence but the 5' primer QGnRH5' does not differ to the chicken sequence:

Forward Primer QGnRH5' 5'-[dATTCTGCAGCCTCTCTCA]-3'

Reverse Primer Q562r 5'-[dTTGGTTTGTTGTTGGCGTTGT]-3'

The second primer Q562r has 3 base pairs that differ from chicken. However, these primers can still amplify chicken GnRH-I mRNA if given the right conditions.

2.9.1.1. Preparation of competitor

The competitor for the GnRH-I mRNA was designed to have a central region of pBluescript SK II+ sequence flanked by the Q562r and QGnRH5' primer sequences. The total number of base pairs in the competitor was 486 and this was designed to be 200 base pairs longer than the standard and sample fragments so that it could be clearly resolved by gel electrophoresis. The primers used to create this molecule was were hybrids between plasmid and GnRH-I mRNA sequence. The GnRH sequence was 5' of the region complimentary to the plasmid (figure 2.9.1.2).

Quail Sequence Plasmid Sequence

ComQGnRH5' 5'-[dATTCTGCAGCCTCTCTCAGTCCCATTCGCCATTCAG]-3'

Quail Sequence Plasmid Sequence

ComQ562r 5'-[dTTGGTTTGTTGTTGGCGTTGTAGGCACCCCAGGCTTTAC]-3'

The PCR products were then sub-cloned by ligating into the Eco RV cloning site of pBluescript SK II+ and transformed in XL1-Blue competent cells using the same method described previously. The plasmids were purified with midipreps or maxipreps and used in the competitive PCR.

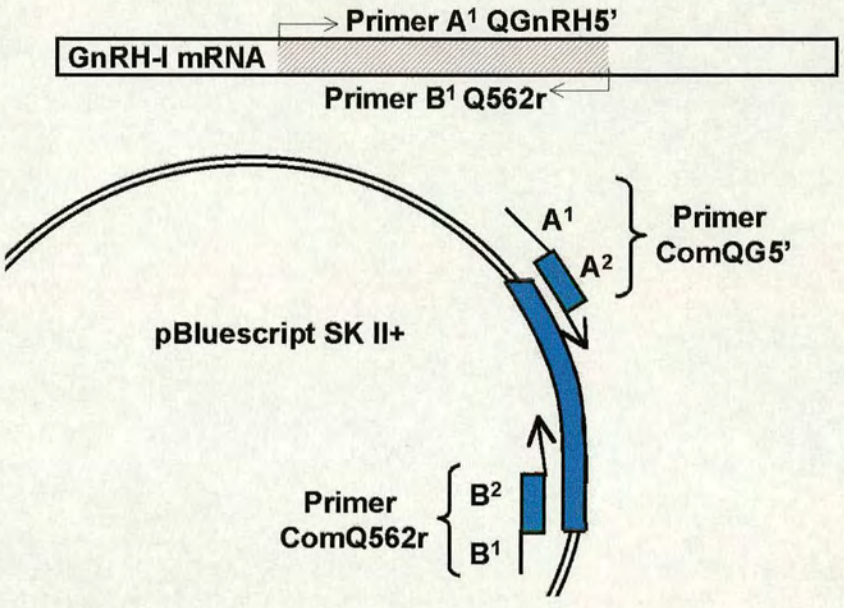


Figure 2.9.1.2. The Competitor Design. The primers used to amplify a small region of the GnRH-I mRNA A^1 and B^1 are added to primers designed to the plasmid pBluescript SK II+ A^2 and B^2. The PCR products can then be sub-cloned to produce large usable quantities of competitor.

This design of competitor was used because it was thought heteroduplexes were less likely to form when the majority of the competitor and standard sequences are different. This and the validation of the assay are discussed in chapter 3.

2.9.1.2. Preparation of standard

The standard was prepared using the products from a PCR on reverse transcribed quail mRNA using the QGnRH5' and Q562r primers. This product was then sub-cloned and the plasmids purified as for the competitor.

2.9.1.3. Q-RT PCR procedure

The neat competitor was diluted serially by a factor of 5 and each dilution run in a PCR with reverse transcribed mRNA samples. The dilution that produced bands of equal intensity for both competitor and samples after gel electrophoresis was taken as the appropriate concentration of competitor for use in the assay. This was done for every set of samples in the distribution study. The competitor and standard concentrations were determined by optical density readings at 260 nm. A dilution curve of seven standards, diluted by a factor of 2, was set up to span the competitor concentration equivalent to the sample concentration of GnRH mRNA, starting with the strongest concentration of standard and with the 4th tube having approximately equal quantities of competitor and standard. The standard dilution series was set up on each occasion. Before each set of samples were analysed a trial curve was produced to ensure that the sample concentration were on the standard curve.

GnRH-I mRNA C RT-PCR Mix and Conditions

10 x Buffer (Mg^{++} 1.5 mM)	4.7 µl per 0.5 ml tube
2 mM dNTPs	4.7 µl per 0.5 ml tube
20 µM QGnRH5'	0.47 µl per 0.5 ml tube
20 µM Q562r	0.47 µl per 0.5 ml tube
Taq polymerase	0.2 µl per 0.5 ml tube
dH_2O	14.66 µl per 0.5 ml tube

Stage 1: [95 ^{0}C for 1 min] x 1 cycle

Stage 2: [95 ^{0}C for 20 sec; 60 ^{0}C for 20 sec; 72 ^{0}C for 20 sec] x 40 cycles

The PCR products were separated by electrophoresis through a 2% agarose gel. The bands were visualised using ethidium bromide under UV light. Quantitative measurements of band intensity were made using a digital picture of the gel (MicroAnalyst) and a specifically designed macro in NIH image (National Institutes of Health, USA). The measurements were then analysed in Excel (Microsoft). The log of the ratio of competitor to standard was graphed against the log of the standard curve. The equation of the line was then used to give a concentration for each sample.

2.10. STATISTICAL METHODS

All LH and PRL, and where possible GnRH-I mRNA data, were statistically analysed with Analysis of Variance (ANOVA) in Minitab. Where ANOVA revealed a significant difference, post hoc Tukey's simultaneous tests were carried out for pair-wise comparisons. GnRH-I mRNA data was analysed with students T-Tests when ANOVA was inappropriate. Graphs with and without trendlines were drawn in Excel (Microsoft).

3. DEVELOPMENT AND VALIDATION OF A QUANTITATIVE REVERSE TRANSCRIPTION PCR ASSAY FOR GnRH-I mRNA

3.1. INTRODUCTION

GnRH-I mRNA in the avian hypothalamus is present in extremely small quantities. This makes conventional mRNA quantification methods such as Northern blots and RNAse protection assays unsuitable. For this reason a quantitative reverse transcription polymerase chain reaction assay (Q RT-PCR) was created.

The exponential nature of PCR is such that small differences in the concentrations of reagents at the start of the reaction can dramatically affect the yield of the final PCR product. This would mean that for accurate quantification all variables such as concentrations of polymerase, dNTPs, Mg, cDNA, primers, temperatures, ramping times, 'primer-dimer' formation and the presence of contaminating DNA's have to be exactly the same in each tube. Even if all of these factors are adequately controlled there is sometimes tube-to-tube variation in amplification efficiency. The reason for this is not certain but may be due to random events in the first few cycles or tiny differences in temperature (Gilliland *et al.,* 1990). The competitive PCR technique used here for quantification bypasses these problems by co-amplifying a competitive template (competitor) that uses the same primers as the target cDNA. A dilution series of the target cDNA of known concentrations (standard) is added to a constant amount of competitor to create a standard curve. In the same PCR assay an equal

amount of competitor is added to each cDNA. The ratio of unknown target to competitor can then be compared with the standard curve to give a relative quantification. As long as the amplification rates of the target cDNA and competitor are equal then the ratio of one to the other remains constant throughout the reaction. The results of amplification tests are reported in this chapter along with dilution tests and repeats of RT and PCR samples to gain an idea of the variation inherent in the assay.

3.2. METHODS

3.2.1. Quail GnRH-I mRNA Sequence

The first step in developing the assay was to sub-clone and sequence the gene encoding GnRH-I in quail in order to identify a suitable region for amplification in the Q RT-PCR. A quail GnRH-I mRNA consensus sequence was obtained by combining the information from 4 independent clones from the PCR of quail genomic DNA and 3' RACE. Primers were designed to chicken GnRH-I cDNA sequence (see chapter 2, section 2.8.1.). Three of these clones were sequenced by Dr Ian Dunn prior to my arrival in the laboratory.

3.2.2. Competitor Design

The first competitor developed was homologous to the target sequence (see chapter 2, section 2.9.1.). This was made by adding a 200 base pair section of plasmid (PBSKII+) into the target sequence, so that it could be distinguished by gel electrophoresis. The homologous competitor fragment method was first used by

Becker-André and Hahlbrock (1989) and subsequently by Gilliand *et al.,* (1993). The advantage of this method is that the similarity between competitor and target ensures that the amplification rates are the same. However, the use of this competitor resulted in the formation of heteroduplexes (figure 3.1.1.).

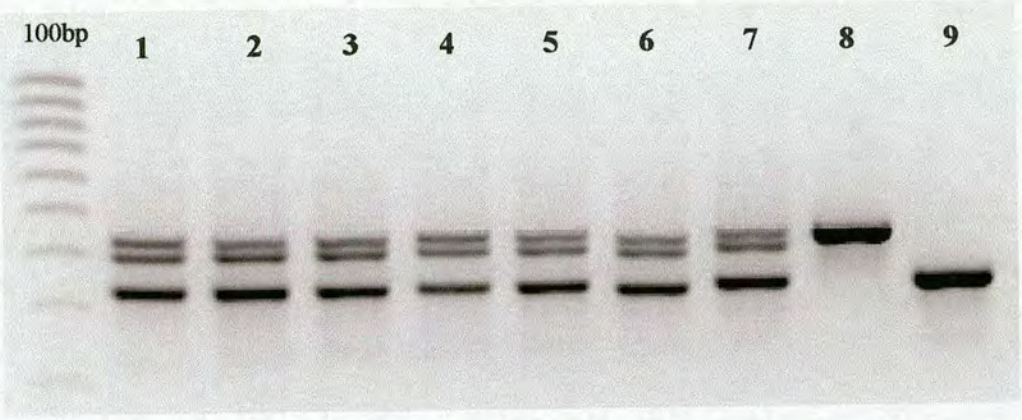


Figure 3.1.1. An example of heteroduplex formation when using a homologous competitor. 2% agarose gel electrophoresis visualising Q RT-PCR products with ethidium bromide under UV light. The top band is the competitor, the bottom the target and the middle a heteroduplex of the two. Lanes 1 to 7 were exposed to a range of temperatures after the end of the PCR reaction before cooling. Lanes 8 and 9 are controls with competitor and target sequence respectively.

Although the heteroduplexes themselves can be used for quantification purposes (Henco and Heibey, 1990) it was decided that to avoid potential problems of interpretation the formation of these heteroduplexes would be prevented. Several attempts were made by changing buffer concentrations of the reaction mixture and electrophoresis gel, adding dimethylsulphate (DMSO), tetramethylammonium chloride (TMAC) or betaine and changing the temperatures of the PCR stages and the number of PCR cycles. Unfortunately none of these techniques were successful in removing heteroduplexes and so a different type of competitor was constructed. This was a heterologous competitor and was made from plasmid DNA flanked by the

same primer sequence as the target (see chapter 2, section 2.9.1.). However, it is essential when using heterologous competitor fragments to check that the amplification efficiency of the competitor is equal (or very similar) to that of the target (Pannetier *et al.*, 1993).

3.2.3. Amplification Efficiencies

Two methods were used to ensure that the GnRH-I mRNA competitor and target sequence (standard) amplified equally. In the first method a 10 µl aliquot was removed from a PCR tube containing 100µl after every two PCR cycles. The second method controlled for any possible effects of stopping and starting the reaction and for any contamination during the removal of aliquots. Here a PCR mixture was divided into separate tubes and run for a varying number of cycles, each tube receiving two more cycles than the previous one. The 'one tube' experiment was repeated with a higher concentration of competitor to standard and with a lower concentration of competitor to standard and also with DMSO, to see if any of these conditions affect the amplification efficiency. The PCR products from each aliquot were then visualised with ethidium bromide on a 2% agarose gel. The intensity of the bands of the competitor and standard were then compared. If the amplification efficiencies are the same for competitor and standard then the ratio of competitor to standard should be equal in each tube. To check this the ratio of competitor to standard was graphed and a line of best fit added, the predicted value for this line where amplification rates are equal is 0. The log competitor and log standard amount of product (as determined by band intensity) were graphed against the number of

PCR cycles and the line of best fit drawn using Excel (Microsoft). If the amplification rates are the same then these two lines should be equal.

3.2.4. Dilution tests

Dilution tests were carried out to establish that the competitive assay was able to accurately detect known differences in GnRH-I mRNA. GnRH-I mRNA was diluted and reverse transcribed and then run in a PCR with a standard curve. This was repeated four times and the resulting data were combined.

In the first dilution series RNA from the POA was diluted with water by ½ and ¼. Thus the total RNA and the GnRH-I mRNA were halved by each dilution. The dilution series was:

1. Undiluted POA RNA,
2. ½ POA RNA + ½ H_20
3. ¼ POA RNA + ¾ H_20

The predicted measurements were of a decrease in GnRH-I mRNA corresponding to the dilutions. The second dilution series diluted the GnRH-I mRNA while keeping the total mRNA the same. This was done by diluting the POA material with reverse transcribed cortex mRNA, which does not contain GnRH-I mRNA. The dilutions for this were as follows:

1. Neat POA RNA,
2. ½ POA RNA + ½ cortex RNA
3. ¼ POA RNA + ¾ cortex RNA

Again the predicted result was a corresponding reduction in GnRH-I mRNA measurement as only the standard mRNA, namely GnRH-I should affect the outcome. The final dilution test controlled for possible increases in background mRNA rather than it just remaining the same. The third dilution series increased the

total mRNA by diluting the POA material with cortex mRNA, while keeping the GnRH mRNA levels the same. The dilutions for this were as follows:

1. ½ POA RNA + ½ H_20
2. ½ POA RNA + ¼ cortex RNA + ¼ H_20
3. ½ POA RNA + ½ cortex RNA

3.2.5. Assay Co-efficient of Variance

To determine of the variation inherent in the assay, two samples were repeatedly assayed. Five aliquots were taken from each mRNA sample and reverse transcribed and used for the PCR. From one of the reverse transcribed mixtures a further five samples were taken and analysed in the assay to measure the variance due to the PCR and subsequent steps.

3.3. RESULTS

3.3.1. Quail GnRH-I mRNA Sequence

The sequence of the quail GnRH-I gene is presented in figure 3.3.1.1. and is 93% identical, over 388 nucleotides, with chicken GnRH-I (figure 3.3.1.2.). The sequence of intron 1 is shown in figure 3.3.1.3. with flanking regions from exon 1 and exon 2. The sequence of intron I contained 2 deletion/insertions compared to the chicken and the remaining regions were 88% (base 1-172), 90% (base 202-243) and 92% (base 252-321) identical to the chicken sequence for intron I. The deduced pre-pro-peptide amino acid sequence is 90% identical to chicken GnRH-I pre-pro-peptide, the majority of these differences are in the region coded by exon 3. It should be noted

that the first 21 bases of the cDNA sequence presented are constrained by the primer used in the PCR amplification and may be include some mismatches.

```
  1  ATTTTCCAGC GGGAAGAGTT GGAGCGATTC TGCAGCCTCT CTCAGCAAGC
 51  AGAATGGAGA AGTCCAGGAA GATCTTGGTT GGTGTTCTCC TGTTTACCAC
101  GTCTGTGGCA ATCTGCTTGG CTCAACACTG GTCTTATGGC CTGCAACCAG
151  GAGGGAAAAG GAACGCTGAA AATCTGGTGG AATCATTTCA AGAGATTGCA
201  AATGAAATGG AAAATCTAAG GGAAGTGAAG GAGGCTGAAT GCCCTGACTC
251  ATACCAGCAT CCCAGGCTGA GCAATCTGAA GGAAACCATG GCAAGTCTGA
301  TCGAAGGAGA AGCCAGAAGA AAGAAGATTT AAATCCACAA CGCCAACAAC
351  AAACCAAGAA TAAAACTCCA AAGCAATGTT GAAAACAG
```

Figure 3.3.1.1. Consensus sequence of quail GnRH-I cDNA generated from both PCR and RT-PCR clones of genomic DNA and cDNA from quail hypothalamus. The coding sequence for the quail GnRH-I decapeptide is underlined and the putative translation initiation site and polyadenylation site are in bold. It should be noted that the first 21 bases of the sequence presented are constrained by the primer used in amplification and may contain mismatches. The primer sequences used for the quantitative RT-PCR assay are marked in blue.

```
  1 attttccagc gggaagagtt ggagcgattc tgcagcctct ctcagcaagc
    |||||||||| |||||||||| |||||||||| |||||||||| |||||||| |
    attttccagc gggaagagtt ggagcgattc tgcagcctct ctcagcaaac

 51 agaatggaga agtccaggaa gatcttggtt ggtgttctcc tgtttaccac
    |||||||||| |||||||||| |||||||||  ||||| |||| |||| ||| |
    agaatggaga agtccaggaa gatcttggtc ggtgtcctcc tgttcaccgc

101 gtctgtggca atctgcttgg ctcaacactg gtcttatggc ctgcaaccag
     ||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    atctgtggca atctgcttgg ctcaacactg gtcttatggc ctgcaaccag

151 gagggaaaag gaacgctgaa aatctggtgg aatcatttca agagattgca
    |||| ||||| |||||||||| ||||| |||| |||||||||| |||||| |||
    gaggaaaaag gaacgctgaa aatctagtgg aatcatttca agagatcgca

201 aatgaaatgg aaaatctaag ggaagtgaag gaggctgaat gccctgactc
    || ||||||| ||| |||| |  |||| | ||  ||||||||| ||||||||||
    aacgaaatgg aaagtctagg agaagggcag aaggctgaat gccctgactc

251 ataccagcat cccaggctga gcaatctgaa ggaaaccatg gcaagtctga
    |||||||||  |||||||||| |  ||||||| |||||||||| ||||| ||||
    ttaccagcat cccaggctga gtgatctgaa ggaaaccatg gcaagcctga

301 tcgaaggaga agccagaaga aagaagattt aaatccacaa cgccaacaac
    |||||||||| |||||||||| ||| |||||| |||||||||| | ||| ||||
    tcgaaggaga agccagaaga aaggagattt aaatccacaa caccatcaac

351 aaaccaagaa taaaactcca aagcaatgtt gaaaacag
    ||||| |||| ||||| | || |||||||||| ||||||||
    aaacccagaa taaaatttca aagcaatgtt gaaaacagtt
```

Figure 3.3.1.2. A line-up of quail GnRH-I cDNA (top) with chicken GnRH-I cDNA. There are 26 differences across the 388 base pairs. The primers used in the Q RT-PCR assay are highlighted in blue. The last base in each exon is in bold, the last three intron /exon boundaries are inferred from the chicken sequence.

```
51  AG
        GTAATGTA CTTGAACAGC ACACCATACC CACTTCACCT TTTTAGTCCT
    TACAGATGCT TGCATTGGAA GGAACCTGCA TTAGAAGGCA CTGCAAAGAG
    CGCTTAGTAT AGAAACTGGC TACACTGGAC CCATCTGTCA AATATCAACA
    GGGATTTTCA GTGAACCTTT GAGGTCCAGC GTTCCTAAAG CCCTGACAGG
    GTGCTGTGAC CCACATGTAG GCACATGCCA CCGGGGTGCA GGAGACCAGA
    GACGATTCTG TAAGACAAAA ATTGAGCTGT GATGACACAG GGTCAGAAAT
    ATGCTTTGTT CTCTCATCAC AG
53                        AA
```

Figure 3.3.1.3. Consensus sequence of the 320 bp intron I from quail GnRH-I generated from PCR clones of genomic DNA. The flanking bases from exon 1 and 2 deduced from the cDNA sequence are in bold.

Evidence that quail mRNA was sequenced, and not contaminating chicken sequence, was demonstrated by the low sequence homology in intron 1 where there are several deletions/insertions in the quail sequence when compared with the chicken sequence. This pattern of conservation is as expected because there is less known functional constraint on intron sequences.

3.3.2. Amplification Efficiencies with Equimolar Competitor and Standard

The first amplification test used a competitor and standard at approximately equimolar concentrations (competitor = 2.3 attomoles and standard = 2.6 attomoles) as determined by UV spectrophotometry (figure 3.3.2.1.).

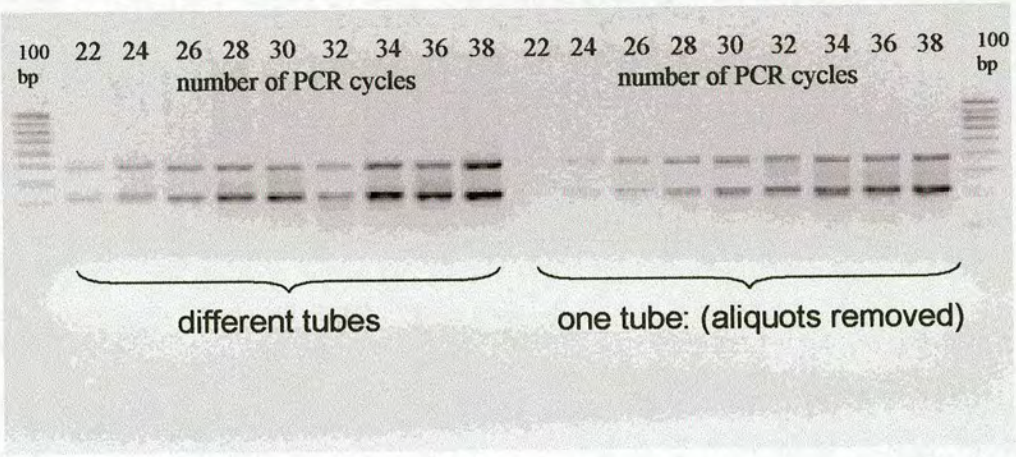


Figure 3.3.2.1. A 2% agarose gel following electrophoresis to test the amplification efficiencies of competitor (top band) and standard (bottom band) in the GnRH-I mRNA Q RT-PCR. From left to right each band has been amplified by two more cycles than the previous lane.

The amplification efficiencies of standard and competitor were similar in both single tube and separate tube runs (figure 3.3.2.2. and figure 3.3.2.3.). The slope of the log competitor plotted against cycle number was 0.0109 and 0.0093 for the standard when aliquots were in different tubes. Aliquots taken from a single tube the competitor and standard also had similar slopes, 0.0657 and 0.0739 respectively. A graph of the ratio of competitor to standard against the number of PCR cycles produced a slope close to 0 for both for the different tube aliquots (-0.0036, graph not shown) and for the single tube aliquots (0.0066, graph not shown). Therefore, both methods demonstrate that the amplification of the competitor and standard amplify at the same rate.

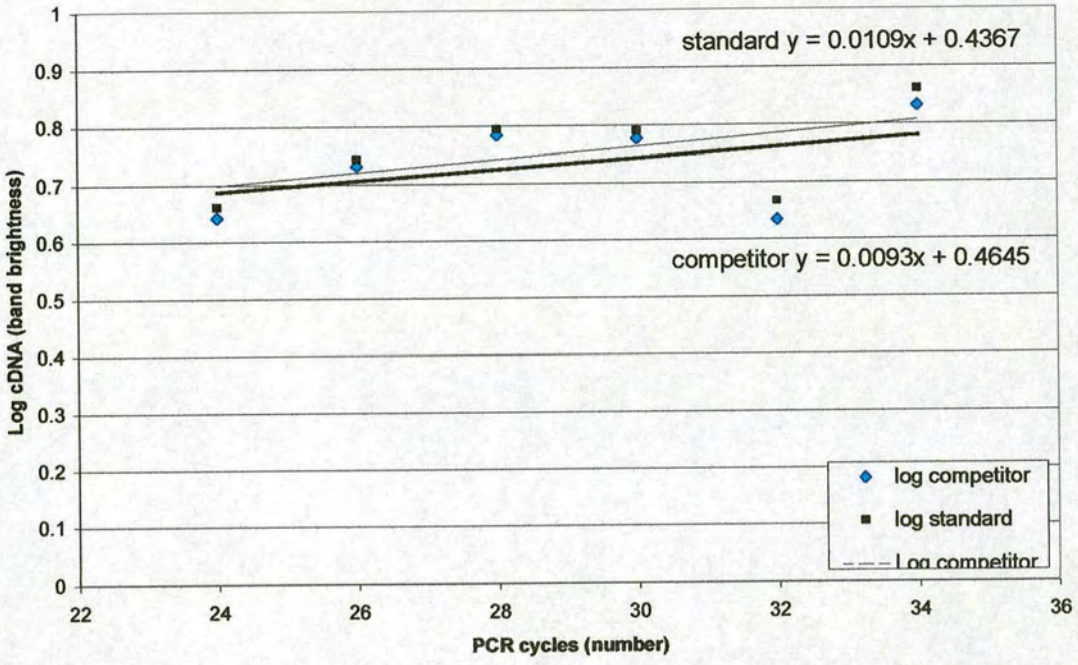


Figure 3.3.2.2. The kinetics of amplification of approximately equimolar GnRH-I standard and competitor across PCR cycles 24 to 34 (competitor = 2.3 attomoles and standard = 2.6 attomoles). Aliquots from a PCR mixture were run in separate tubes for differing numbers of cycles. For the corresponding gel see figure 3.3.1.

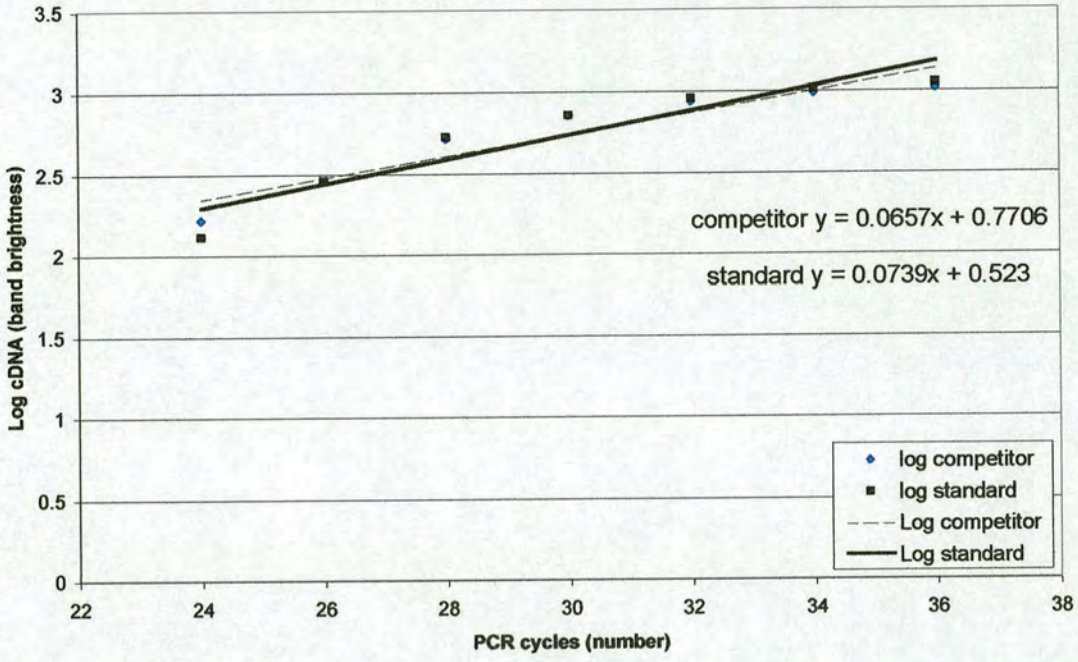


Figure 3.3.2.3. The kinetics of amplification of the equimolar GnRH-I mRNA standard and competitor across PCR cycles 24 to 36 (competitor = 2.3 attomoles and standard = 2.6 attomoles). Aliquots were taken from the same tube every 2 cycles. For the corresponding gel see figure 4.3.1.

3.3.3. Amplification with Different Concentrations of Standard to Competitor

In the second amplification test the competitor and standard were used at different concentrations. Firstly the competitor was higher concentration than the standard (competitor = 2.8 attomoles and standard = 0.6 attomoles) and secondly the competitor was at a lower concentration than the standard (competitor = 0.4 attomoles and standard = 2.5 attomoles). The amplification efficiencies were similar for both reactions (figure 3.3.3.1. and figure 3.3.3.2.). The slopes were very similar (0.0022 and 0.0043) when the competitor concentration was greater than standard and the ratio of standard to competitor produced a slope of 0.0059. When the competitor was lower than the standard the slopes were also very similar (0.0945 and

0.0971) and a graph of the ratio of standard to competitor against number of PCR cycles produced a slope of 0.0009. This shows that the ratio of competitor to standard was the same throughout the PCR.

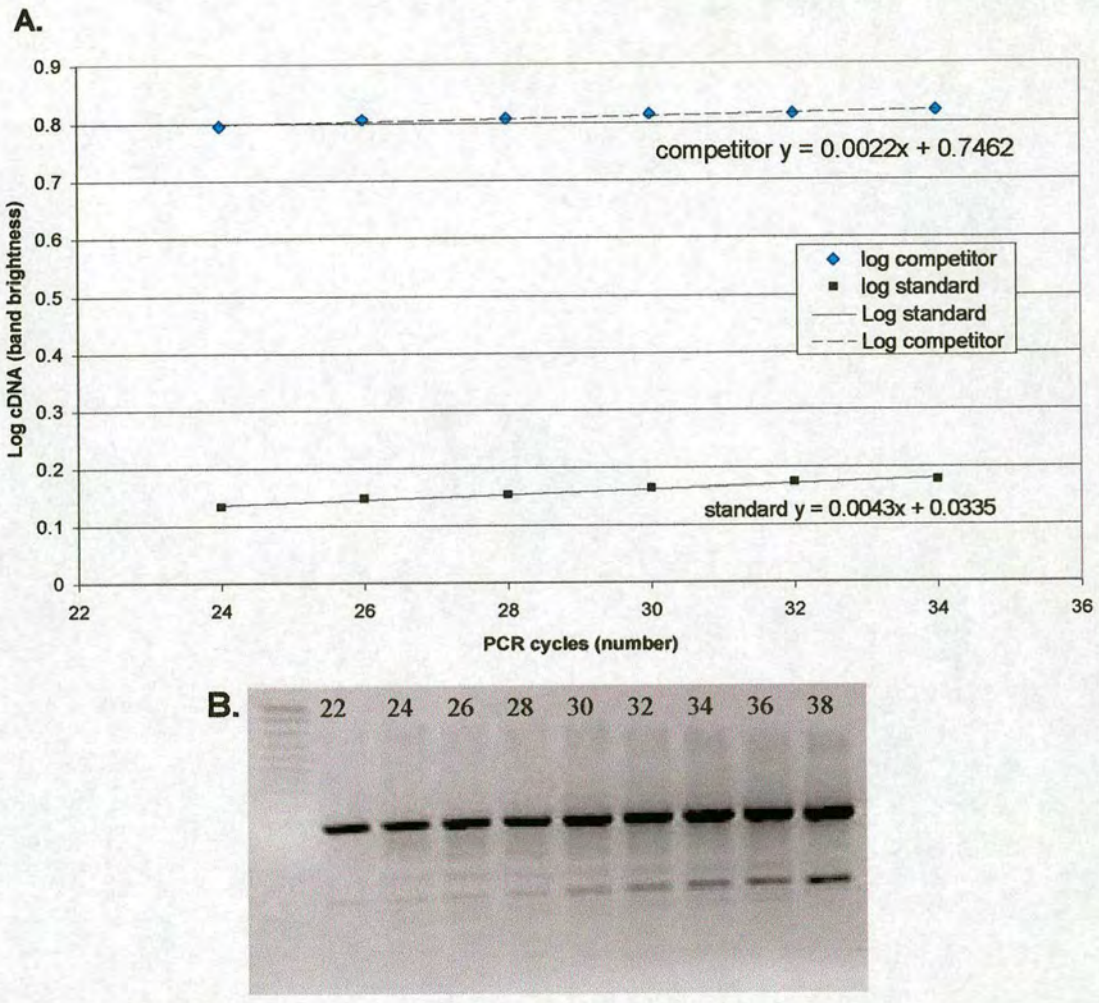


Figure 3.3.3.1. **A.** The kinetics of amplification with a higher concentration of GnRH-I mRNA competitor than standard across PCR cycles 24 to 34 (competitor = 2.8 attomoles and standard = 0.6 attomoles). Aliquots were taken from the same tube every 2 cycles. **B.** 2% agarose gel following electrophoresis of the amplification of GnRH-I mRNA competitor (top band) and standard (bottom band). From left to right each band has been amplified by two more cycles than the previous lane.

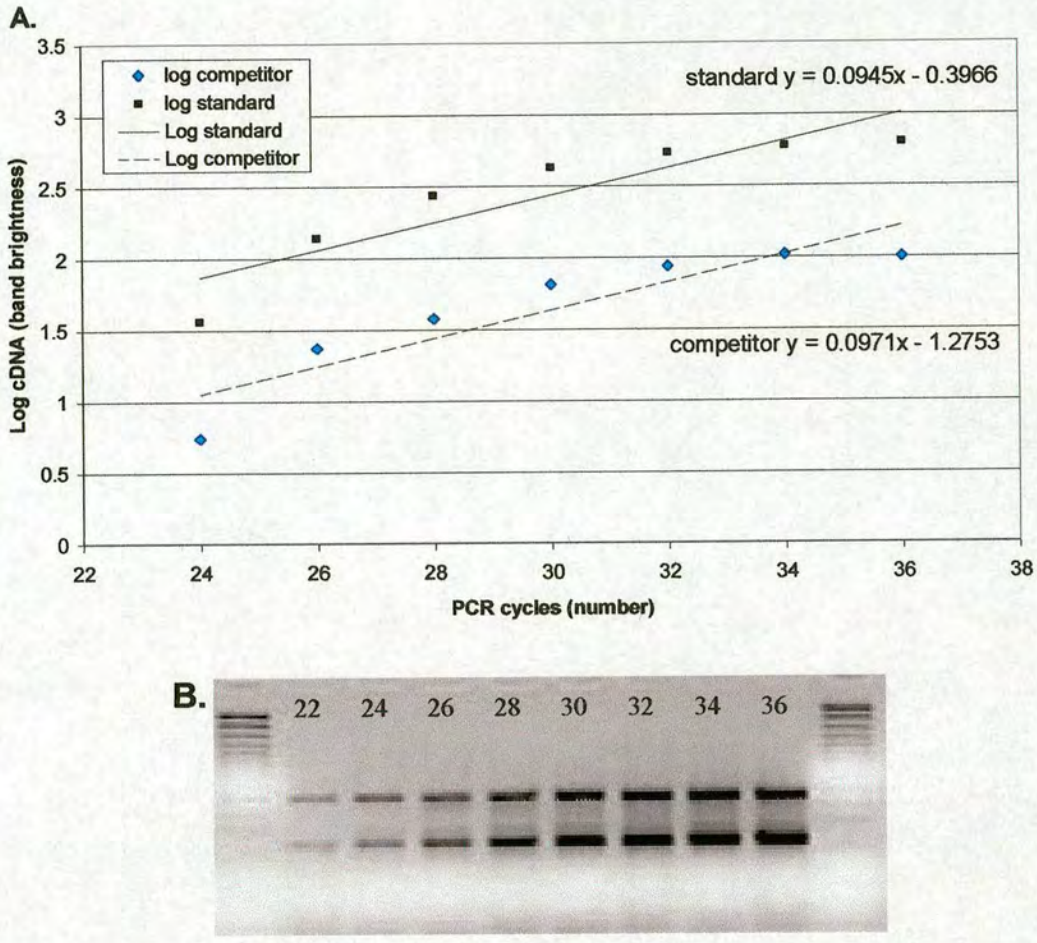


Figure 3.3.3.2. **A.** The kinetics of amplification with a higher concentration of GnRH-I mRNA standard than competitor across PCR cycles 22 to 34 (competitor = 0.4 attomoles and standard = 2.5 attomoles). Aliquots were taken from the same tube every 2 cycles. **B.** 2% agarose gel following electrophoresis of the amplification of GnRH-I mRNA competitor (top band) and standard (bottom band). From left to right each band has been amplified by two more cycles than the previous lane.

3.3.4. Amplification Efficiencies with DMSO

The third and final amplification test was conducted to establish if there might be an effect of DMSO on amplification efficiency. The reaction mixture started with 0.7 attomoles of competitor and 2.2 attomoles of standard. There was no effect of DMSO as efficiencies were alike both for competitor and standard (figure 4.3.4.1.). The slopes of the lines were 0.056 for competitor and 0.0547 for standard. The slope of

the graph of the ratio of competitor to standard against number of PCR cycles was very close to 0 at 0.0011.

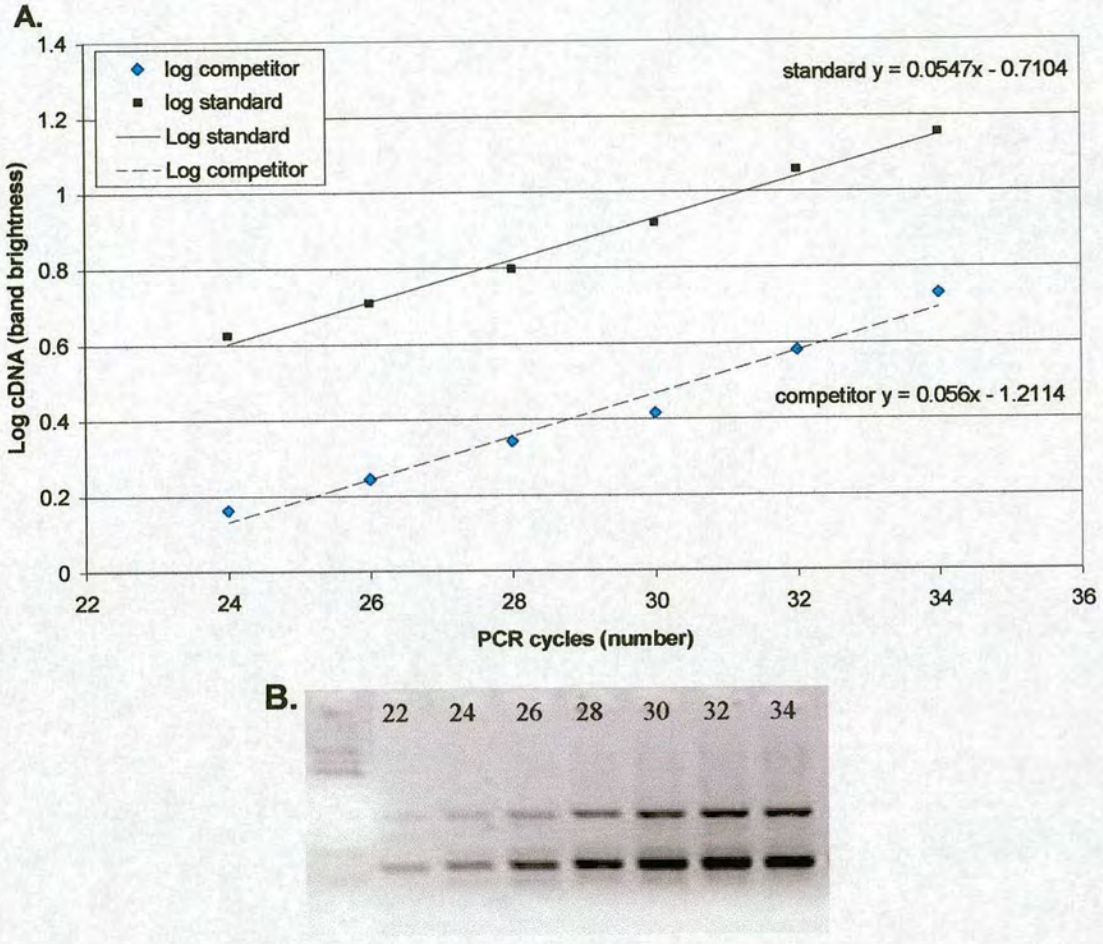


Figure 3.3.4.1. **A.** The kinetics of amplification of GnRH-I mRNA standard and competitor with 8% DMSO across PCR cycles 24 to 34 (competitor = 0.7 attomoles and standard = 2.2 attomoles). Aliquots were taken from the same tube every 2 cycles. **B.** 2% agarose gel following electrophoresis of the amplification of GnRH-I mRNA competitor (top band) and standard (bottom band). From left to right each band has been amplified by two more cycles than the previous lane.

3.3.5. Validation Experiments: Dilution Comparisons

The Q RT-PCR standard curve for all dilution series is shown in figure 3.3.5.2. In all three dilution series the expected curve was calculated by dividing the number of

moles in the top concentration of POA RNA by the appropriate dilution factor. In all three cases the expected and observed lines were very similar and the expected results fell between the standard error of the mean of the observed results (figures 3.3.5.3., 3.3.5.4. and 3.3.5.5.). An example PCR gel is shown in figure 3.3.5.1.

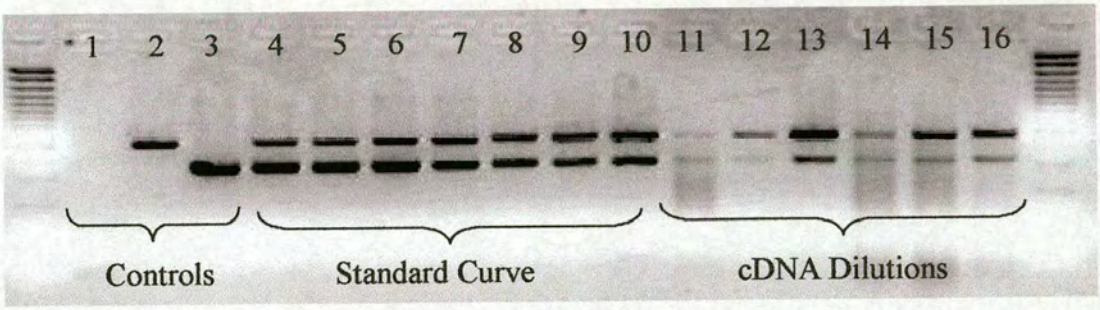


Figure 3.3.5.1. 2% agarose gel following electrophoresis of the amplification of GnRH-I mRNA standard curve lanes 1 to 10, and dilutions of cDNA (top band) with competitor (bottom band) lanes 11 to 16. Dilution series; lane 11 = neat POA cDNA, 12 = ½ POA cDNA and ½ H_20, 13 = ¼ POA cDNA and ¾ H_20, 14 = ½ POA cDNA and ½ cortex cDNA, 15 = ¼ POA cDNA and ¾ cortex cDNA and 16 = ½ POA cDNA, ¼ cortex cDNA and ¼ H_20. Controls; lane 1 = H_20, 2 = competitor and 3 = standard. DNA weight markers used are 100bp+.

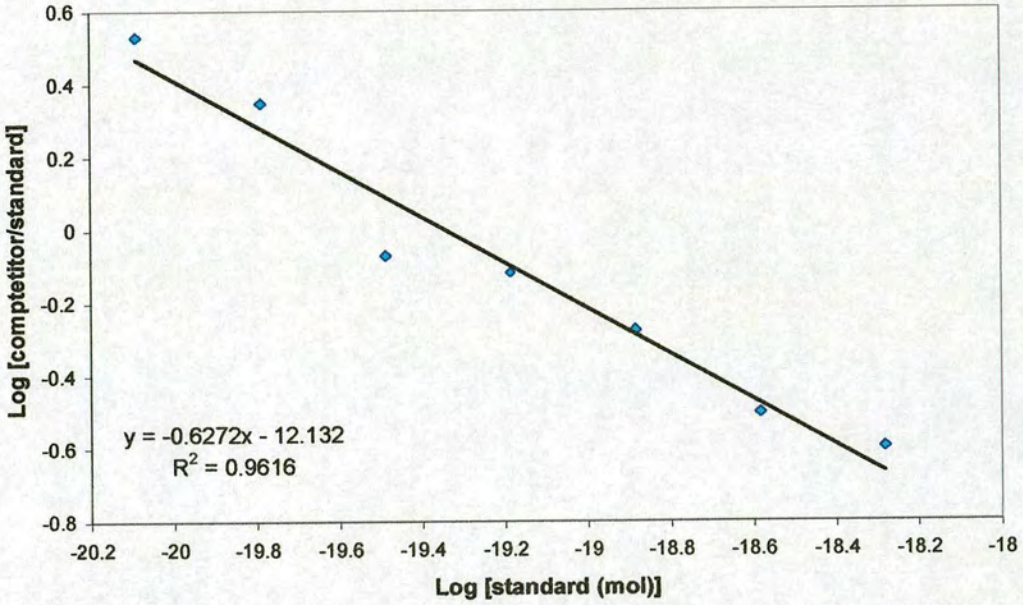


Figure 3.3.5.2. Standard curve for Q RT-PCR for the dilution series experiments.

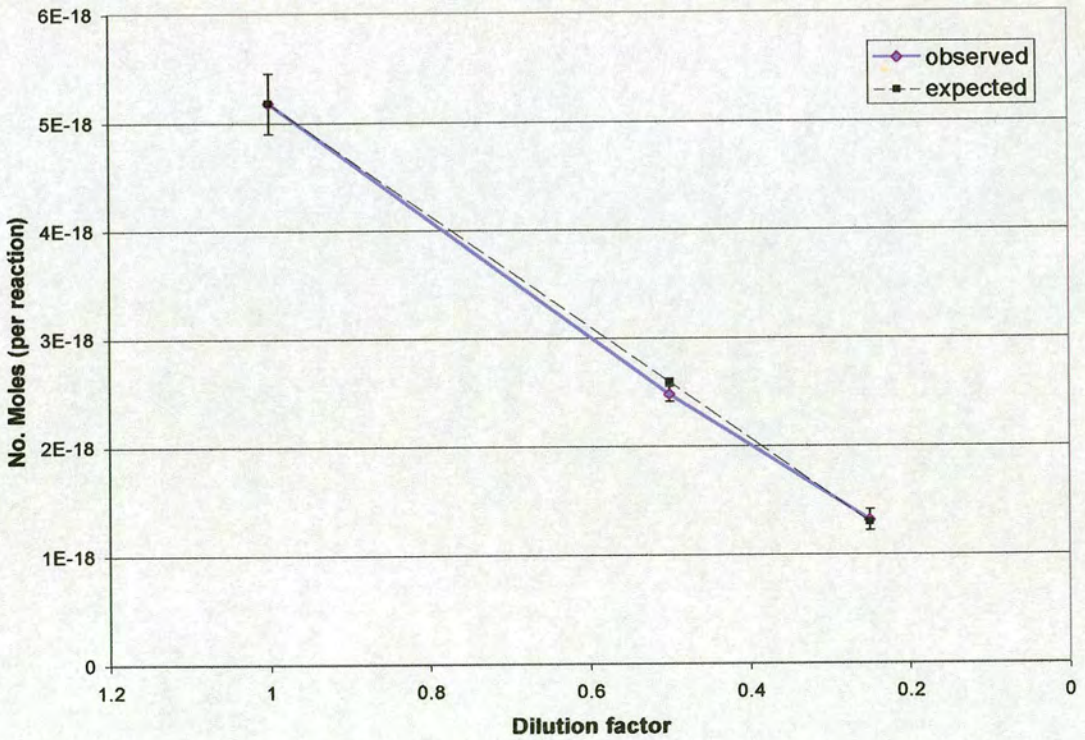


Figure 3.3.5.3. GnRH-I mRNA levels in a top concentration of POA cDNA and dilutions to ½ and ¼, diluted with H_20. Average ± SE MEAN.

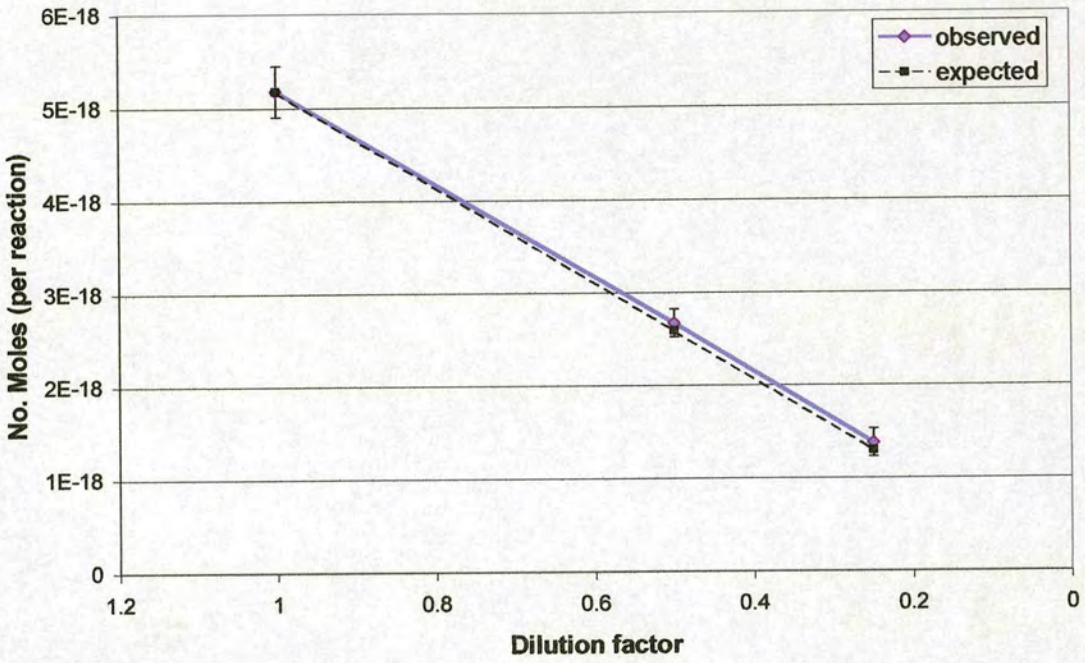


Figure 3.3.5.4. GnRH-I mRNA levels in a top concentration of POA cDNA and dilutions to ½ and ¼, diluted with cortex cDNA. Average ± SE MEAN.

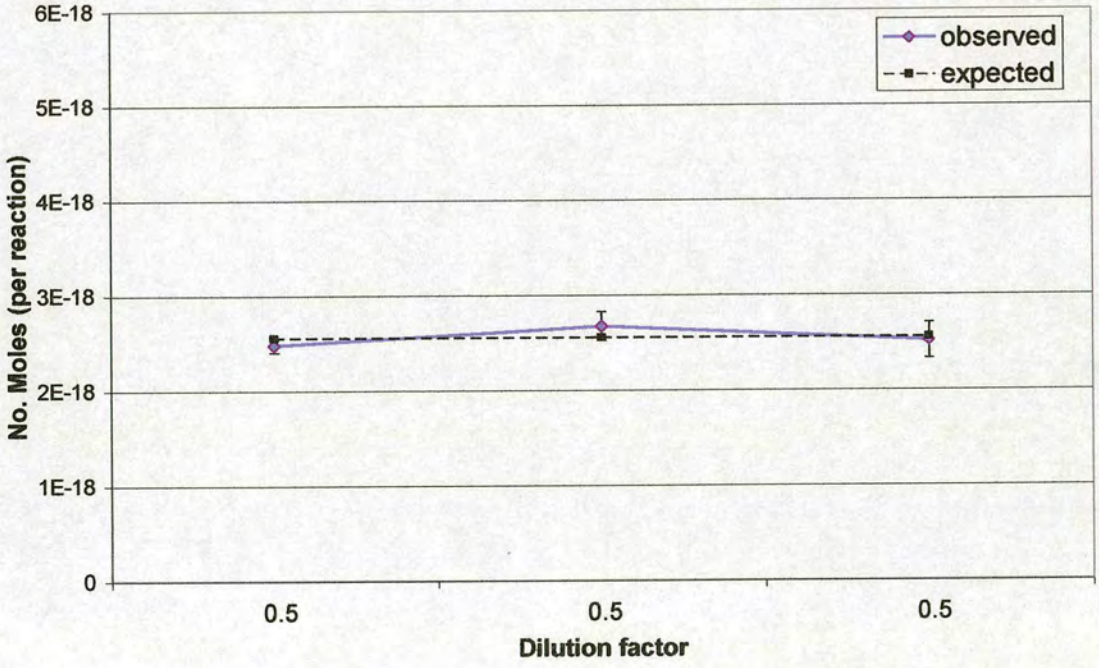


Figure 3.3.5.5. Equal GnRH-I mRNA concentrations in increasing quantities of total RNA. This was diluted with cortex cDNA, H_20 and a 50:50 mixture of them both. Average ± SE MEAN.

3.3.6. Validation Experiments: Coefficient Of Variance

The results of repeat PCR reactions show quite a high level of variation is occurring between runs of the same mRNA samples, with an average coefficient of variance at 22.6 %. The coefficient of variance at the PCR stage is 15.9 % (Table 3.3.6.). The overall intra-assay co-efficient is 19.2 %.

	Sample 1 RT	Sample 1 PCR	Sample 2 RT	Sample 2 PCR
	8.87E-19	1.01E-18	1.75E-18	1.62E-18
	1.04E-18	9.49E-19	1.83E-18	1.77E-18
	1.01E-18	1.48E-18	1.11E-18	1.89E-18
	1.56E-18	8.98E-19	2.04E-18	1.49E-18
	9.94E-19	1.21E-18	1.93E-18	1.92E-18
Average	1.10E-18	1.11E-18	1.73E-18	1.74E-18
ST DEV	2.63E-19	2.40E-19	3.67E-19	1.81E-19
SE MEAN	1.18E-19	1.06E-19	1.64E-19	8.08E-20
Co efficient of Variation (%)	23.97	21.36	21.18	10.39

Table 3.3.6. GnRH-I mRNA levels (moles per POA) in two repeated samples. The first is five samples taken from the mRNA prior to reverse transcription (sample RT) and the next five are samples taken from one of the reverse transcription mixtures samples PCR).

3.4. PROBLEMS ENCOUNTERED

The assay described here has, even with a heterologous competitor design, on occasions produced a heteroduplex band. It has been possible to remove this by heating the PCR reaction mixture to 95^0C for 5 to 10 minutes and then cooling on ice. It is therefore recommended that the PCR mix used for this assay is in large enough quantity to be run twice on gel electrophoresis.

3.5. DISCUSSION

The Japanese quail (*Coturnix japonica*) GnRH-I mRNA sequence has high homology to chicken GnRH-I. A close similarity between the quail and chicken GnRH-I sequences was expected because of southern blot analyses and their close evolutionary relationship (Dunn *et al.*, 1993).

The target GnRH-I mRNA sequence and the competitor used in the assay have very similar amplification efficiencies, even though they possess different sequences between their respective primer binding sites. The standard and competitor amplify at similar rates whether they were equimolar or at differing concentrations and also when DMSO was added to the reaction mixture. These finding are consistent with previous studies that have shown that the sequence of the primers has the greatest effect on amplification efficiency (Wang *et al.*, 1989). By diluting POA cDNA by specific amounts it was possible to test the competitive PCR assays ability to measure relatively small changes in GnRH-I mRNA levels. The change in mRNA levels was determined accurately when the background mRNA was stayed the same or increased with the values being very close to those predicted. Thus the GnRH-I mRNA Q RT-PCR can measure at least a four fold difference in target mRNA levels between different samples. Unfortunately the assay has quite a high coefficient of variation, this should be considered with negative results as the high variation could reduce its ability to detect changes in GnRH-I mRNA. The next step was to use the assay in a biological context to test that it was able to detect natural levels of variation. This was confirmed in comparisons between tissue types (chapter 5) and in a series of photostimulation experiments (chapters 6 to 9).

4. DISTRIBUTION AND QUANTIFICATION OF GnRH-I mRNA LEVELS IN REPRODUCTIVE AND NON-REPRODUCTIVE TISSUES.

4.1. INTRODUCTION

It was hypothesised that in addition to the hypothalamus, GnRH-I gene expression might occur in avian gonads as has been observed in mammals (Bahk *et al.*, 1995; Botte *et al.*, 1998) and fish (von Schalburg and Sherwood, 1999), mast cells as in doves (Silverman *et al.*, 1994), immunological tissues as in rat spleen lymphocytes (Azad *et al.*, 1991), the pituitary (Pagesy *et al.*, 1992; Kakar and Jennes, 1995) and possibly in other non-reproductive tissues as has been observed in humans (Kakar and Jennes, 1995) and chickens (Sun *et al.*, 2001). This study was conducted to determine the distribution of GnRH-I mRNA in reproductive and non-reproductive tissues in quail to compare the quantities of these transcripts with the levels in the brain.

4.2. EXPERIMENTAL DESIGN

The methodology used for the Q RT-PCR assay in this chapter differed slightly to that described in chapter 2. The cDNA from different tissues was titrated against 5 five-fold dilutions of quantities of competitor. It was not appropriate to create a standard curve with which to compare ratios of competitor to standard with competitor to unknown because of the wide range of concentrations across tissues. The standard

curve would have had to be unrealistically long, encompassing many orders of difference in concentration or would have had to be extrapolated to such a degree that the results would have been inaccurate.

The first experiment was performed as a preliminary study to determine which tissues contained detectable GnRH-I mRNA. For this RNA from 2 male and 2 female, 18 month old breeding quail were assayed (18L:6D). Tissue samples were taken from the: pre-optic area (POA), basal hypothalamus (BH), cerebellum, cortex, pituitary, testes, ovarian stroma, theca and granulosa cell layers from mature ovarian follicles, spleen, liver, kidney and small intestine. The quality of RNA extracted was assessed by formaldehyde gel electrophoresis and the efficiency of reverse transcription was checked by PCR assay with prolactin receptor (PRL-R) primers. A PCR using quail GnRH-I mRNA primers was then used to determine which tissues contained GnRH-I mRNA.

In the second experiment 4 males and 4 females of 18 months of age were used and RNA from the POA, BH, gonad, kidney and spleen were assayed. RNA and reverse transcription were checked as in the previous experiment. GnRH-I mRNA was quantified as a concentration in moles per μl of RNA and in moles per whole tissue, for example, one whole testis.

4.3. RESULTS

4.3.1. Distribution of GnRH-I mRNA

The total RNA extracted was shown to be good quality by gel electrophoresis, in all tissues except for the granulosa layer of the follicles (figure 4.3.1.). The efficiency of the reverse transcription step was confirmed as a strong PRL-R band, produced in all tissues except for the granulosa (figure 4.3.2.). GnRH-I mRNA was not detectable in the cerebellum, cortex, spleen, liver, kidney, small intestine, ovarian granulosa, ovarian theca or pituitary (blank gels not shown). The spleen and kidney were used as negative controls in the subsequent experiment. GnRH-I mRNA was detectable in the pre-optic area, basal hypothalamus, testes and ovarian stroma (see figure 4.3.3.).

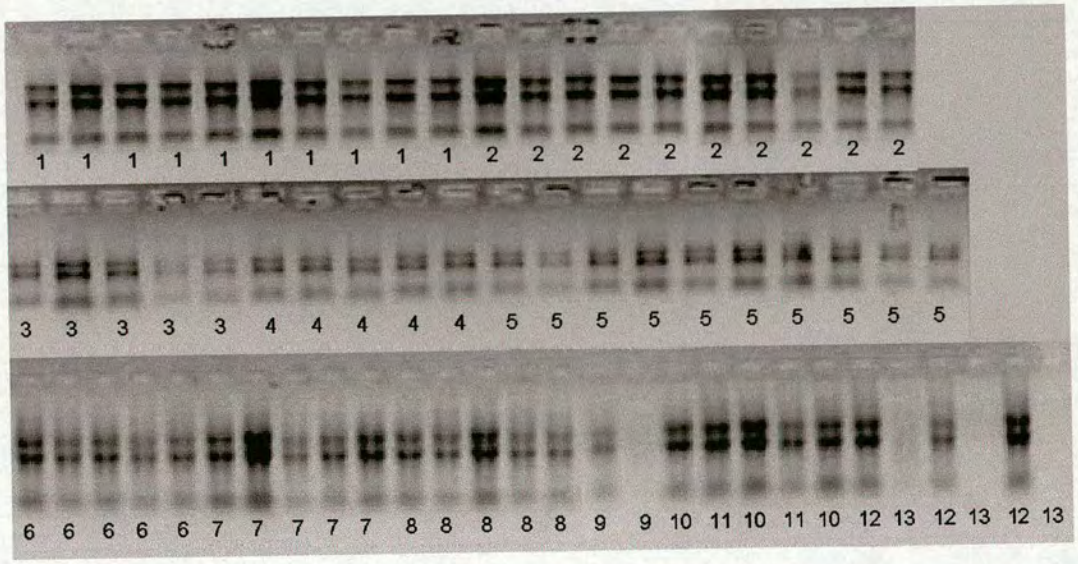


Figure 4.3.1. Electrophoresis of RNA from various tissues in a 1% formaldehyde gel, visualised with ethidium bromide under UV light. 1 = POA, 2 = BH, 3 = cortex, 4 = cerebellum, 5 = small intestine, 6 = kidney, 7 = liver, 8 = spleen, 9 = pituitaries, 10 = ovarian stroma, 11 = testes, 12 = ovarian theca and 13 = ovarian granulosa.

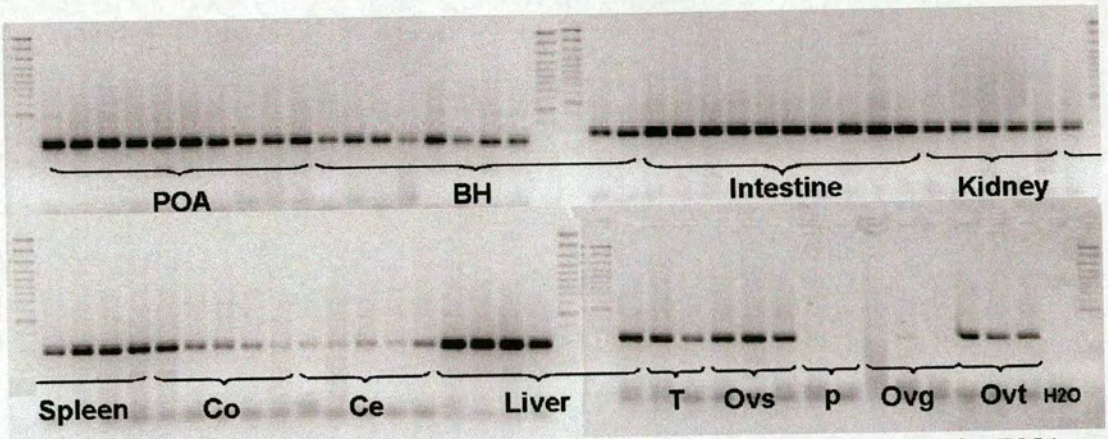


Figure 4.3.2. Electrophoresis of PRL-R amplified from reverse transcribed mRNA on a 2% gel, visualised with ethidium bromide under UV light. POA preoptic area, BH basal hypothalamus, Co = cortex, Ce = cerebellum, T = testes, Ovs = ovarian stroma, P = pituitaries, Ovg = ovarian granulosa and Ovt = ovarian theca.

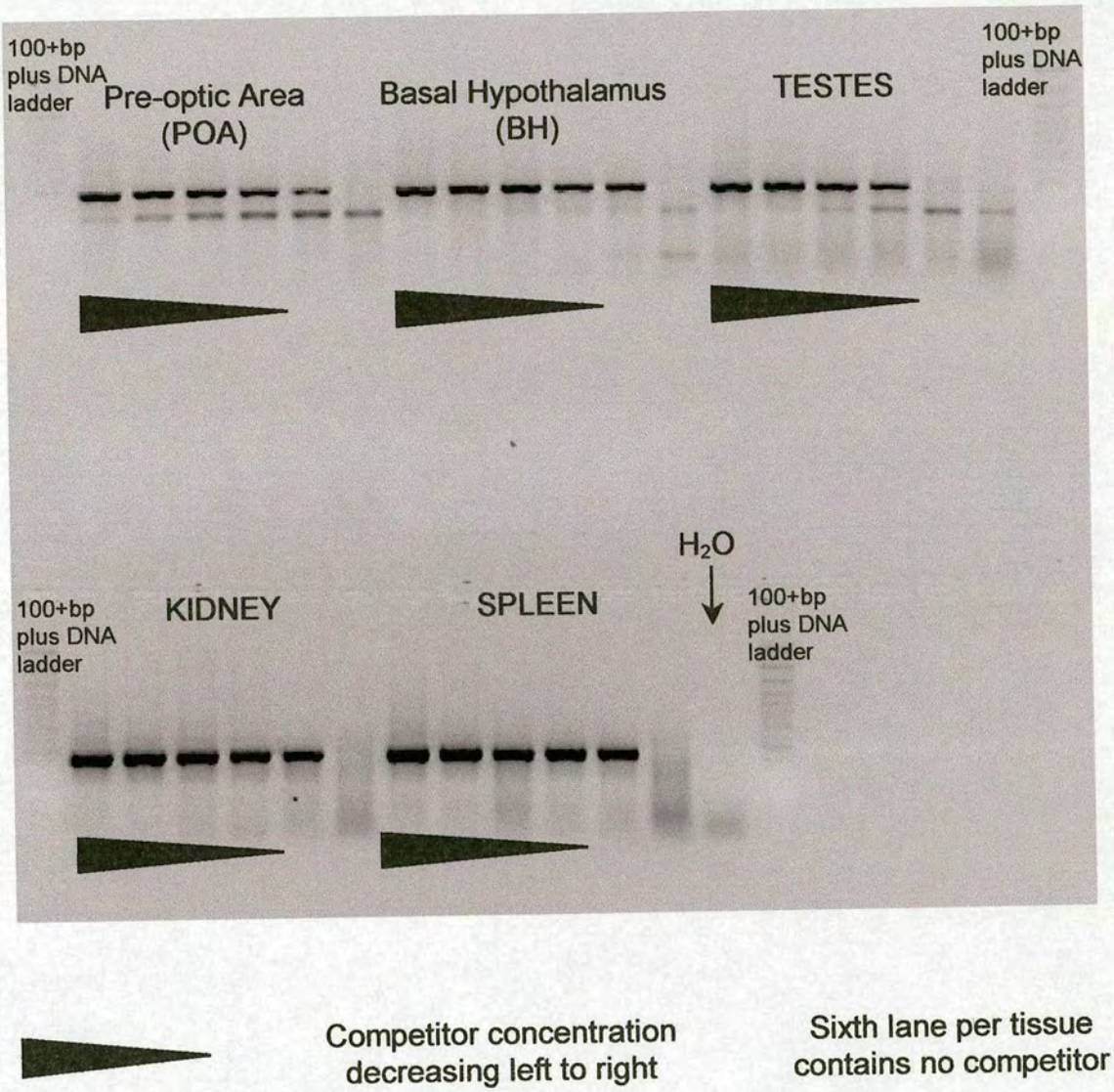


Figure 4.3.3. An example QC- PCR assay of GnRH-I mRNA in quail POA, BH, testes, kidney and spleen run on a 3% agarose gel (upper DNA band = competitor and lower = native GnRH-I).

4.3.2. Quantification of GnRH-I mRNA

4.3.2.1. Total GnRH-I mRNA content of tissues (moles/tissue)

The total amount of GnRH-I mRNA in the POA was $2.27\pm0.97x10^{-17}$ moles in the males and $2.03\pm0.57x10^{-17}$ moles in females (d.f. 7, F 0.04, P=0.84) and in the BH $1.60\pm1.2x10^{-19}$ moles in males and $2.99x10^{-21}$ moles in females (d.f. 7, F 1.71, P=0.24)(figure 4.3.2.1.). The amount of GnRH-I mRNA in the BH of males and females was extremely low. The total GnRH-I mRNA content was $5.03\pm1.4x10^{-17}$ moles in the testes and $3.51\pm1.6x10^{-18}$ moles in the ovarian stroma (d.f. 7, F 10.97, P=0.016). The total amount of GnRH-I mRNA in the testes was greater than in the POA (d.f. 11, F 5.7, P=0.038) or the BH (d.f. 11, F 28.51, P=0.000). The total amount of GnRH-I mRNA in the ovarian stroma was greater than in the POA (d.f. 11, F 5.53, P=0.041) but not the BH (d.f. 11, F 8.51, P=0.015). The testes and ovaries were fully developed. The mean weight of the testes and ovarian stroma were 1.8 g and 0.24 g respectively.

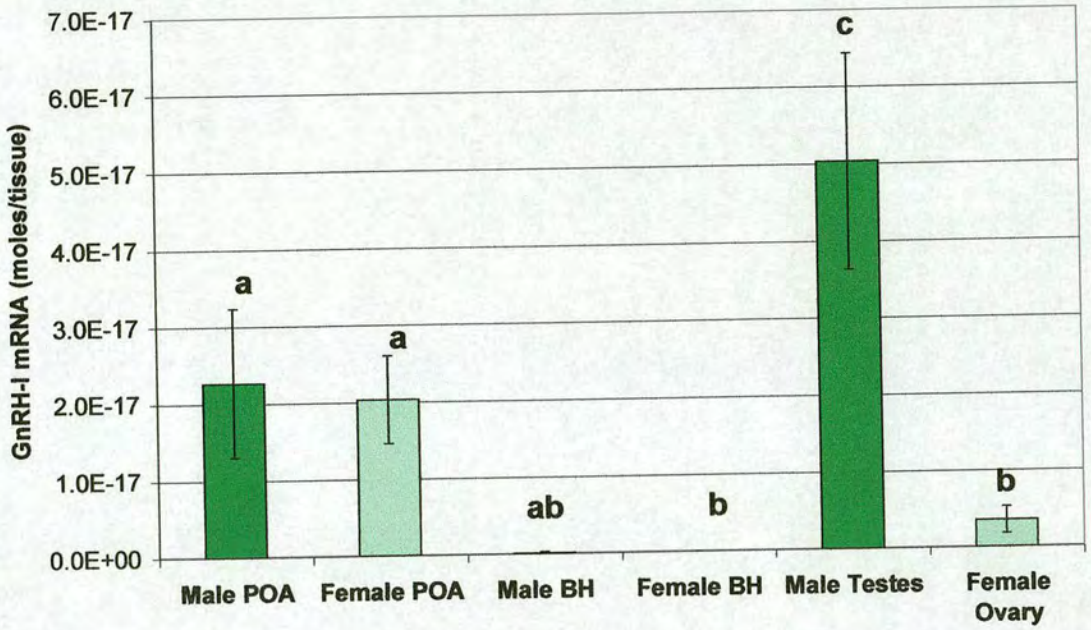


Figure 4.3.2.1. Comparison of amounts of GnRH-I mRNA in POA, BH and Gonads, in males (dark green) and females (light green), expressed in moles per whole tissue. Values with different subscripts are significantly different. For significance levels see text. N = 4 for all groups, average ± SE MEAN.

4.5.2.2. Concentration of GnRH-I mRNA in tissues (moles/μg of RNA)

The concentration of GnRH-I mRNA in the POA was $1.66\pm0.71\times10^{-18}$ moles/μg of RNA in the males and $1.52\pm0.53\times10^{-18}$ moles/μg of RNA in the females (figure 4.3.2.2.). In the BH the GnRH-I mRNA concentration was $3.84\pm2.9\times10^{-20}$ moles/μg of RNA in the males and $6.36\pm0.0\times10^{-22}$ moles/μg of RNA in the females. The concentration of GnRH-I mRNA in the testes was 4.28±0.82x1-0^{-20} moles/μg of RNA and $2.87\pm1.0\times10^{-20}$ moles/μg in the ovarian stroma (d.f. 7, F 1.14, P=0.326). The concentration of GnRH-I mRNA was greater in the POA than the BH (d.f. 11, F 14.58, P=0.002), testes (d.f. 11, F 6.75, P=0.027) and ovarian stroma (d.f. 11, F 6.87,

P=0.026). The concentration of GnRH-I mRNA found in the BH was not significant to that in the testes (d.f. 11, F 1.05, P=0.33) or ovarian stroma (d.f. 11, F 0.16, 0.70).

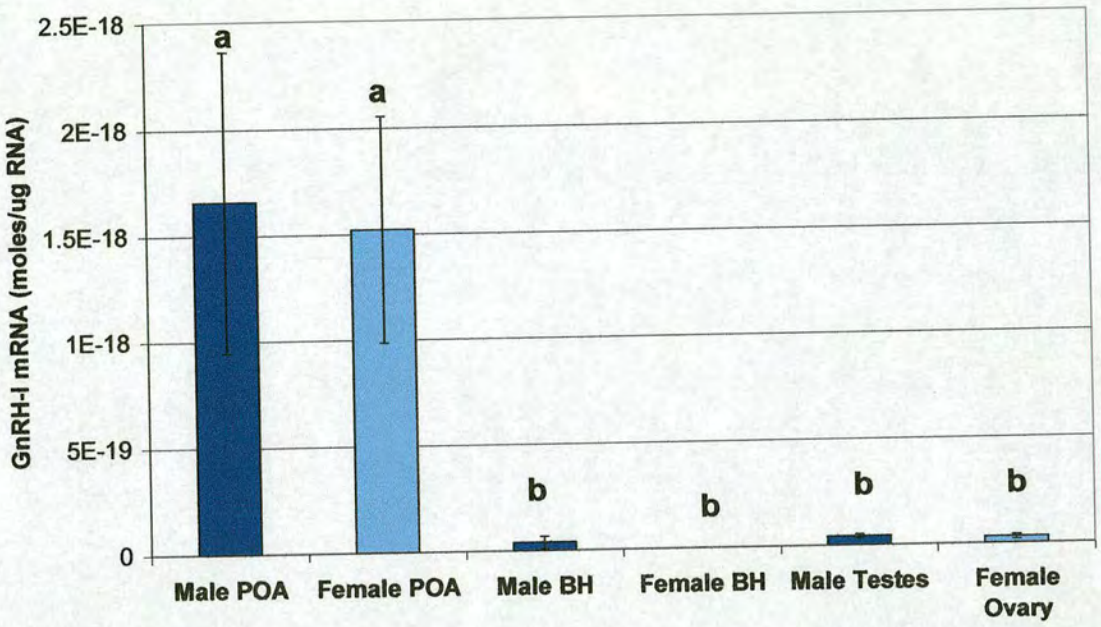


Figure 4.3.2.2. Comparison of concentrations of GnRH-I mRNA in POA, BH and Gonads, in males (dark blue) and females (light blue), expressed in moles per μg of RNA. Values with different subscripts are significantly different. For significance levels see text. N = 4 for all groups, average ± SE MEAN.

4.4. DISCUSSION

GnRH-I mRNA transcripts were found in the quail hypothalamus and gonads but not in any other tissues. This is the first report of GnRH-I mRNA in the ovaries of an avian species and the first report of GnRH-I mRNA in the quail testes.

GnRH-I mRNA was found in the anterior hypothalamus (dissected region described as POA, see chapter 2) where it has previously been quantified in chickens and in very low levels in the BH (Dunn *et al.*, 1996; Dunn and Sharp, 1999, Sun *et al.*, 2001).

There was no evidence of sex differences in the content of the hypothalamus in quail as observed in rats (Malik *et al.,* 1991). The expression of GnRH-I in the BH was about 1/100th of that observed in the POA and its presence in the BH has several potential explanations. It is possible that detectable levels of GnRH-I mRNA are transported through the BH within GnRH-I neurone axons to the median eminence (Mohr *et al.*, 1991). Alternatively a small number of cell bodies may be contained within the BH, although these have not been detected using immunocytochemistry in Japanese quail (Foster *et al.,* 1988). In this study GnRH-I mRNA was not found elsewhere in the brain, this is concordant with immunocytochemical distribution studies of GnRH in chicken (for review see Dunn and Millam, 1998).

There was no GnRH-I mRNA detectable in the pituitary in this study. This is concordant with work on rats (Azad *et al.*, 1991) where the anterior pituitary was used as a negative control for a study on the expression of GnRH transcripts in tissues of the immune system. However, contrary to this, Pagesy *et al.* (1992) found GnRH-I mRNA to be present in rat pituitaries.

GnRH expression has been reported in many tissues outside of the hypothalamus in birds, mammals and fish, particularly the gonads and in mammals in the placenta. In some of these areas GnRH is known to have a function. In the testes of rats GnRH is secreted from the Sertoli cells and acts on the Leydig cells, which have GnRH receptors (Sharpe *et al.*, 1981). Receptors for GnRH are also present in the granulosa cells of the ovary and GnRH mRNA has been demonstrated using RT-PCR to be

present in the ovary of rats (Oikawa *et al.*, 1990). GnRH mRNA was subsequently detected by in situ analysis in the granulosa and theca of the rat ovary (Clayton *et al.*, 1992). In the rat ovary GnRH may be involved in the regulation of follicle atresia (Billig *et al.*, 1994). In salmonids at least 3 forms of GnRH are expressed in the ovary and testes during gametogenesis (von Schalburg and Sherwood, 1999). It seems likely that GnRH is a paracrine factor in the gonads across the vertebrates although its precise role may vary in respective to reproductive activity.

The total amount of GnRH-I mRNA in the developed gonads was higher than in the hypothalamus. This is the second report of a quantitative comparison of gonadal and hypothalamic levels of GnRH-I mRNA in vertebrates (Sun *et al.*, 2001), and supports the evidence that GnRH plays a key role in the gonads as well as the hypothalamus. The large amount of GnRH-I mRNA in the testes as a whole was due to the large size of the testes in comparison to the POA, BH and ovarian stroma.

The absence of GnRH-I mRNA from the granulosa cells requires further conformation. It could be a consequence of the low levels of RNA isolated or a failure of the reverse transcription step.

In chicken, GnRH-I mRNA has been detected in many tissues outside of the hypothalamic pituitary gonadal-axis (Sun *et al.*, 2001). The functional significance of this is uncertain. GnRH-I mRNA was not detected in non-reproductive tissues in quail. There may be several reasons for the failure to observe GnRH-I transcripts in

quail other than the gonads and hypothalamus of quail. There may be differences in distribution in quail and chicken or more likely the Q RT-PCR assays developed to measure GnRH-I mRNA may differ in sensitivity. Observations by Sun *et al,* (2001) of GnRH-I mRNA in non-reproductive tissues in chickens may be explained by the extreme sensitivity of the RT-PCR assay used to measure it. A small number of GnRH-I mRNA transcripts may be produced in many tissues but have no physiological significance. However, the quail Q RT-PCR assay developed here was sufficiently high to measure approximately 1800 GnRH-I mRNA transcripts, as determined by OD. Tissues in which GnRH-I mRNA was not detected must, therefore, contain considerably less than 1800 GnRH-I mRNA transcripts and have on average less than one GnRH-I mRNA transcript per cell.

5. CHANGES IN HYPOTHALAMIC GnRH-I mRNA DURING PHOTOINDUCED FIRST DAY RELEASE OF LH

5.1. INTRODUCTION

The release of LH from the pituitary is controlled by the release of GnRH-I from the median eminence (Jennes and Conn, 1994; Conn *et al.,* 1995, Perera and Follett, 1992). Meddle and Follett (1995; 1997) were unable to co-localise gene transcription factor cFos-lir antigens (FRAs) within GnRH-ir cell bodies in the hypothalamus for up to three days after photostimulation. This was confirmed by Péczely and Kovács (2000) in the mallard. Meddle and Follett (1997) demonstrated that cells within the quail basal hypothalamus and median eminence are activated at hour 18 from dawn of the first long day, using cFos immunocytochemistry. This is approximately the same time as the first detectable increase in plasma LH. The cells with cFos-lir in the median eminence were glial, as determined by their co-localisation with glial fibrillary acidic protein (GFAP). Glial cells are known to ensheath the terminals of the GnRH-I neurones in the outer layer of the median eminence (Kozlowski and Coates, 1985, Witkin *et al.,* 1997) and there is considerable evidence to suggest that these glial cells retract to allow the release of GnRH-I into the portal blood vessels. Electron microscope work by Prevot *et al.,* (1999) revealed that during diestrus II in rats, when LH levels are basal, no GnRH-I nerve terminals were in contact with the pericapillary space. However, in proestrus when the preovulatory GnRH-I surge occurs approximately 12% of the GnRH-I nerve terminals made contact with the

pericapillary space. This provides evidence that dynamic plastic changes occur in the external zone of the median eminence to allow the release of GnRH-I from the nerve terminals.

The probable control of the nerve terminals of the GnRH-I neurones through glial function does not exclude other forms of control within the GnRH-I neurone. There may also be changes in GnRH-I synthesis. However, Meddle and Follett (1995; 1997) were not able to co-localise cFos in quail GnRH-I neurones after photostimulation suggesting it is possible that there is no change in GnRH-I gene transcription at this time. This observation led them to hypothesise that a store of GnRH-I in GnRH-I neurones is rapidly released as a consequence of glial cell retraction following photoinduction resulting in GnRH-I induced LH secretion.

Most observations are consistent with the idea that the initiation of photoinduced GnRH-I release is independent of increased GnRH-I synthesis. There is no change in hypothalamic content after photostimulation in Japanese quail (Creighton and Follett, 1987), starlings (Dawson *et al.,* 1985), deer mice (Koryotko *et al.,* 1997) and hamsters (Ronchi *et al.,* 1992; Urbanski *et al.,* 1991; Yellon, 1994) though a change has been observed in hamsters (Steger *et al.,* 1984; Porkka-Heiskanen *et al.,* 1997; Pickard and Silverman, 1979) horses (Hart *et al.,* 1984) and sheep (Ebling *et al.,* 1987).

However, measurements of GnRH peptide are difficult to interpret as they represent a balance between pro-GnRH synthesis, processing and rate of turnover, in addition to changes in GnRH release.

There is some immunocytochemical evidence of increased GnRH synthesis in photostimulated birds. In Japanese quail the density of GnRH immunoreactive nerve fibres and intensity of staining is greater in birds exposed to long days for 24 weeks than in reproductively inactive birds held on short days. (Foster *et al.* 1988; Teruyama and Beck, 2000).

Direct evidence for an effect of photostimulation on GnRH synthesis comes from a study in the domestic chicken (Dunn and Sharp, 1999). They found that GnRH-I mRNA content of the chicken hypothalamus increases after one week of photostimulation. This observation suggests that GnRH-I neurones respond to increased photoperiod with an increase in GnRH-I transcription and/or GnRH-I mRNA stability. It is not known whether this increase is a necessary part of, or even associated with, the initial stages of photoinduction.

Hypotheses of how GnRH-I neurones respond to photoperiodic induction are summarised in figure 5.1.

Hypothesis A. GnRH-I peptide release is controlled by a photoinduced retraction of glial cells around GnRH neurone terminals in the median eminence. There is no new synthesis of GnRH-I, existing stores are released as a consequence of glial retraction. This hypothesis predicts that there should be no increase in GnRH-I mRNA during the first day LH release and no GnRH-I mRNA in short day birds.

Hypothesis B. GnRH-I peptide release is controlled by glial cells at the terminal. These release a store of GnRH-I which has built up because of the continuous production of GnRH-I. In this hypothesis synthesis of GnRH-I does not increase in response to a long photoperiod. Here GnRH-I mRNA is expected to be the same in short and long birds during the first day release of LH.

Hypothesis C. GnRH-I peptide released is increased by the removal of an in inhibitory factor acting on translation or some other part of the protein synthesis pathway. Glial cell retraction is not involved. Here GnRH-I mRNA is expected to be the same in short and long day birds during the first day release of LH.

Hypothesis D. The first long day of photostimulation co-ordinates the release of GnRH-I peptide from the GnRH-I neuronal network. GnRH-I peptide release becomes synchronised and produces a pulse of GnRH-I secretion. GnRH-I mRNA is predicted to remain the same on the first long day of photostimulation. (GnRH-I release is known to be pulsatile (Wilson and Sharp, 1975)).

Hypothesis G. GnRH-I mRNA is expected increases after the critical day length is exceeded. An increase in GnRH-I release should occur shortly after an increase in GnRH-I mRNA.

Hypothesis H. Photoinduction stimulates stored GnRH-I release as well as increasing GnRH-I peptide synthesis. GnRH-I mRNA is expected to be present on

short days and to increase during the first long day. An increase in GnRH-I release should occur before an increase in GnRH-I mRNA.

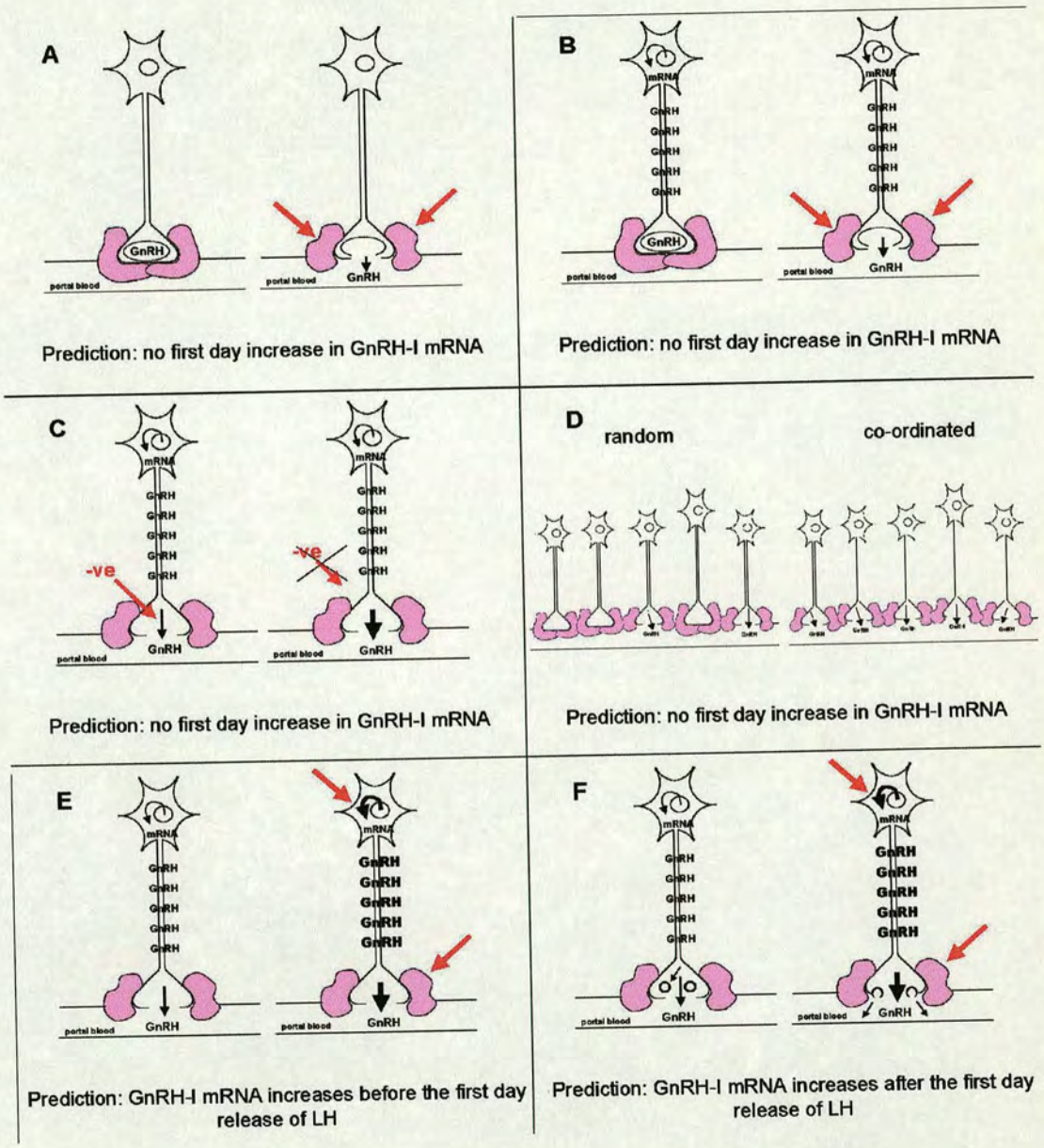


Figure 5.1. The main hypotheses of GnRH-I neurone control during the first day of photoperiodic induction. The red arrows indicates possible delivery sites of photoperiodic information, the straight black arrows indicate GnRH-I movement, the curved black arrows indicate the transcription of GnRH-I mRNA and elipses represent stores of GnRH-I peptide

Hypothalamic GnRH-I mRNA was measured to determine the timing of changes in GnRH-I mRNA levels in relation to photoperiodic induction during exposure to a first long day to distinguish between these hypotheses.

5.2. EXPERIMENTAL DESIGN

A first day LH release is not observed in all quail and the proportion of the population that do respond increases if they have previously been photostimulated (Follett *et al.,* 1977). To ensure that all birds show a first day LH response it was necessary to pre-treat them by exposing them to a cycle of long and short days in advance of the experiment and then only those exhibiting the first day release of gonadotrophins were used in the experiment.

The plasma LH and hypothalamic GnRH-I mRNA levels in photostimulated birds were compared with short day controls at one-week (n=9), 36 hours (n=7), 20 hours (n=8) and 16 hours (n=8) after dawn of the first long day. Photostimulated birds for the 'one-week' and '36 hour' groups were exposed to photoperiods of 20L:4D. All controls were retained on 8L:16D. The statistical comparison between photostimulated and control groups were made using one-way analysis of variance on MINITAB®. The comparisons of GnRH-I mRNA levels can only be made between experimental and control groups of the same time point. This is because the competitive PCR assay gives a relative and not an absolute quantity for GnRH-I RNA. Each of the four time points were run on different days.

5.3. RESULTS

5.3.1. The selection of birds with a first day release response

In order to select those birds in the Roslin quail colony which exhibited the first day release of LH, 46 male and 34 female birds were transferred from short days of 8L:16D to 20L:4D. Blood samples were taken at hours 0, 18 and 36 from dawn of that first long day (figure 5.3.1.). During these initial experiments it was found that serially bled control birds had lower levels of LH by the second bleed as compared with the controls, this was not significant (d.f. 14, T -0.9981, P=0.92). It is possible that the of stress of being handled was lowering plasma LH levels. Although this was below significance, to prevent any possible effect of stress, birds were handled regularly prior to all subsequent experiments. The percentage of the population that exhibited the first day release of LH was 83%. In the birds that did respond there was no significant difference 18 hours from dawn as compared with time 0 (d.f. 29, T 2.27, P=0.22). There was a significant increase in LH 36 hours from dawn of the first long day (d.f. 29, T 7.16, $P \leq 0.0001$).

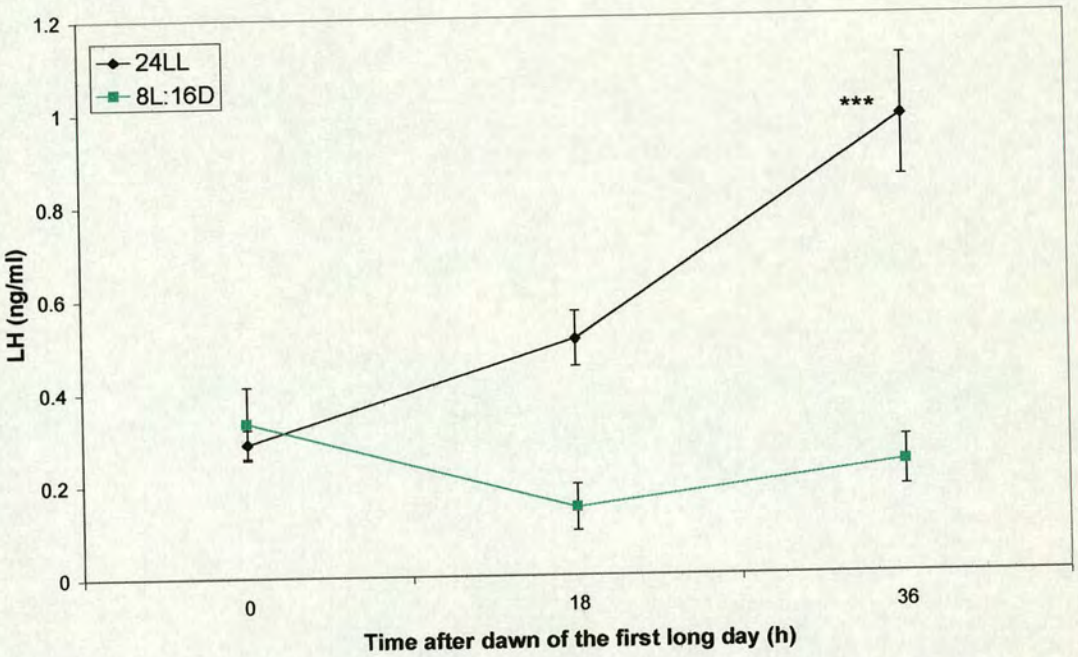


Figure 5.3.1. Plasma LH levels in serially bled photostimulated (20L:4D, black line) and short day (8L:16D, green line) control birds; Average ± SE MEAN, *P≤0.05, ** P≤0.01, *** P≤0.001 relative to time 0.

5.3.2. Change in GnRH-I mRNA during the initiation of photoinduced LH release

Plasma LH was higher in quail after one-week (d.f. 17, T -9.09, P≤0.0001) and 36 hours (d.f. 13, T 4.44, P≤0.001) . There was no evidence of an increase in plasma LH after 20 hours (d.f. 15, T -1.60, P=0.75) after dawn of the first long day (20L:4D) (figure 5.3.2.1.) or 16 hours (d.f. 15, T -0.43, P=0.999) from dawn of the first long day.

GnRH-I mRNA was also significantly higher than in short day controls one week (d.f. 15, T 2.71, P≤0.05), 36 hours (d.f. 13, T 2.82, P≤0.05) and 20 hours (d.f. 15, T 3.05, P≤0.01) from dawn (figure 5.3.2.2.). The hypothalamic GnRH-I mRNA content in the photostimulated birds was approximately double that in the control birds after

1 week, 36h and 20h from dawn of the first long day. There was no difference in GnRH-I mRNA between photostimulated and short day control birds, 16 hours from dawn of the first long day (d.f. 15, T 0.22, P=0.83).

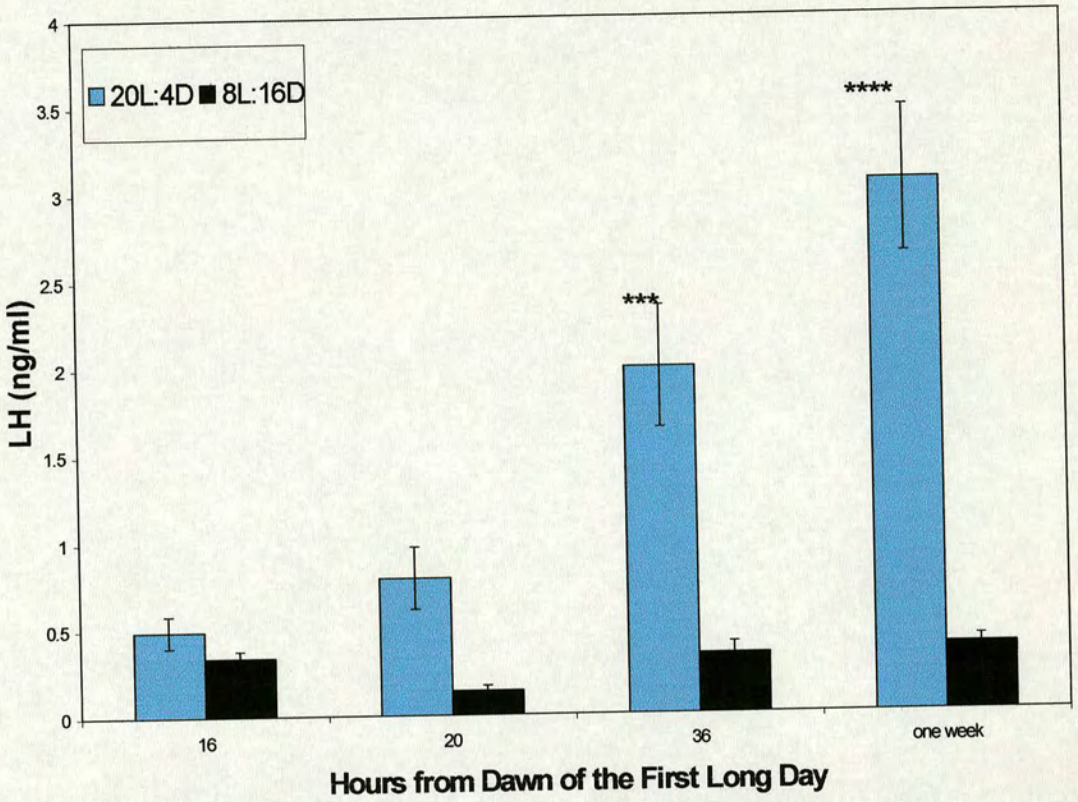


Figure 5.3.2.1. Plasma LH levels in photostimulated (20L:4D) and short day (8L:16D) control birds; one week (n=9), 36 hours (n=7), 20 hours (n=8) and 16 hours (n=8) from dawn of the first long day. Mean ± SE MEAN, *** P≤0.001, **** P≤0.0001.

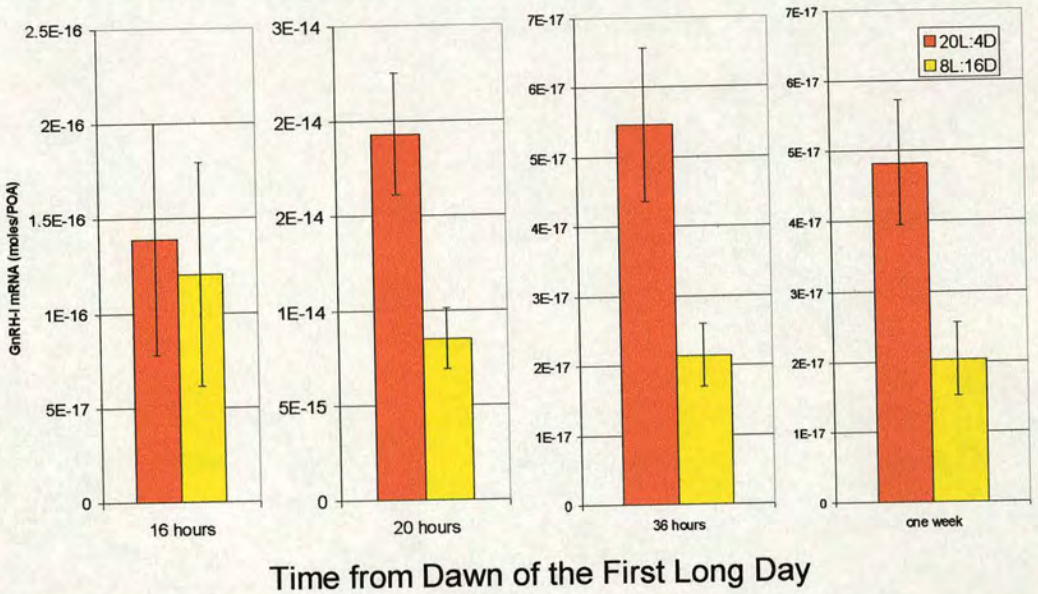


Figure 5.3.2.2. GnRH-I mRNA levels in photostimulated (20L:4D) and short day (8L:16D) control birds; one week (n=9), 36 hours (n=7), 20 hours (n=8) and 16 hours (n=8) from dawn of the first long day. Average ± SE MEAN, * $P \leq 0.05$, ** $P \leq 0.01$.

5.4. DISCUSSION

The significant increase in GnRH-I mRNA in quail after one week of exposure to long days (figure 5.3.2.1.) confirms observations in chicken (Dunn and Sharp, 1999).

GnRH-I mRNA increases at approximately the same time as the first increase in LH (figures 5.3.2.1. and 5.3.2.2.). However, LH in photostimulated quail is not significantly different to LH in the short day controls 20 hours from dawn of the first long day. This is surprising as the literature reports increases in LH at hour 20 in intact birds as well as castrates (Follett *et al.*, 1977). The observation that LH increases at approximately the same time as LH suggests that a change in GnRH-I peptide synthesis is a part of the mechanism controlling photoinduced LH release and supports previous studies in photostimulated chickens (Dunn and Follett, 1999),

hamsters (Porkka-Heiskanen *et al.,* 1997) and Salmon (Amano *et al.,* 1995; Amano *et al.,* 1999). GnRH-I neurones must therefore, respond to increased photoperiod by increases in either GnRH-I gene transcription and/or GnRH-I mRNA stability. That this change occurs so early after dawn of the first long day implies that a change in GnRH-I synthesis could be an early event in photoperiodic induction of gonadotrophin secretion. These findings allow hypotheses A, B, C and D to be rejected. However, although they can be discounted as the principal mechanisms regulating GnRH-I release, glial cell retraction and GnRH-I pulse co-ordination GnRH-I release may still play a role secondary to an increase in GnRH-I peptide synthesis. Unfortunately, the work reported here has not been able to pinpoint the exact timing of the increase in GnRH-I mRNA in relation to the increase in LH on the first long day of photostimulation, but LH continues to increase beyond the increase in mRNA. GnRH-I mRNA may rise before or after the increase in LH and so it is not possible to distinguish between hypotheses E and F. In order to clarify this further studies are necessary into the timing of these events. Most crucially the levels of GnRH-I mRNA and plasma LH at hour 18 and possibly 17 and 19 from dawn of the first long day should be studied. However, it is not known if the resolving power of the assay will be great enough to resolve such differences at these times.

6. THE EFFECT OF PHOTOSTIMULATION ON HYPOTHALAMIC GnRH-I mRNA: THE 'CARRY-OVER' EFFECT

6.1. INTRODUCTION

If quail maintained on short days are exposed to one long day and then returned to short days the photoinductive effects of that one long day, on gonadotrophin secretion persist for more than a week. For example, if castrated quail are exposed to 20 hours of light (20L:4D) and then returned to short days (6L:18D) the photoperiodic induction of LH secretion peaks approximately three days later and then decreases slowly reaching basal levels over the next 14 days (Nicholls *et al.*, 1983, Perera and Follett, 1992; Follett and Meddle and Follett, 1997). The effect is the same in intact quail although both the magnitude of the increase in LH and the duration of increased secretion is less than in castrated quail (Gledhill and Follett, 1976; Follett and Robinson, 1980).

It is likely that the carry over effect is caused by either a slow change in the re-entrainment of the circadian system or in the neural pathways that transduce the photoperiodic information. The carry over effect is not mediated solely at the level of the anterior pituitary since deafferentiation of the quail basal hypothalamus leads to a rapid disappearance of LH from the circulation (Davies and Follett 1975). Further, there is no change in pituitary sensitivity to GnRH after photostimulation (Follett *et al.*, 1977). Follett *et al.*, (1998) considered a long-lasting change in the circadian

system to be a very 'attractive' hypothesis because the carry over effect has been well documented in plants, which obviously do not have neural circuitry. If this were the case then it could be hypothesised that the basic photoperiodic mechanism evolved in a common ancestor of animals and plants. There are no measurable changes in hypothalamic GnRH content during photoinduction in quail (Bicknell and Follett, 1977; Creighton and Follett, 1987). This observation suggests that the prolonged photoinduced LH release must be due to GnRH control mechanisms of the GnRH neuronal cell bodies, perhaps at the GnRH nerve terminals. However, measurement of GnRH content is a poor indicator of release dynamics (Perera and Follett, 1992) as it is possible that there was an increase in GnRH synthesis, or change in GnRH degradation rate that compensates for the increased secretion. To address this problem Perera and Follett (1992) measured GnRH release *in vitro* from explanted hypothalamic blocks taken from quail at different times after photostimulation. They found that GnRH release was significantly higher on days 1 and 3 in birds that had received one long day of photostimulation and returned to short days, than in short day controls. GnRH secretion was back to basal levels by day 14 of the experiment. Unfortunately this experiment is not, in itself conclusive as there are a number of inherent problems. Firstly, the hypothalamic blocks were cultured in pairs and could not be cultured for longer than 8 hours and secondly, there is controversy over whether *in vitro* studies accurately reflect *in vivo* phenomena (Perera and Follett, 1992; Millam *et al.,* 1998). The development of a GnRH-I mRNA Q RT-PCR assay provides an the opportunity to investigate whether the continued release of LH after one long day depends upon an increase in GnRH-I mRNA. To do this GnRH-I

mRNA levels were measured at various times after one long day of photostimulation. This was then repeated with fewer time points to confirm the original findings.

6.2. EXPERIMENTAL DESIGN

On day one of the first experiment, photosensitive male quail on short days (8L:16D) were photostimulated with one long day of 20L:4D (figure 6.2.1.). The birds were then returned to short days of 8L:16D and hypothalamic tissues and blood were sampled across the following week. Controls were kept on short days of 8L:16D and not exposed to a long day. The first hypothalamic samples were taken on day 2 of the experiment from both photostimulated (n=8) and control (n=8) birds. Then on days 3, 5 and 8 hypothalamic samples were taken from photostimulated groups (all n=8). The final tissue samples were taken from photostimulated (n=8) and control (n=8) birds on day 15. Trunk blood samples were taken from all birds killed to collect hypothalami. Birds were killed and blood sampled 1 hour after dawn. Brachial vein samples were taken from photostimulated and control groups killed on day 15 from days 0, 1, 3, 6, 10 and 13 (n=8) at 1 hour from dawn on every day. GnRH-I mRNA was measured in hypothalamic samples and LH measured in the blood samples.

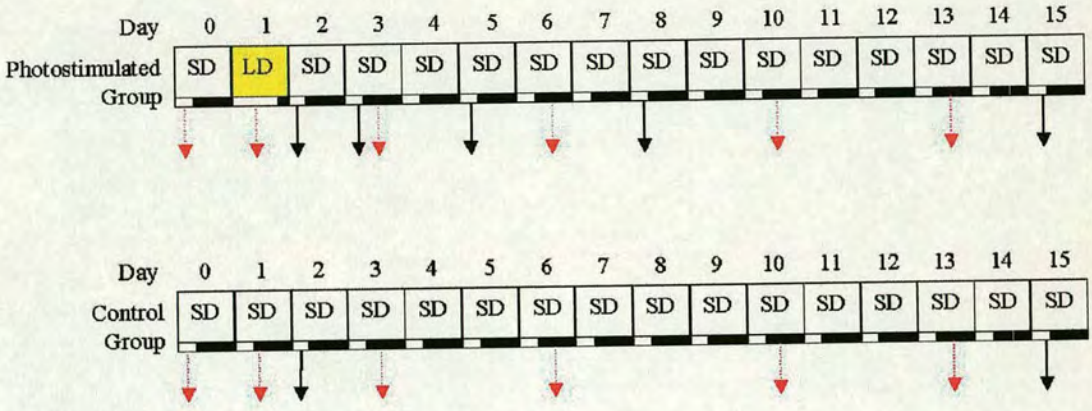


Figure 6.2.1. The experimental design for the first "carry over" experiment investigating the effects of one long day of photostimulation on GnRH-I mRNA in male quail. ↓ = hypothalamic sampling, ↓ =blood sampling points, SD = short day (8L:16D) and LD = long day (20L:4D).

In the second confirmatory experiment photosensitive short day male and female quail were also exposed to one long day of photostimulation (20L:4D). In this case hypothalami and brachial vein blood samples were taken from stimulated and short day controls on the 2nd and 4th days of the experiment (n=6 for females and n=2 for males in all four groups).

6.3. RESULTS

Plasma LH concentrations were significantly different to the control at time 0 after photostimulation (d.f. 47, F 2.85, $P \leq 0.05$) reaching a maximum on day 3 and then gradually falling back to the same mean average as day 0 by day 13 (figure 6.3.1 0.6±0.2 vs. 0.1±0.01 ng/ml, d.f. 15, $P \leq 0.05$). The controls are also significantly different from each other (d.f. 47, F 5.48, $P \leq 0.001$) with maximum levels on day 6.

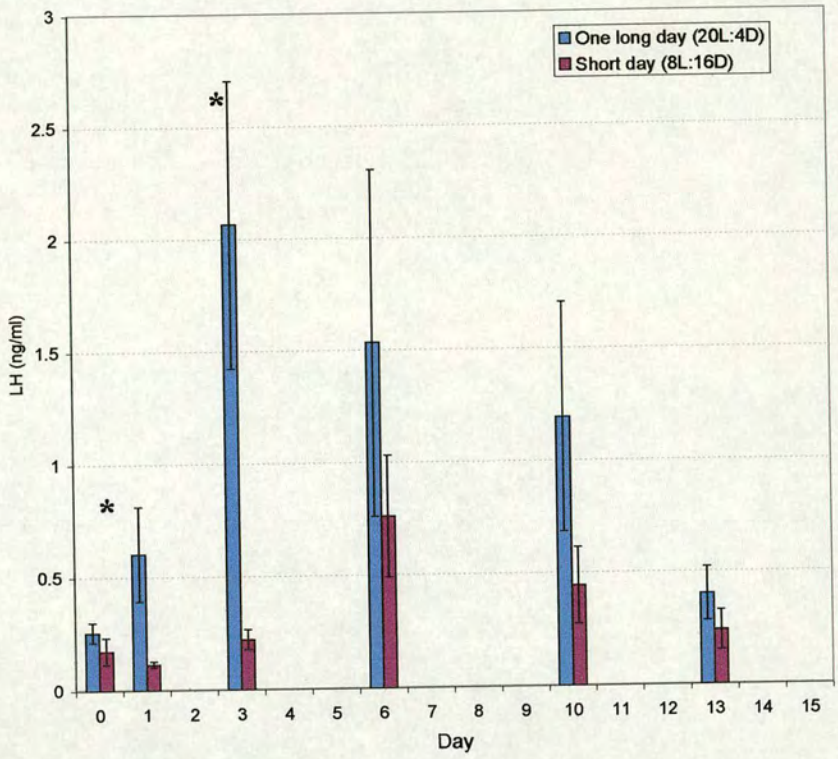


Figure 6.3.1. Plasma LH levels from male quail held on short days (8L:16D) and after exposure to one long day (20L:4D) on day 1. Control birds were held on short days (8L:16D) throughout the study. N=8 for control and stimulated groups,* $P \leq 0.05$, average ± SE MEAN.

Hypothalamic GnRH-I mRNA content in quail exposed to one long day was significantly higher than control birds one hour after dawn on day 2 ($1.08E^{-16} \pm 1.63E^{-17}$ vs. $4.42E^{-17} \pm 5.3E^{-18}$ moles/POA, d.f. 15, T-5.9, $P \leq 0.0001$) but not on days 3, 5, 8 or 15 (figure 6.3.2.). Hypothalamic GnRH-I mRNA content was higher on day 2 than day 3 (d.f. 15, T-5.6, 0.0001), 5 (d.f. 15, T -4.2, 0.01), 8 (d.f. 15, T-6.0, 0.0001) and 15 (d.f. 15, T-6.4, 0.0001). There was no significant difference between testes weights from day 2 and 15, photostimulated and control quail (figure 6.3.3.).

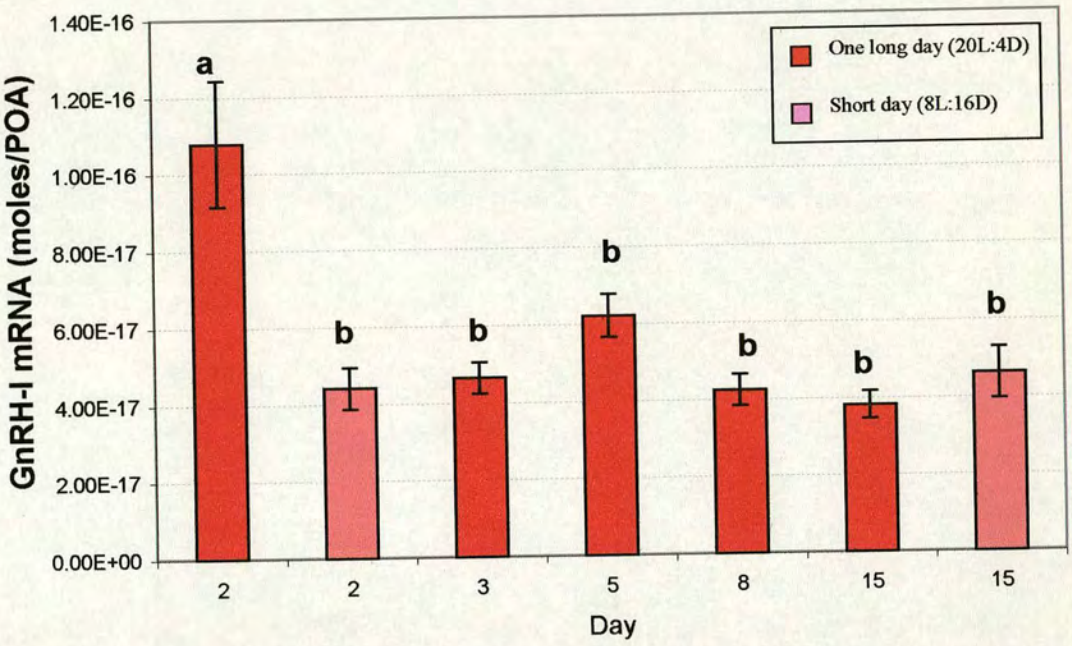


Figure 6.3.2. Hypothalamic GnRH-I mRNA content in male quail exposed to one long day of 20L:4D on day 1 and then returned to 8L: 16D on days 2, 3, 5, 8 and 15 and short day controls (8L:16D) on days 2 and 15. Values with different subscripts are significantly different. N=8 for each group, average ± SE MEAN.

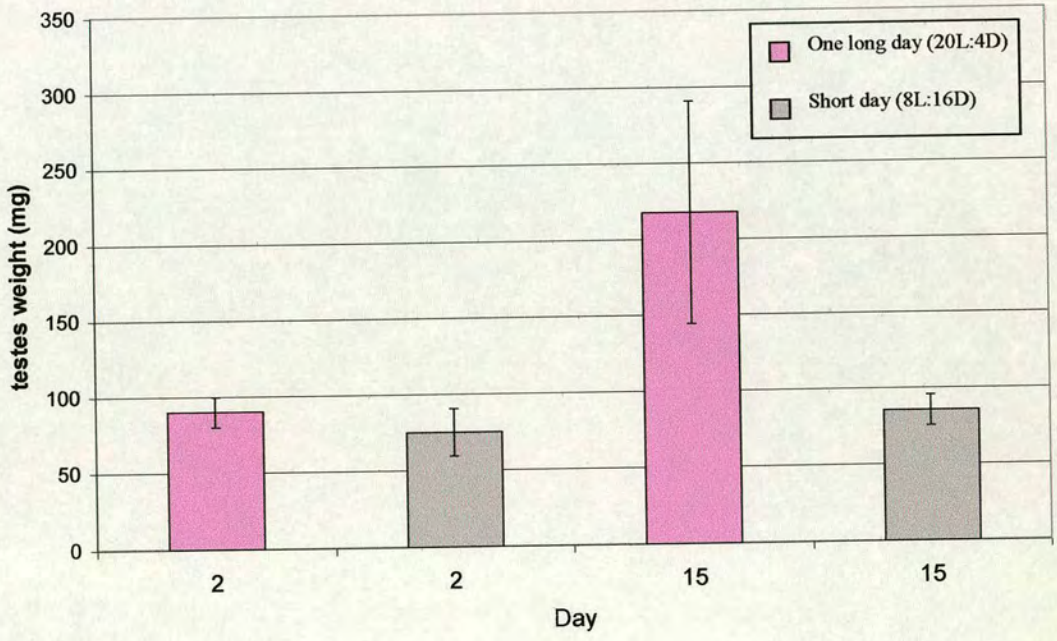


Figure 6.3.3. Testes weights in quail exposed to one long day of 20L:4D on day 1 and then returned to 8L:16D and short day controls (8L:16) on days 2 and 15. N=8 for each group, average ± SE MEAN.

The confirmatory experiment verified the findings of the first experiment. Plasma LH concentrations were at their highest on day two and were significantly higher than short day controls (4.0±0.2 vs 0.6±0.09 ng/ml, d.f. 15, T 5.02, P≤0.001, figure 6.3.4.). Hypothalami GnRH-I mRNA content was significantly higher on the second day after one long day than in the short day controls ($4.3E^{-17}\pm7.2E^{-18}$ vs. $1.7E^{-17}\pm4.6E^{-18}$ moles/POA, d.f. 15, T 4.06, P≤0.01 figure 6.3.5.). GnRH-I mRNA content then decreased to short day control values by day four when LH is still significantly elevated (1.3±0.24 vs 0.4±0.12 ng/ml, d.f. 15, T 1.37, P≤0.05). There was no significant difference in either plasma LH or GnRH-I mRNA between males and females. There were no significant differences in testes or ovary weights in photostimulated or control birds on days 2 and 4 (figure 6.3.6.).

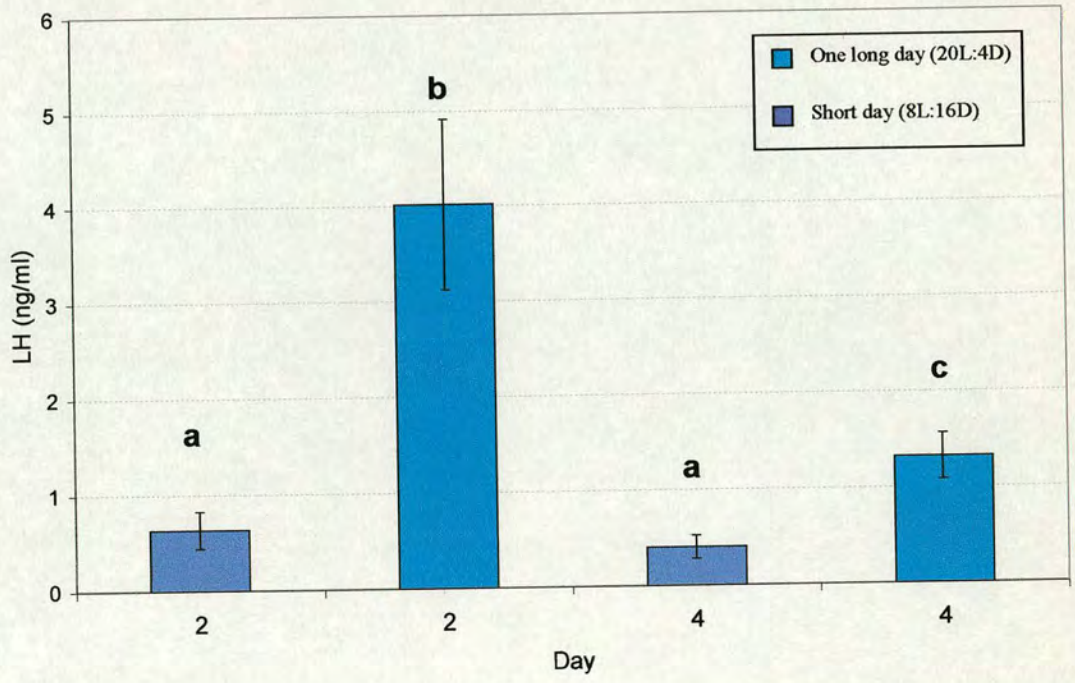


Figure 6.3.4. Plasma LH levels in quail exposed to one long day of 20L:4D on day 1 and then returned to 8L:16D and short day controls (8L:16) on days 2 and 4. Values with different superscripts are significantly different. Each group comprises 6 females and 2 males, average ± SE MEAN.

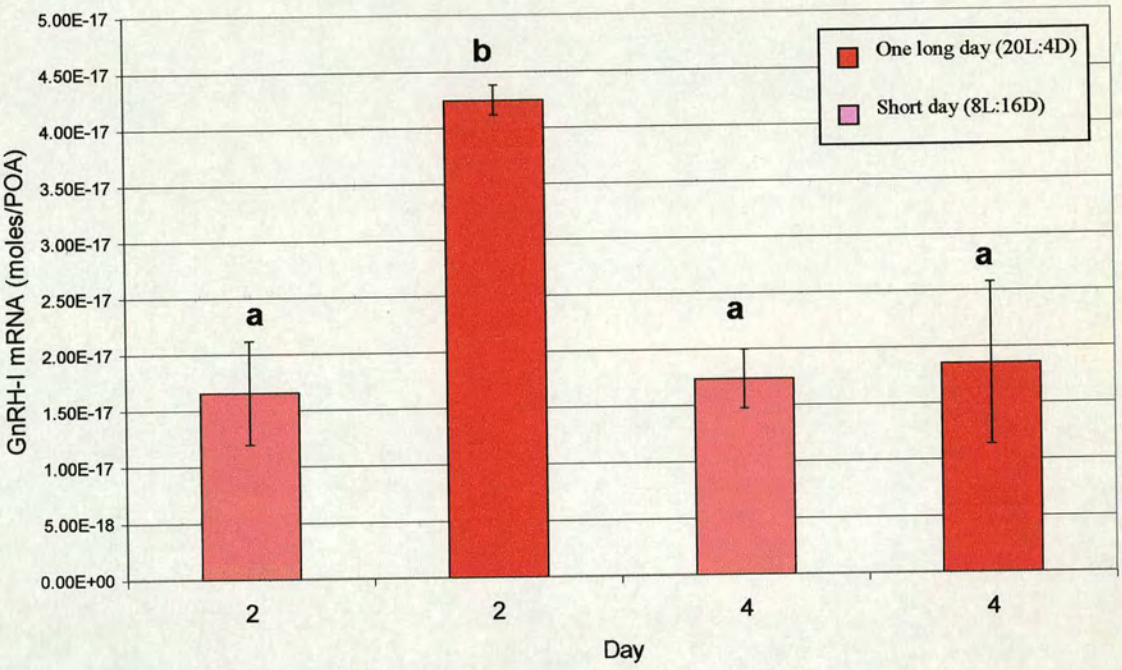


Figure 6.3.5. Hypothalamic GnRH-I mRNA contents in birds exposed to one long day of 20L:4D on day 1 and then returned to 8L:16D and short day controls (8L:16) on days 2 and 4. Values with different superscripts are significantly different. Each group comprises 6 females and 2 males, average ± SE MEAN.

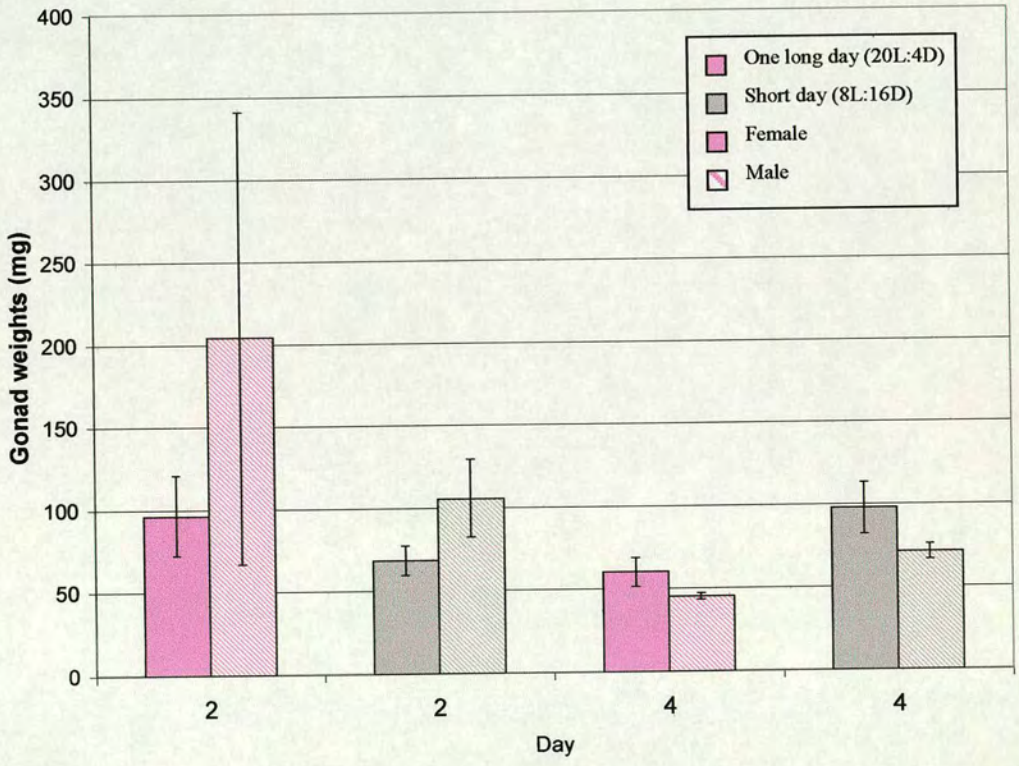


Figure 6.3.6. Gonad weights in male and female birds exposed to one long day of

20L:4D on day 1 and then returned to 8L:16D and short day controls (8L:16) on days 2 and 4. Each group comprises 6 females and 2 males, average ± SE MEAN.

6.4 DISCUSSION

The initial rise in plasma LH concentration after one long day of photostimulation was mirrored by an increase in GnRH-I mRNA. Plasma LH was still high one hour from dawn on the second day of the experiment on a day when the birds had been returned to 8L:16D. The increased GnRH-I gene transcription does not remain high for as long as the increase in LH secretion. GnRH-I mRNA rapidly returns to levels that are not significantly different from controls by day 3 (49 hours from dawn of the first long day). Therefore, the carry over effect of one long day on plasma LH could be caused by a number of possible events downstream of the neural 'decision-making' system. However, the carry over effect could, at least in part, be a property of the central nervous system, either of the GnRH neurones themselves, or of some component higher in the photoperiodic transduction system. This is suggested by the *in vitro* work of Perera and Follett (1992) and experiments reported by Nicholls *et al.,* (1983). Nicholls *et al.,* found that if castrated quail were treated with testosterone at the time of exposure to the single long day then the LH increase was blocked but then increased after the inhibitory effects of testosterone treatment has dissipated and thereafter follows the same time course as the control birds not treated with testosterone. A store of readily releasable GnRH-I could exist in the GnRH neurones which continues to be released after photoinduced synthesis of GnRH-I has ceased. Alternatively, photoinduced GnRH-I mRNA may continue to be translated in to pro-GnRH-I and this continues to maintain a releasable pool of GnRH. The progressive

depletion of this pool would become exhausted resulting in a progressive decrease in plasma LH (figure 6.4.1.).

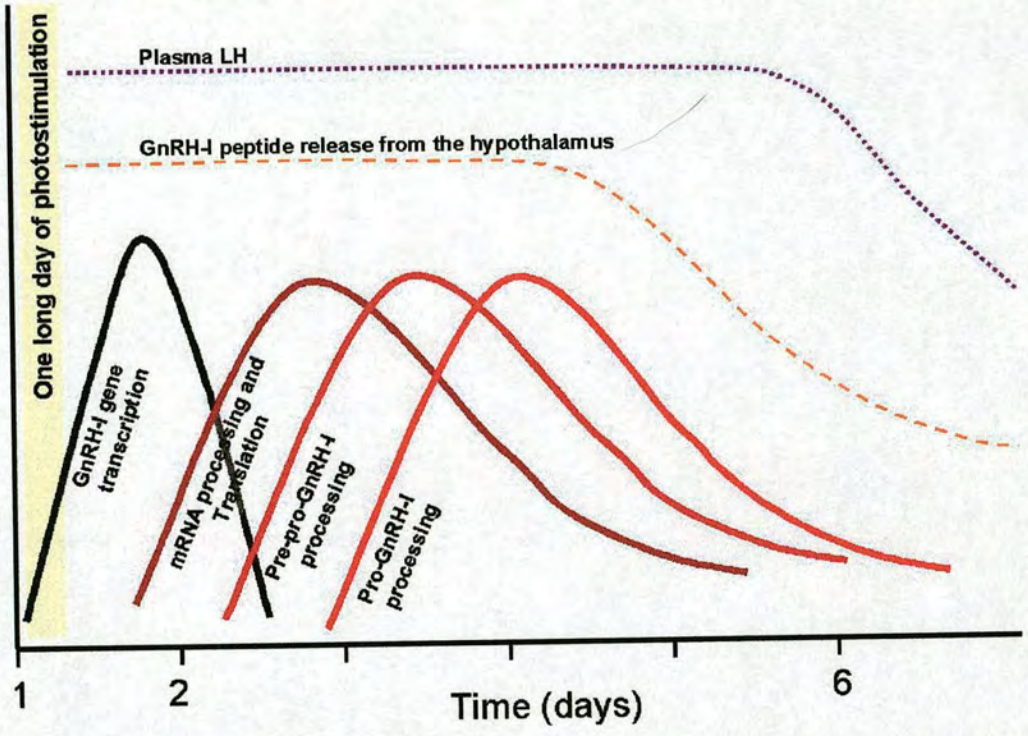


Figure 6.4.1. A hypothesis to explain the 'carry over' effect of one long day of photostimulation on plasma LH. Plasma LH remains elevated for several days after hypothalamic GnRH-I mRNA transcription has fallen to basal levels. This is due to the continued transcription of genomic DNA, RNA processing, translation and subsequent processing of GnRH-I pre-propeptide which gradually decline as each component in the pathway is exhausted.

Unfortunately the LH controls in this experiment are significantly different. The reason for this is unknown but it is considered here to be an artefact possibly due to accidental exposure to changes in light or temperature during the experiment.

7. THE EFFECT OF PHOTOSTIMULATION ON HYPOTHALAMIC GnRH-I mRNA: THE DEVELOPMENT OF RELATIVE PHOTOREFRACTORINESS

7.1. INTRODUCTION

The physiological mechanisms controlling relative photorefractoriness have not yet been discovered. In relatively photorefractory Japanese quail there is no change in plasma LH levels in birds held on fixed long days (Gibson, Follett and Gledhill, 1975; Urbanski and Follett, 1982) and there is no change in hypothalamic GnRH content (Foster *et al.,* 1988). However, due to the release and synthesis dynamics of GnRH, a measure of the total hypothalamic GnRH content may not provide an accurate reflection GnRH neuronal activity.

There is evidence to suggest that a decrease in GnRH neuronal activity or 'hypothalamic drive', is involved in the development of relative photorefractoriness. In 1988, Follett implanted castrated quail with testosterone to suppress plasma LH while exposing them to long days for 9 weeks to induce photorefractoriness or maintaining them on short days to ensure the birds were fully photosensitive. On removing the testosterone implants and exposing both groups to long days LH levels increased significantly less in photorefractory birds than the fully photosensitive controls. As well as demonstrating a reduction in hypothalamic drive this experiment established that there is no 'fatigue' of components in the hypothalamo-pituitary axis in the relatively photorefractory system. This has been suggested in one of several

hypotheses to explain the development of photorefractoriness (for review see Dawson, 2001).

In 1993, Sharp elaborated the 'steroid-independent hypothalamic drive' hypothesis as an explanation of how the development of photorefractoriness and its dissipation could be mediated through photoinduced stimulatory and inhibitory inputs to the GnRH-I neurones (figure 7.1.1).

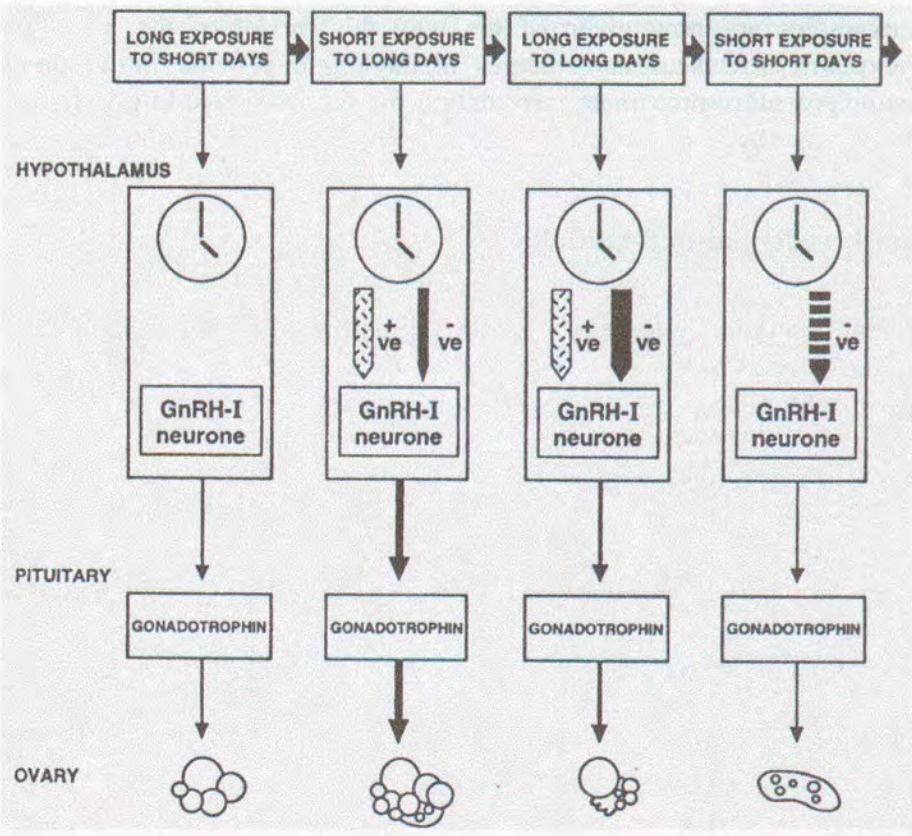


Figure 7.1.1. A model of the avian photoperiodic response. The circle represents a biological clock from which positive (hatched arrow) and inhibitory (black arrow) inputs to the GnRH-I neurones are generated. Photorefractoriness occurs when the inhibitory input is greater than the stimulatory input. The dissipation of the inhibitory input (broken black arrow) represents the dissipation of photorefractoriness (Taken from Sharp, 1996)

Three non-mutually exclusive hypotheses are suggested for the site of control of GnRH-I release in photorefractory quail. GnRH-I release may be controlled at the GnRH-I terminals by glial sheath retraction, or by a decrease in GnRH-I gene expression or by an increase in intra cellular turnover or degradation of GnRH-I mRNA, prepropeptide or peptide.

To determine whether GnRH-I gene expression is reduced in relatively photorefractory quail, GnRH-I mRNA was measured in groups of photorefractory and photosensitive quail. A reduction in GnRH-I mRNA would imply that a change in GnRH-I gene expression is involved in the development of photorefractoriness.

It has been proposed that the development of relative photorefractoriness may be associated with increased plasma prolactin (Sharp and Sreekumar, 2001). Plasma PRL was measured in quail taken for GnRH-I mRNA measurements to confirm that concentrations of the hormone were increased in relatively photorefractory birds held on long day lengths.

If Japanese quail, other than the 'Follett' strain (see chapter 2, section 2.1.), are held indefinitely on short days they will come into breeding condition (Sharp and Sterling, 1985). A group of short day breeding quail are included in this study to establish whether GnRH-I mRNA is increased in breeding birds in the absence of photostimulation.

7.2. EXPERIMENTAL DESIGN

Male and female quail, approximately 18 months old, were held on long days for a year prior to the start of the experiment. These birds were predicted to be fully photorefractory as exposure of quail to long days for 8 weeks is known to induce relative photorefractoriness (Robinson and Follett, 1982; Follett and Pearce-Kelly, 1990). To confirm the photorefractory state a group of control birds (n=7, 5 males and 2 females) was moved from 18 to 13 hours of light for three weeks (figure 7.2.1.). This photoperiod is stimulatory for short day photosensitive birds but is non-stimulatory for photorefractory birds. If these birds were photorefractory plasma LH was predicted to be lower than in photorefractory control birds maintained on 18L:6D. It is well documented that 13 hours of light will stimulate LH release in fully photosensitive birds (Robinson and Follett, 1982; Follett and Pearce-Kelly, 1990).

The remaining birds were split into three groups; long day (LD) photorefractory (n=10, 6 males and 4 females), LD photosensitive (n=16, 8 males and 8 females) and short day (SD) photosensitive (n=14, 5 males and 9 females)(figure 7.2.1.). The long day photorefractory birds were held on 18L:6D for the duration of the experiment. Photorefractoriness was dissipated in the latter two groups by placing them on short days for 6 weeks. The LD photosensitive group was then placed on 18L:6D for 3 weeks and the SD photosensitive group were kept on 8L:16D for 3 weeks.

There were 13 quail which came into breeding on short days (7 males and 6 females).

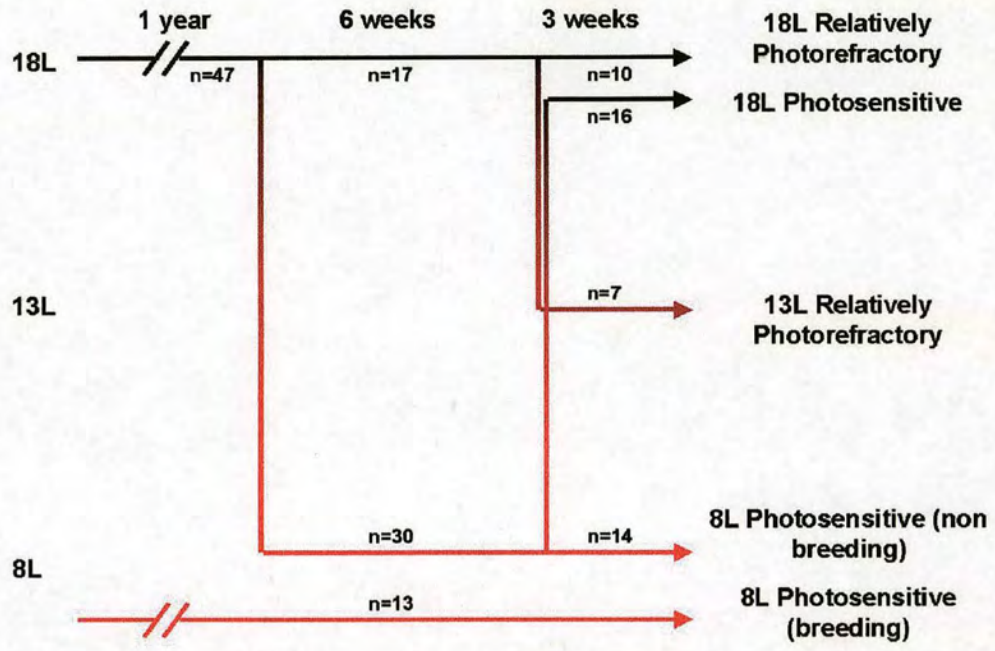


Figure 7.2.1. The experimental outline of the photorefractoriness experiment. The orange line represents the photosensitive groups while the black line represents the photorefractory groups.

7.3. RESULTS

7.3.1. Plasma LH in photosensitive and relatively photorefractory quail

Measurements of plasma LH were lower in the birds that were photorefractory at the beginning of the experiment. However, there was no significant difference between any of the LH levels (Figure 7.3.1.). There was no overall significant difference in plasma LH levels between male and female groups.

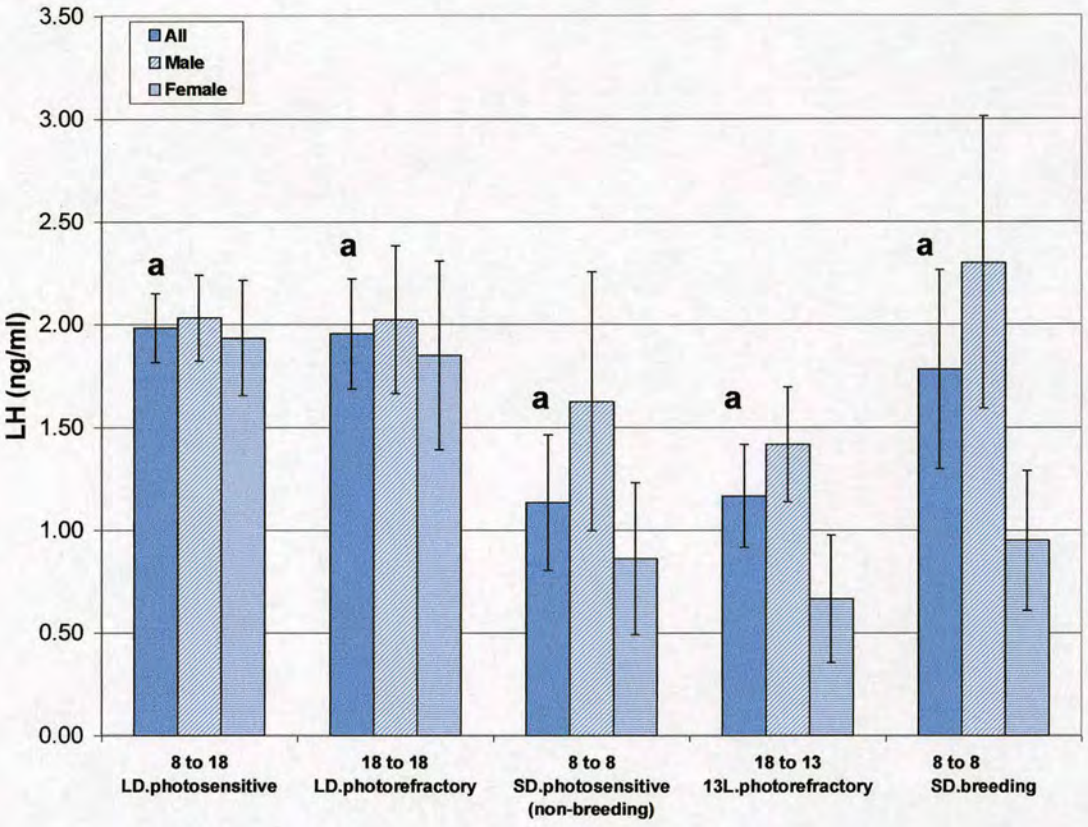


Figure 7.3.1. LH plasma concentration in photosensitive quail exposed to long (18L:6D) or short (8L:16D) days, photorefractory quail exposed to long days (18L:6D) or to a 13 h photoperiod and a group of birds which had come into breeding condition while held on short days (8L:16D). Values with different superscripts are significantly different. Average ± SE MEAN.

7.3.2. GnRH-I mRNA levels in photosensitive and relatively photorefractory quail

Hypothalamic GnRH-I mRNA was significantly different across all groups when analysed together (d.f. 59, F 3.44, P≤0.05). The post hoc analysis using Tukey's tests found that the only groups that differed significantly were LD photosensitive and SD birds (d.f. 29, T -3.19, P≤0.05). However, when using the Least Squares Means and two sample T-Tests there were more groups that significantly differed. Hypothalamic GnRH-I mRNA in relatively photorefractory birds transferred from 18L to 13L was

close to being significantly depressed at P=0.065 (d.f. 16, T 2.02, P=0.065)(figure 7.3.2.). Most importantly, however, was the observation that an amount of GnRH-I mRNA in LD photorefractory birds was significantly less than in LD photosensitive birds (d.f. 25, T 2.19, P=0.05). The LD photosensitive birds had significantly greater GnRH-I mRNA levels than SD photosensitive (d.f. 29, T 3.78, P≤0.01) and SD photorefractory birds on 13L (d.f. 22, T 3.57, P≤0.01). There was no significant difference in GnRH-I mRNA hypothalamic content between male and female hypothalami.

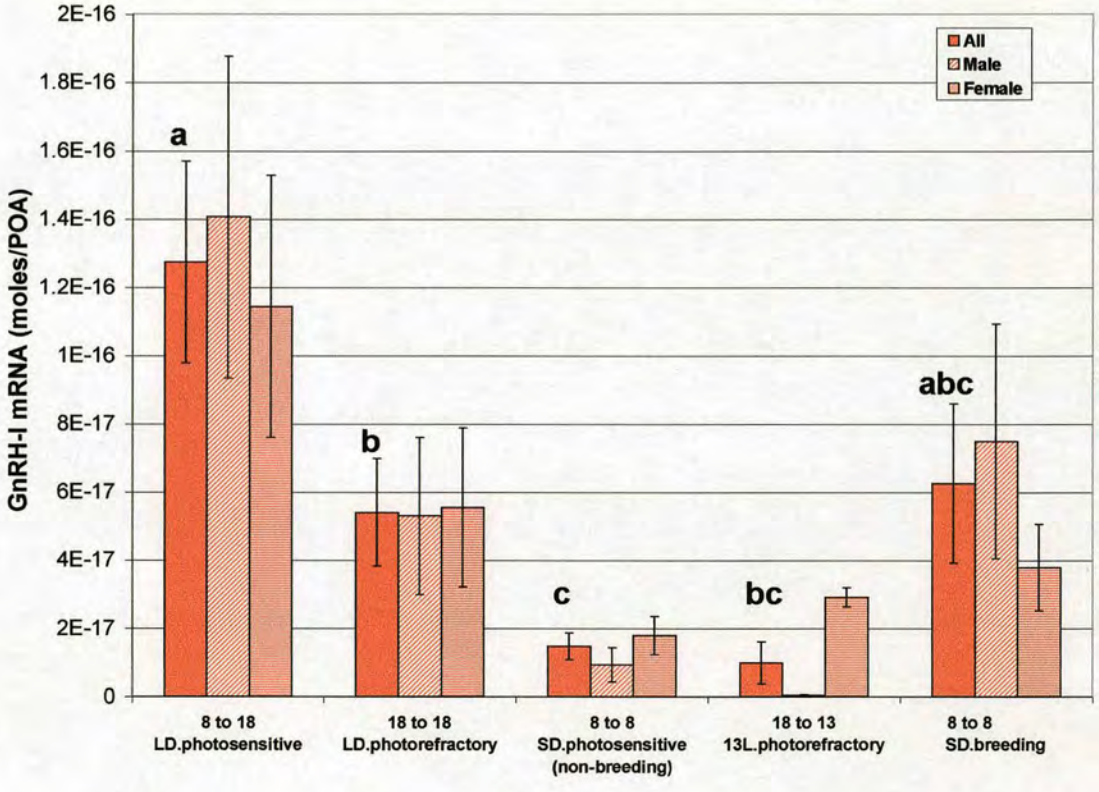


Figure 7.3.2. Hypothalamic GnRH-I mRNA in male and female, photosensitive quail exposed to long (18L:6D) or short (8L:16D) days, photorefractory quail exposed to long days (18L:6D) or to a 13 h photoperiod and a group of birds which had come into breeding condition while held on short days (8L:16D). Values with different superscripts are significantly different. Average ± SE MEAN.

7.3.3. Prolactin levels in photosensitive and relatively photorefractory quail

Plasma prolactin in the photorefractory quail held on long days was not significantly different from the values in long or short day photosensitive birds (figure 7.3.3.). There was no significant difference between the prolactin plasma concentrations in the photosensitive 8L:16D and photorefractory 13L:11D groups and no significant difference in plasma prolactin concentrations in males and females.

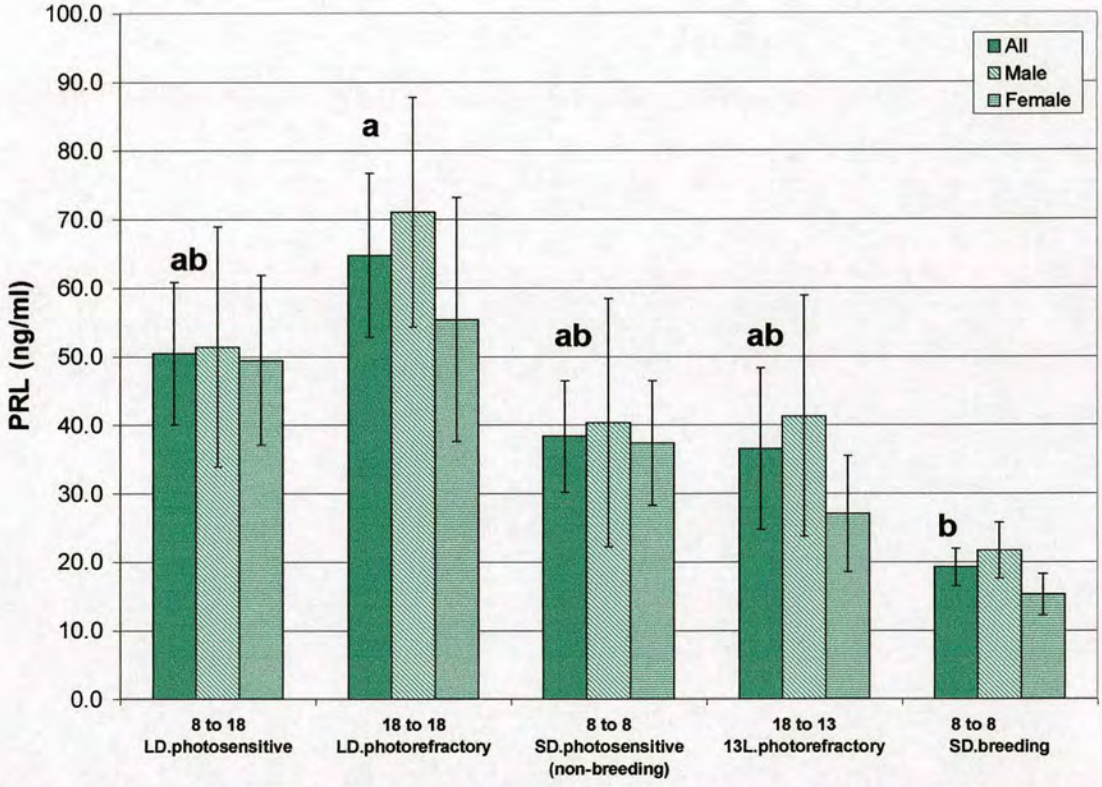


Figure 7.3.3. Plasma PRL levels in photosensitive quail exposed to long (18L:6D) or short (8L:16D) days, photorefractory quail exposed to long days (18L:6D) or to a 13 h photoperiod and a group of birds which had come into breeding condition while held on short days (8L:16D). Values with different superscripts are significantly different. Average ± SE MEAN.

7.3.4. Quail Breeding on short days

Plasma LH in quail breeding on short days was not significantly different from values in relatively photorefractory birds held on 18L:6D (figure 7.3.1.). The mean plasma LH in SD breeding birds was higher than the SD non-breeding and 13L photorefractory groups and lower than the LD photosensitive quail but again there was no significant difference between these groups.

Hypothalamic GnRH-I mRNA was higher in SD breeding birds than in SD non-breeding birds and 13L photorefractory groups, lower than LD photosensitive and approximately the same as the LD photorefractory group, but there was no significant difference between them (figure 7.3.2.).

Prolactin levels were significantly lower in the short day breeding birds than in LD photorefractory (d.f. 21, T -3.3, $P \leq 0.05$) groups but were not significantly different from LD photosensitive, SD non-breeding or 13L photorefractory birds (figure 7.3.3.).

7.4. DISCUSSION

Hypothalamic GnRH-I mRNA was lower in relatively photorefractory birds maintained on 18L:6D than in photosensitive birds held on the same photoperiod but the plasma LH concentrations in the two groups were not significantly different. This is the first evidence that a decrease in hypothalamic GnRH mRNA is correlated with the relative photorefractory condition. GnRH-I mRNA measurement also provides a

means of determining the relatively photorefractory state which earlier studies could only do by lowering day length to 13L and measuring some aspect of reproductive function, e.g. plasma LH or cloacal gland size. It is also the first example of GnRH-I mRNA levels not mirroring basal levels of LH levels and photoperiod.

This work provides tentative evidence that a change in GnRH gene expression is associated with relatively photorefractoriness. It could prove useful to repeat this experiment with a greater number of birds and also in castrates to control for the steroid background. Castrating quail is known to increase GnRH peptide in the brain and reduces variation and increases levels of plasma LH (see introduction pages 15 and 20). It may also be useful to repeat this experiment at the time that relative photorefractoriness develops, after 9 weeks of photostimulation. The birds used in this research had been on long days for more than a year and the decreases in GnRH-I message may be related to age per se. However, this seems unlikely since a control of this possibility was included in the experimental design i.e. quail of the same age, subjected to short days to dissipate photorefractoriness and returned to long days.

Plasma PRL concentrations were high in relatively photorefractory quail, consistent with the view that there might be a causal relationship with the development or maintenance of relative photorefractoriness (Sharp and Sreekumar, 2001). This possibility was investigated in the experiments described in chapter 8.

Plasma LH and GnRH-I mRNA in short day breeding quail were not different from any other group in the study. The very large variation in both LH and GnRH-I mRNA could account for this observation.

8. THE EFFECT OF PROLACTIN ON HYPOTHALAMIC GNRH-I mRNA: A ROLE IN THE DEVELOPMENT OF RELATIVE PHOTOREFRACTORINESS?

8.1 INTRODUCTION

The anti-gonadal properties of prolactin are well-documented and is known to act in part by suppressing plasma LH in mammals (rats, Fang *et al.*, 1974; humans, Moult *et al.*, 1982; Winters and Troen, 1984; rabbits, Larsen and Odell, 1987) and also in birds (quail, Camper and Burke 1977a, Zadworny *et al.*, 1989; chickens, Lea *et al.*, 1981; Sharp and Pedersen, 1988; turkeys, Camper and Burke 1977b; Rozenboim *et al.*, 1993; starlings, Dawson *et al.*, 1985; ring doves, Buntin et al., 1999). This observation coupled with the finding that plasma prolactin is at its highest seasonal values during the development of relative photorefractoriness has led to the hypothesis that prolactin may be involved with the development of relative photorefractoriness (Sharp and Sreekumar, 2001). Work by Dawson and Sharp (1998) suggests that this is the case for absolutely photorefractory birds such as starlings. They found that, although VIP-immunised starlings in which plasma prolactin is suppressed, become photorefractory, the rate of gonadal regression was reduced, indicating that prolactin is not the causative agent of photorefractoriness but acts as a modulator of the timing of photorefractoriness. This observation is consistent with other studies on birds where an increase in prolactin is associated with ovarian regression but does not induce photorefractoriness (El Halawani *et al.*,

1980; Dawson and Goldsmith, 1985; Hiatt *et al.*, 1987). Similarly studies on mammals using bromocriptine to suppress photo-induced prolactin do not prevent gonadal regression/recrudescence (Smith *et al.*, 1987, Tortonese, 1999).

The inhibitory actions of prolactin may occur at all levels in the hypothalamo-pituitary-gonadal axis. The steroidogenic action of LH is reduced by prolactin in turkeys (Camper and Burke 1977b, Zadworny *et al.*, 1989) and although responsiveness of the pituitary to GnRH is not decreased in ring doves treated with prolactin (Buntin *et al.*, 1999) LH release and LH β-subunit gene expression is suppressed by prolactin in turkey pituitary cells *in vitro* (You *et al.*, 1995).

At the hypothalamic level the development of absolutely photorefractoriness in several avian species is associated with decreased GnRH neuronal activity (Dawson *et al.* 1985; Bluhm *et al.*, 1991; Saldanha *et al.*, 1994; Hahn and Ball, 1995) at a time when prolactin secretion is high (Ebling *et al.,* 1982; Goldsmith and Nicholls, 1984; Hiatt *et al.,* 1987). A causal relationship between high plasma prolactin and reduced GnRH gene expression is suggested by the finding that high plasma prolactin in incubating chicken is associated with reduced GnRH-I gene expression (Dunn *et al.*, 1996). Treatment of laying turkeys with intravenous prolactin decreases the hypothalamic GnRH-I and -II content (Rosenboim *et al.,* 1993) but if the prolactin is delivered directly to the brain of laying turkeys by intracerebroventricular infusion LH is not suppressed (Youngren *et al.*, 1991). This would imply that prolactin exerts its suppressive effect on GnRH in the brain indirectly but there is still further complexity as not all birds respond to prolactin in the same way. For example, in ring

doves intracerebroventricular prolactin -injection suppresses LH (Buntin and Tesch, 1989; Buntin and Figge, 1989; Buntin *et al.*, 1999) but has no significant effect on hypothalamic GnRH content (Buntin *et al.*, 1999). However, as previously mentioned, it is possible that there were changes in preproGnRH-I turn over not measurable by the steady-state techniques used, associated with postulated inhibitory action of prolactin on GnRH-I neuronal function.

The effects of prolactin on reproductive function at the termination of photoinduced breeding have only been studied in a few absolutely photorefractory birds, including the starling and turkey. The development of photorefractoriness in relatively photorefractory quail and chickens differs from the development of absolute photorefractoriness in starlings and turkeys in that there is no associated decrease in plasma LH (Juss, 1993). The aim of the work in this chapter was to establish the effects of intravenous prolactin administration on plasma LH, GnRH-I mRNA and plasma prolactin levels in relatively photorefractory quail. This was performed to establish whether a causal relationship exists between high levels of prolactin and the relatively photorefractory condition and to determine whether prolactin -injection might exert its effects through suppression of GnRH-I gene expression.

Prolactin measurements were also made as there is some evidence that the hormone exerts an inhibitory effect on its own secretion (Hall and Chadwick, 1984). Further support for this view comes from the finding that intravenous and intracerebroventricular prolactin -injection exerts a suppressive effect on prolactin

secretion in turkeys with low prolactin levels (Youngren *et al.,* 1991; Rozenboim *et al.,* 1993).

8.2. EXPERIMENTAL DESIGN

Photosensitive female quail were used for the first two experiments maintained on short days (8L:16D) until day three, when they were transferred to long days for six days. The first experiment used a stimulatory photoperiod of 13L:11D. This low level of stimulation was used because it was thought that longer day lengths might induce such a strong stimulatory effect on GnRH-I secretion that any inhibitory effect of administered prolactin would be masked. However, there was no significant increase in hypothalamic GnRH-I mRNA after transfer to 13L:11D (see results). Consequently, a second experiment was carried out using a stimulatory day length of 20L:4D. A third experiment was conducted to determine whether exogenously administered prolactin affected GnRH-I mRNA in laying females maintained throughout on long days of 20L:4D. Laying females were injected with either prolactin + vehicle (n=10) or vehicle alone (n=10) according to the schedule described below.

Ovine prolactin (Sigma-Aldrich, Poole, Dorset) was dissolved in 0.01 M sodium bicarbonate and diluted x 10 with 0.9% saline to a final concentration of 125 IU/mg (equivalent to 4 mg/kg). There is a possibility of cross reaction between ovine and exogenous PRL this has been calculated at x% in previous studies (ref). Control birds received bicarbonate/saline vehicle solution only. To accustom birds to handling and injection, all birds received twice daily (at 0800 and 1500) subcutaneous injections of

vehicle. Each experiment was performed over six days. On the first day, all groups received injections of vehicle, and blood samples were taken. On the second day, experimental groups received twice-daily injections of prolactin. Prolactin and vehicle injections were then administered for a further three days, with a second blood sample being taken on the fifth day. On the sixth day, birds received one further injection in the morning, before being killed at 10:00, when trunk blood and hypothalamic POA tissue samples were taken for plasma LH and prolactin and GnRH-I mRNA measurements respectively.

8.3. RESULTS

8.3.1. The effect of exogenous prolactin in female quail photostimulated with a 13 h photoperiod

8.3.1.1. Plasma LH

Plasma LH levels on day one of the experiment ranged between 0.68 and 1.40 ng/ml (figure 8.3.1.1.). However, there was no significant difference between them due to either day length (d.f. 31, F 0.20, P=0.657) or treatment (d.f. 31, F 0.03, P=0.864). There was no interaction between day length and treatment on either day 1, 5 or 6 of the treatment period. There was a significant increase in LH in response to the 13 h photoperiod on day five (d.f. 31, F 10.87, $P \leq 0.01$) and day six (d.f. 31, F 18.98, $P \leq 0.001$). However, there was no effect of prolactin -injection on plasma LH in either the short day control or the 13L photostimulated group on day five (d.f. 31, F 0.96, P=0.336) or day six (d.f. 31, F 0.94, P=0.340).

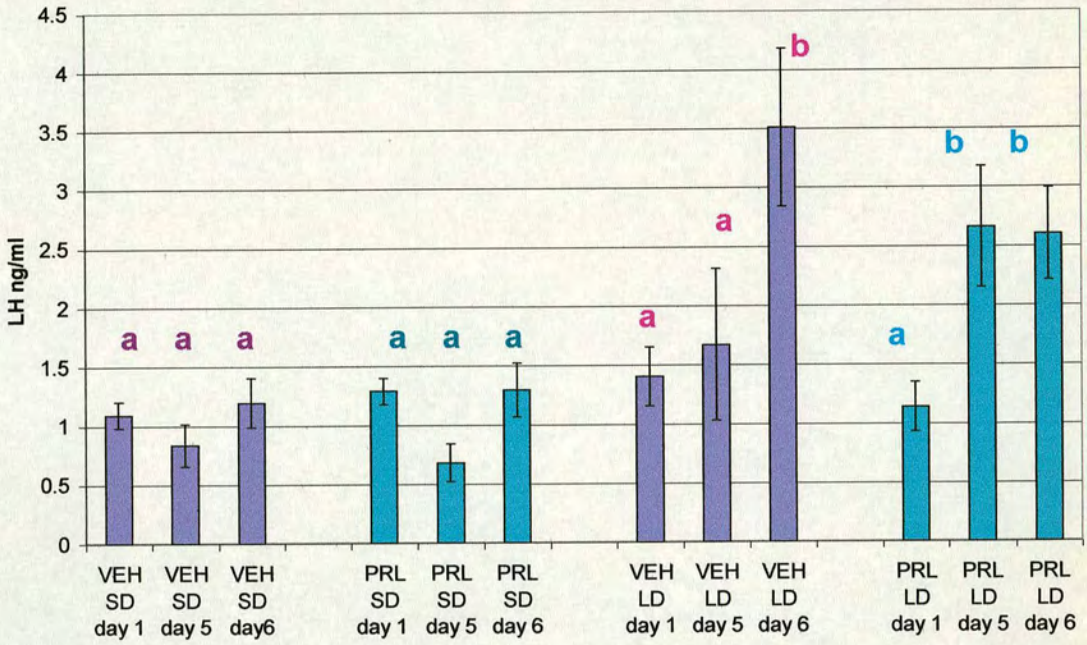
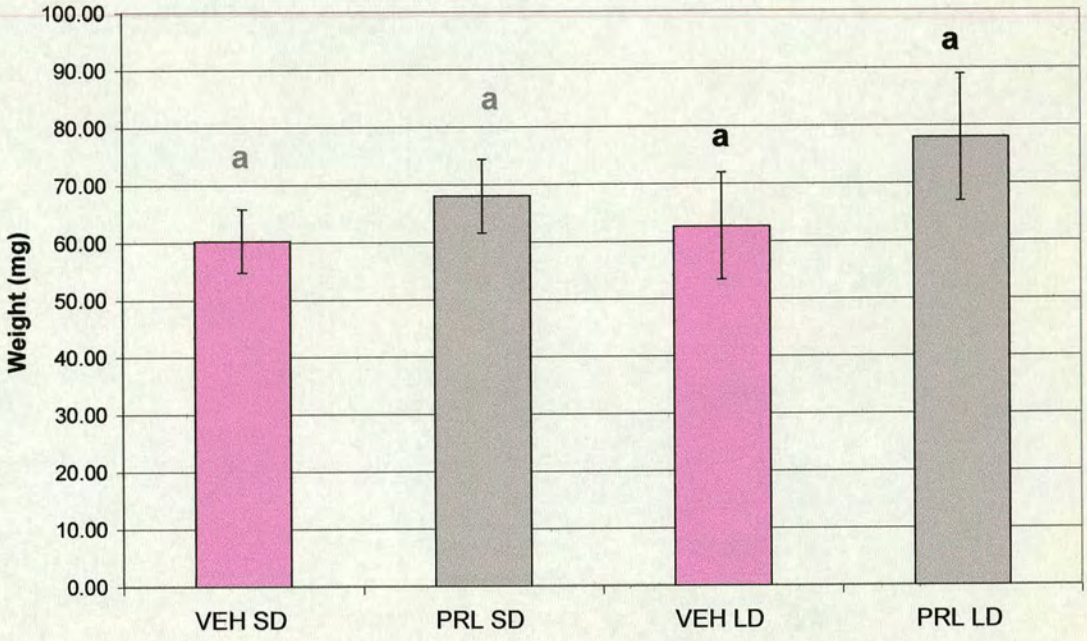


Figure 8.3.1.1. Plasma LH in female quail injected twice daily with either PRL or vehicle after transfer from short days to 13L:11D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.1.2. Ovary weights

Two-way ANOVA revealed no significant difference in ovary weights between the long day and short day groups and there was no detectable effect of prolactin - injection on ovarian weights in long or short day groups (figure 8.3.1.2.).



8.3.1.2. Ovarian weights in female quail injected twice daily with either PRL or vehicle after transfer from short days to 13L:11D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.1.3. GnRH-I mRNA

There was no interaction between day length and treatment group and no difference in hypothalamic GnRH-I mRNA levels across the four groups of birds (figure 8.3.1.3.). The 13L photoperiod was not a strong enough stimulus to produce a significant difference in hypothalamic GnRH-I mRNA levels even after 6 days.

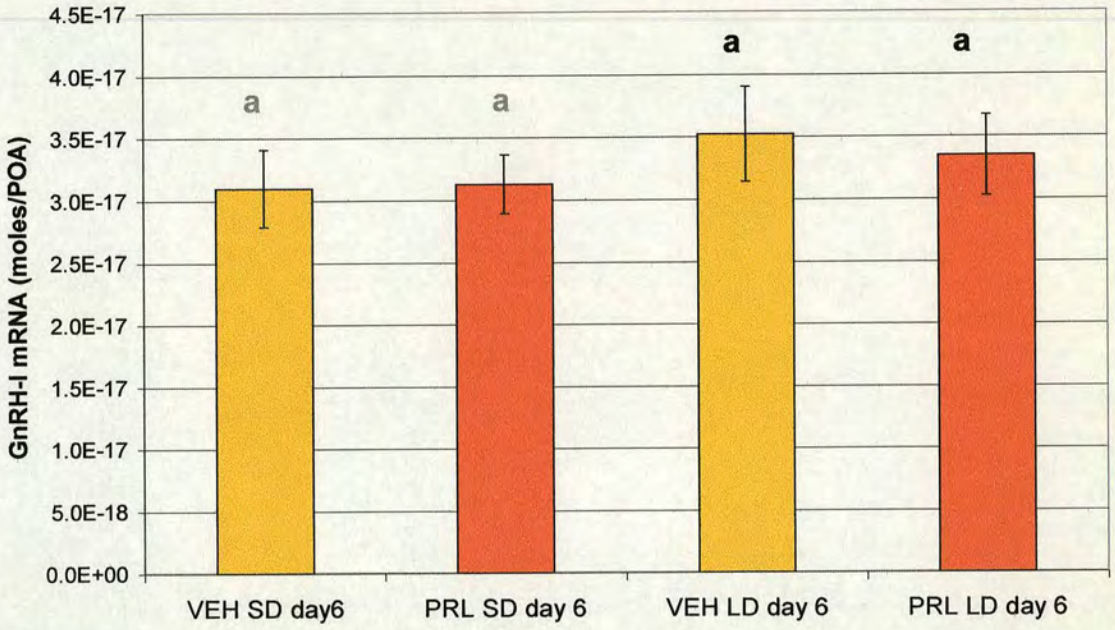


Figure 8.3.1.3. Hypothalamic GnRH-I mRNA levels in female quail injected twice daily with either PRL or vehicle after transfer from short days to 13L:11D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.1.4. Plasma prolactin

Plasma prolactin concentrations on the first day of the experiment in all groups ranged between 4.5 and 10.5 ng/ml (figure 8.3.1.4.). By day five an interaction between day length and treatment was apparent and so it was permissable to conduct post hoc tests. These were done in Minitab® using the least squares means and pair-wise comparisons (Tukey) options in the two-way analysis of variance (ANOVA) statistic. This revealed on day five a significant increase in endogenous prolactin in prolactin -injected photostimulated birds as compared to short day vehicle-injected, short day prolactin -injected and long day vehicle-injected birds (d.f. 15, T-Value 3.21, $P \leq 0.05$, d.f. 15, T-Value 4.26, $P \leq 0.01$ and d.f. 15, T-Value 4.44, $P \leq 0.001$ respectively). On the sixth day there was also an interaction between day length and

treatment, so post hoc analyses were used again. This revealed that in the prolactin - injected photostimulated group endogenous prolactin was significantly depressed (d.f. 15, T-Value -3.43, P≤0.05). There was no significant increase in prolactin levels in the photostimulated vehicle-injected group compared with the non-photostimulated vehicle-injected group (d.f. 15, F 2.49, P=0.084).

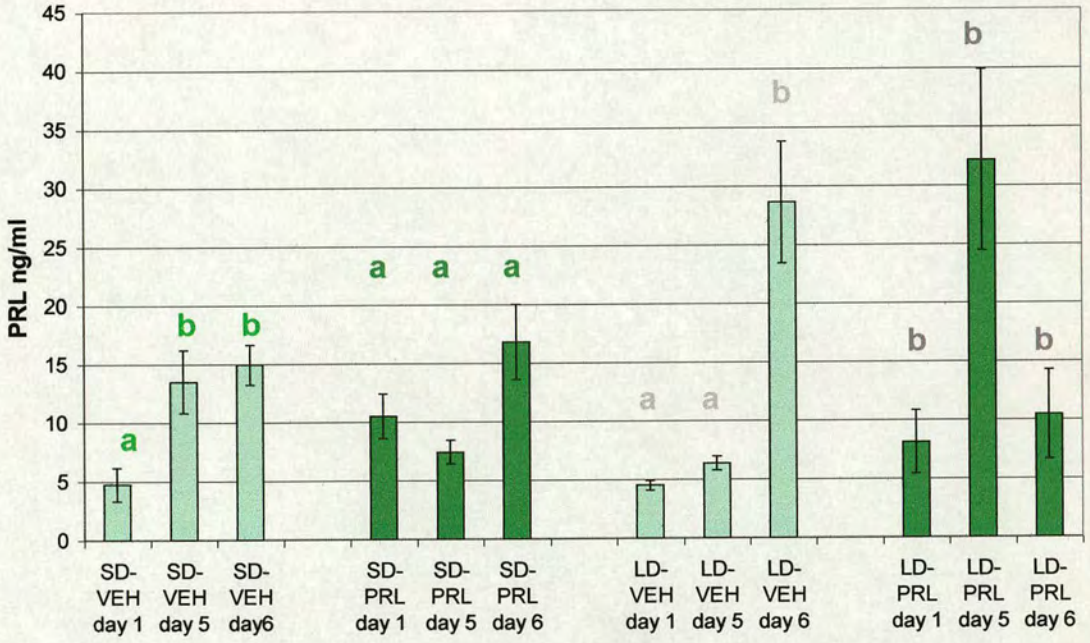
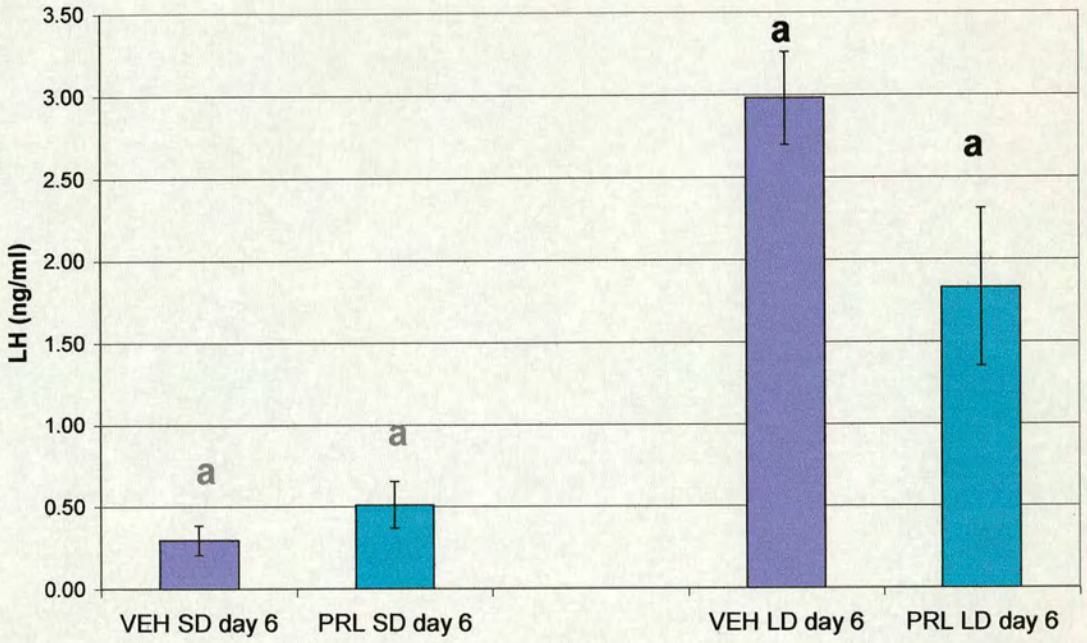


Figure 8.3.1.4. Plasma PRL levels in female quail injected twice daily with either PRL or vehicle after transfer from short days to 13L:11D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.2. The effect of exogenous prolactin in female quail photostimulated with a 20 h photoperiod

8.3.2.1. Plasma LH

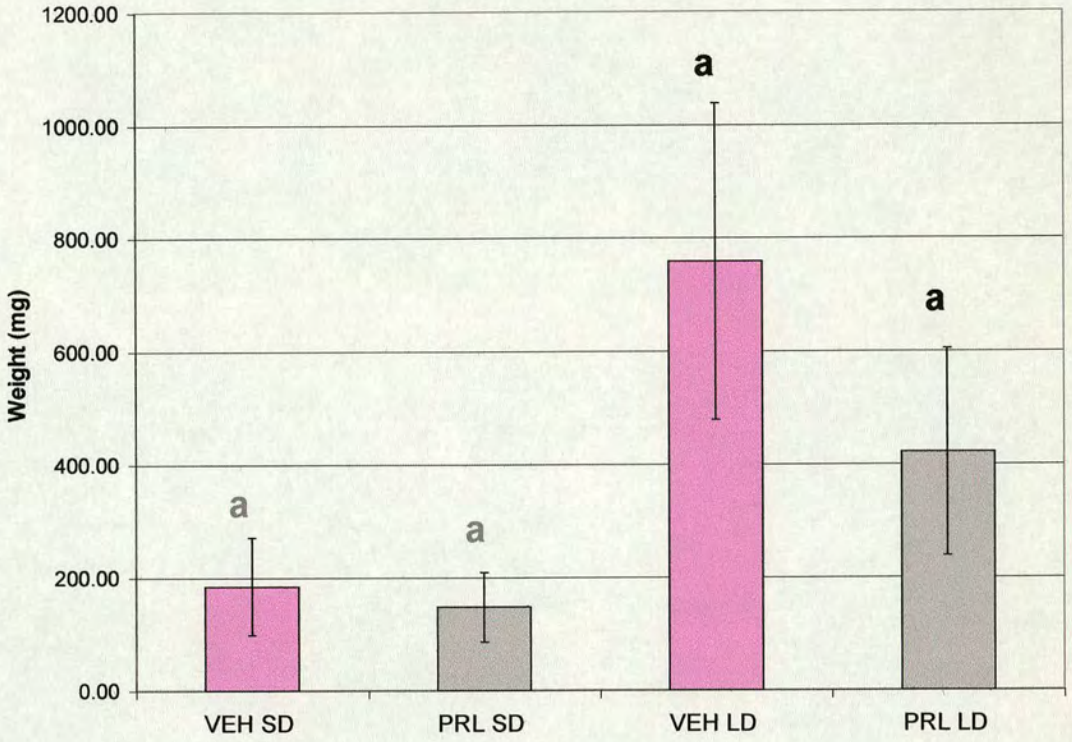
Analysis of plasma LH concnetration using a two-way ANOVA revealed an interaction between day length and prolactin treatment (figure 8.3.2.1.). The pair wise comparisons produced by Tukey simultaneous tests revealed that there was an effect of day length, with significant differences between short day vehicle-injected and long day vehicle-injected (d.f. 15, T-Value 5.88, $P \leq 0.0001$), short day vehicle-injected and long day prolactin -injected (d.f. 15, F 3.05, $P \leq 0.05$) and short day prolactin -injected and long day vehicle-injected (d.f. 15, F -5.36, $P \leq 0.0001$). However, the difference between short day prolactin -injected and long day prolactin -injected groups was not significant (d.f. 15, F 2.56, $P=0.074$). Prolactin injection did not significantly depress plasma LH, although this approached significance (d.f. 15, F -2.63, $P=0.0633$). For this reason a further experiment was conducted to investigate the effect of prolactin treatment on reproductive function in laying quail.



8.3.2.1. Plasma LH levels in female quail injected twice daily with either PRL or vehicle after transfer from short days to 20L:4D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.2.3. Ovarian Weights

There was no significant interaction between day length and treatment type on ovarian weights. There was no significant effect of prolactin injection in ovarian weights on the long day or short day groups. There was a significant increase in ovary weight in long day birds compared to short day birds (d.f. 30, F=5.83, $P \leq 0.05$)(figure 8.4.2.).



8.3.2.2. Ovarian weights in female quail injected twice daily with either PRL or vehicle after transfer from short days to 20L:4D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.2.3. GnRH-I mRNA

An analysis of GnRH-I mRNA by ANOVA revealed an interaction between treatment and day length. The subsequent pair-wise comparisons revealed a significant increase in GnRH-I mRNA after 6 days of photostimulation with 20L:4D in comparison to short day controls (d.f. 15, T-Value 2.76, $P \leq 0.05$). There was also a significant depression of GnRH-I mRNA in prolactin -injected long day birds as compared with vehicle-injected long day birds (d.f. 15, T-Value -2.72 , $P \leq 0.05$)(figure 8.3.2.3.).

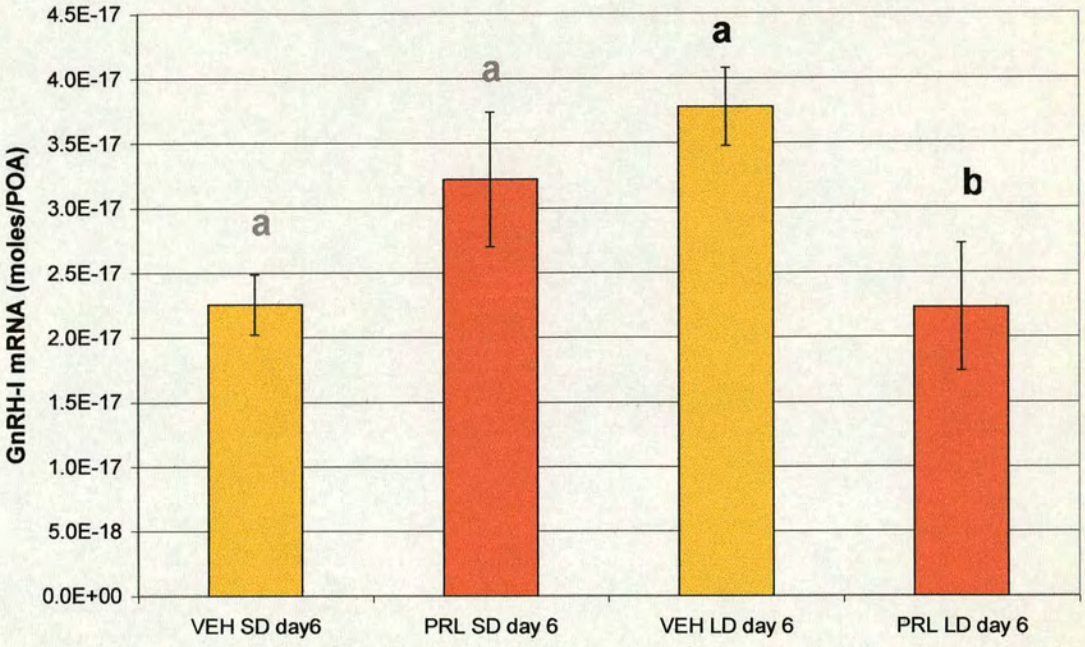


Figure 8.3.2.3. Hypothalamic GnRH-I mRNA levels in female quail injected twice daily with either PRL or vehicle after transfer from short days to 20L:4D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.2.4. Plasma prolactin

Plasma prolactin was significantly depressed by injection with ovine prolactin (figure 8.3.2.4.) (d.f. 29, F 5.37, P≤0.05). However, a two way analysis using ANOVA revealed no significant effect of day length and no interaction between day length and treatment.

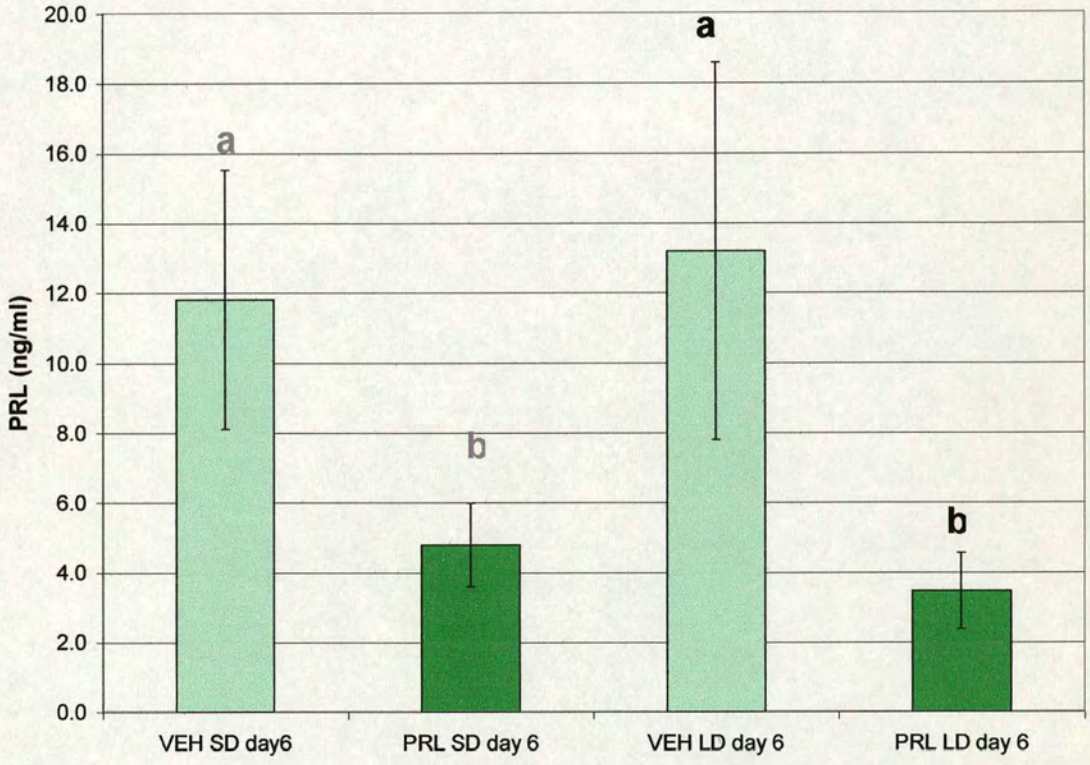


Figure 8.3.2.4. Plasma PRL levels in female quail injected twice daily with either PRL or vehicle after transfer from short days to 20L:4D for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected, SD = short day, 8L:16D and LD = long day, 13L:11D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.3. The effect of exogenous prolactin on reproductive function in breeding female quail maintained on a 20 h photoperiod

8.3.3.1. Plasma LH

A two-way ANOVA revealed no significant effect of prolactin -injection on LH levels of laying quail (d.f. 38, F=3.61 P=0.065)(figure 8.3.3.1.).

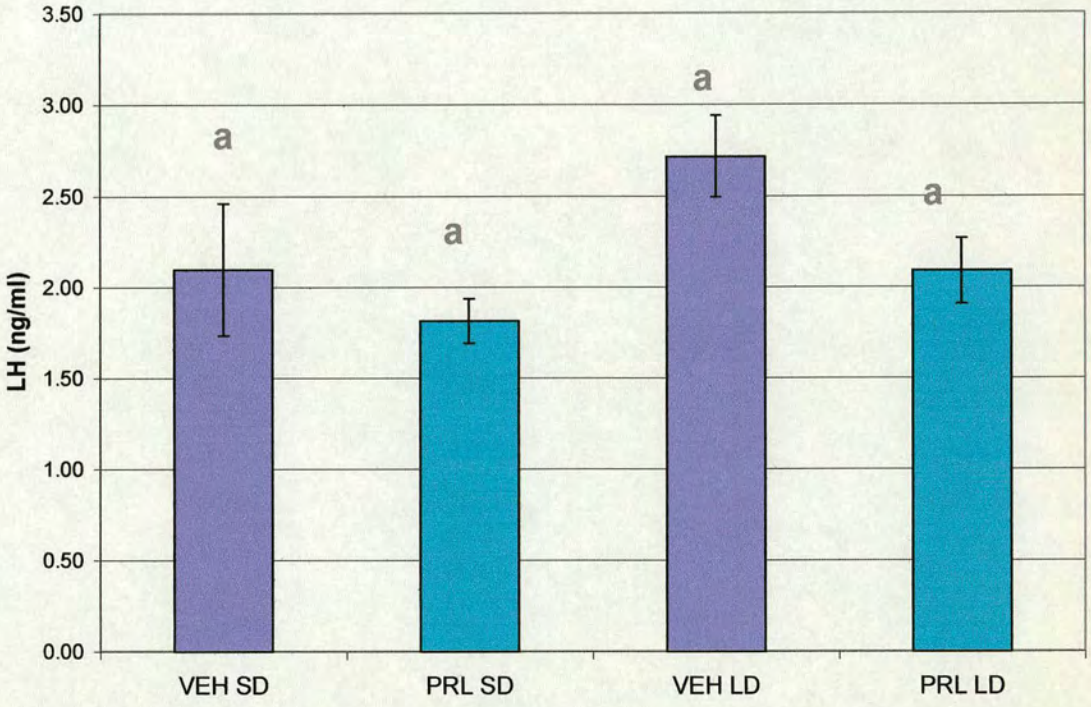


Figure 8.3.3.1. Plasma LH levels in female quail injected twice daily with either PRL or vehicle for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected and LD = long day, 20L:4D. Within each treatment group values with different superscripts are significantly different. Average ± SE MEAN.

8.3.3.2. Ovarian Weights

There was no significant difference between ovarian weights between prolactin -injected and vehicle-injected long day groups of laying quail (figure 8.3.3.2.).

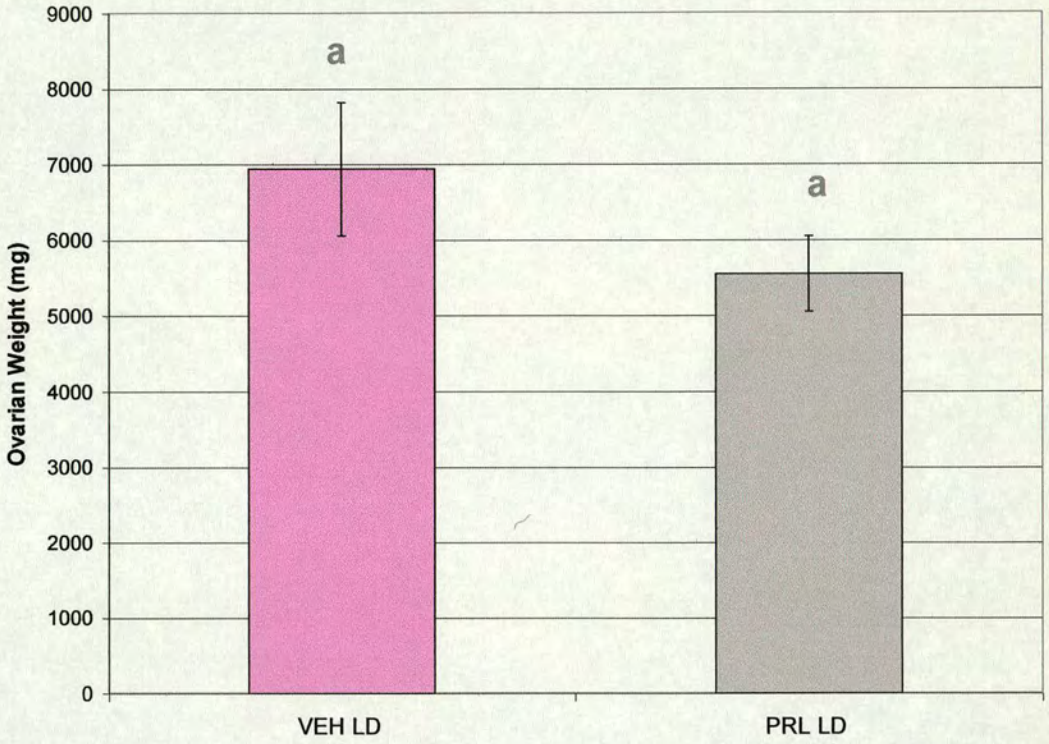


Figure 8.3.3.2. Ovarian weights in female quail injected twice daily with either PRL or vehicle for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected and LD = long day, 20L:4D. Values with different superscripts are significantly different. Average ± SE MEAN.

8.3.3.3 GnRH-I mRNA

Hypothalamic GnRH-I mRNA is significantly suppressed in laying quail injected with prolactin (3.34E-17 moles/POA vs 4.37E-17 moles/POA, d.f. 19, F 5.83, $P \leq 0.05$)(figure 8.3.3.3.).

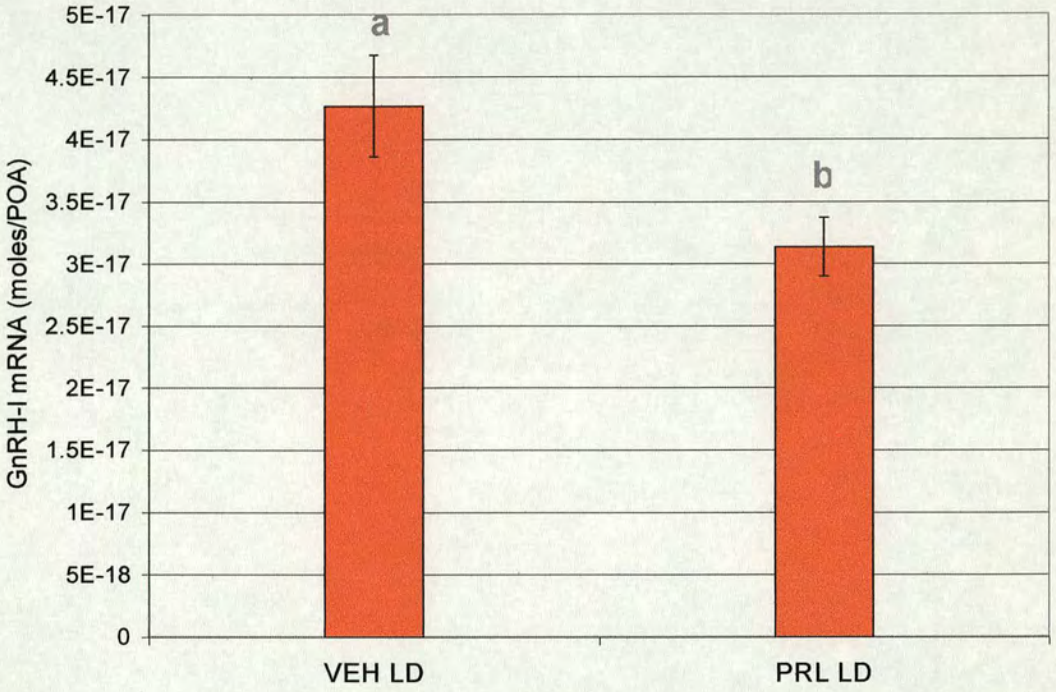


Figure 8.3.3.3. Hypothalamic GnRH-I mRNA levels in female quail injected twice daily with either PRL or vehicle for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected and LD = long day, 20L:4D. Values with different superscripts are significantly different. Average ± SE MEAN.

8.3.3.4. Plasma prolactin

A two-way ANOVA revealed an effect of time and prolactin treatment. Ovine prolactin -injection significantly depressed endogenous prolactin (d.f. 38, F15.10, $P \leq 0.001$)(figure 8.3.3.4.).

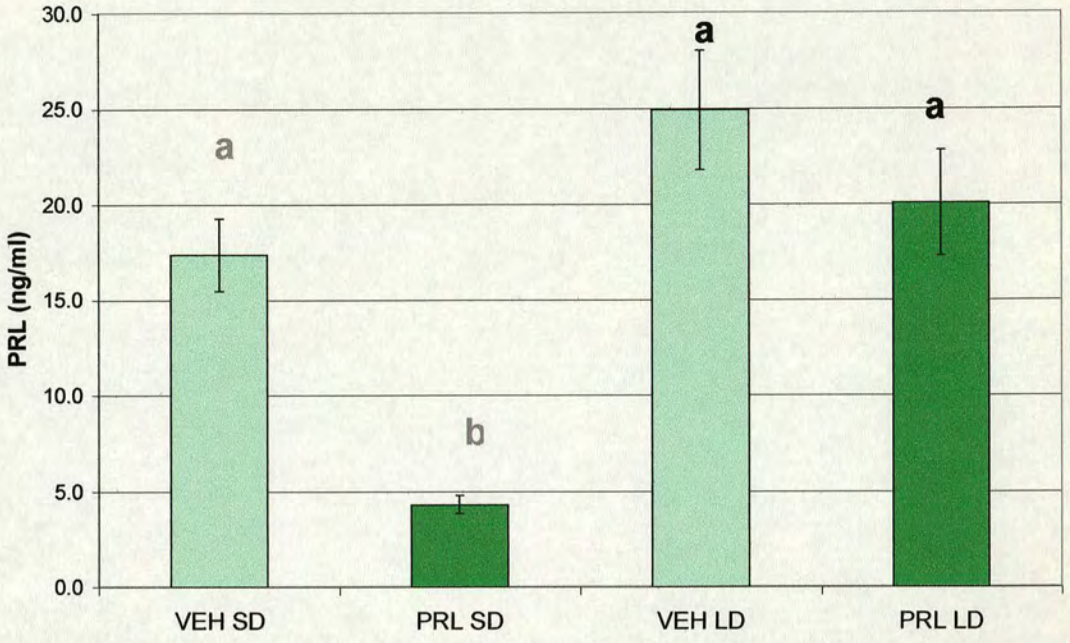


Figure 8.3.3.4. Plasma PRL levels in female quail injected twice daily with either PRL or vehicle for 6 days. VEH = vehicle-injected, PRL = ovine PRL + vehicle-injected and LD = long day, 20L:4D. Values with different superscripts are significantly different. Average ± SE MEAN.

8.4. DISCUSSION

8.4.1. Depression of GnRH-I mRNA by prolactin -injection

In the first experiment there was no detectable increase in LH or prolactin after exposure to one long day of 13 hours of light. This was expected because a first day release of LH is not seen unless quail are exposed to at 14.7 hours of photostimulation or more (Follett *et al.,* 1977). The LH levels on days 5 and 6 were significantly elevated in the birds photostimulated with 13 hours of light. This observation agrees with previous studies (Follett and Maung, 1978). However, there was no detectable effect of prolactin injection on day five or six. This suggests that the prolactin -injection did not induce relative photorefractoriness in these quail as

13L is non-photostimulatory in relatively photorefractory quail (see chapter 7). However, caution must be taken in interpreting these results as there is no direct evidence that the injected ovine PRL was biologically active.

There was also no significant increase in hypothalamic GnRH-I mRNA after 6 days exposure to a 13 h stimulatory photoperiod and there was no effect of prolactin treatment on short day control or experimental birds exposed to a 13 h stimulatory photoperiod on GnRH-I mRNA. Although disappointing in the context of the effect of prolactin -injections, this result is interesting when compared with the first day release experiments (see general discussion). However, the possibility exists that changes in GnRH-I mRNA did occur but that they were too small for the Q RT-PCR assay to detect.

In quail that photostimulated with 20 hours of light there was a significant increase in LH on days 5 and 6 of photostimulation. Prolactin treatment suppressed this LH response but only at the level of significance of $P \leq 0.06$. No firm conclusion can be drawn from this study on the effects of prolactin on plasma LH. It was predicted that the 20 hour highly stimulatory photoperiod would override inhibitory effects of prolactin and this appears to be the case when measuring LH. Even though there was no clear suppressive effect of prolactin on LH there was a significant suppression of hypothalamic GnRH-I mRNA in prolactin -injected birds on 20 hours of photostimulation. This is the first evidence that the inhibitory effects of prolactin could be mediating their effect by directly affecting GnRH-I gene transcription.

In the laying quail on 20L a significant depression of GnRH-I mRNA was also observed in prolactin -injected birds. However, there was no significant depression of LH or significant decrease in ovary weights in prolactin -injected compared to vehicle-injected breeding females. This is somewhat surprising as a study in quail by Camper and Burke (1977a) found that ovarian weights were depressed after injection with prolactin for 7 days. However, they observed great variation between individuals in response to prolactin -injection and reported one experiment in which prolactin treatment did not affect ovarian weight.

The experimental observations in this chapter provides further evidence that prolactin exerts a negative feedback action on its own secretion in laying quail and in photosensitive non-breeding birds.

In summary the work presented in this chapter provides the first evidence that hypothalamic GnRH-I mRNA is suppressed by prolactin. This suggests that a photoinduced increase in prolactin may play a role in maintenance of relative photorefractoriness in Japanese Quail. However, the failure of prolactin treatment in inducing relative photorefractoriness in photosensitive quail transferred to a marginally stimulatory photoperiod of 13 h, suggests that increased prolactin secretion may not be the only factor responsible for the development of relative photorefractoriness.

9. GENERAL DISCUSSION

9.1. THESIS OBJECTIVE

The aim of this thesis was to determine whether a change in GnRH-I gene expression is involved in the photoperiodic control of GnRH-I release in Japanese quail. GnRH-I gene expression, as measured by changes in hypothalamic GnRH-I mRNA content, was shown to change in response to changes in photoperiodic conditions. More importantly this change in GnRH-I mRNA was often dissociated from the changes in LH so that LH continued to be high or increasing after GnRH-I mRNA message has returned to basal levels. LH is known increase for at least 9 days of photostimulation with 20L:4D and achieves a five-fold increase in intacts (Follett, 1976). However, GnRH-I mRNA levels are approximately doubled after 20 hours of photostimulation and remain doubled after 36 hours and one week of photostimulation. This divergence between plasma LH and GnRH-I mRNA gene transcription is also seen in the carry-over experiment where plasma LH continues to increase at a time when GnRH-I mRNA has returned to basal levels.

9.2. THE CONTROL OF PHOTOSTIMULATED GnRH-I RELEASE FROM GnRH NEURONES.

The question of how the rapid photoperiodically induced release of GnRH-I peptide is controlled was addressed in studies using the Japanese quail first day release model of LH. The first rise in plasma LH occurred at hour 18 from dawn of the first long day, yet all of the photoperiodic information necessary for a first day release of LH has been received by 14.7 hours from dawn (Follett *et al.,* 1977). The question

arises, therefore, as to what changes in neural activity occurs in the quail photoperiodic transduction pathways between hour 14.7 and hour 18?

Meddle and Follett (1997) measured an increase in cFos-lir in glial cells in the median eminence and neuronal cells in the infundibular nucleus 18 hours from dawn of the first long day but did not see an increase in cFos-lir in GnRH-I cell bodies. This suggests that the first day release of GnRH-I from the median eminence is controlled directly or indirectly by glial cells and not by changes in GnRH-I gene expression. However, changes in GnRH-I mRNA levels have been observed in chicken where hypothalamic GnRH-I mRNA increases after one week of photostimulation (Dunn and Sharp, 1999). Photoperiodic changes in GnRH-I mRNA have also been reported in photostimulated salmon (Amano *et al.,* 1995; Amano *et al.,* 1999) and Siberian Hamsters (Porkka-Heiskanen *et al.,* 1997). Though conflicting with these studies, no difference in GnRH mRNA was observed between long and short day Syrian Hamsters using in-situ hybridisation techniques (Brown *et al.,* 2001). However, as mentioned in chapter 3 the sensitivity of semi-quantitative RT-PCR assays is higher than in situ hybridisation techniques.

This thesis shows that GnRH-I mRNA levels are increased on the first day of photostimulation in quail. The time-course experiments in chapter 5 show an increased hypothalamic GnRH-I mRNA content after exposure to one week of long days and also after 36 hours of photostimulation, as compared to birds on short days. This finding was supported by the comparison of short day and long day vehicle injected birds in the PRL injection experiments in chapter 8 where GnRH-I mRNA

was significantly higher in LD than SD birds after 6 days of photostimulation (20L:4D).

An increase in GnRH-I mRNA in the POA was first detected by hour 20 from dawn of the first long day. Therefore, the work reported in this thesis provides the first evidence that Japanese quail GnRH-I mRNA neurones respond to increased photoperiod by increases in either GnRH-I gene transcription and/or GnRH-I mRNA stability. This is the earliest photoperiodic change in GnRH-I neurones so far reported for any species. That this change occurs so early in photoperiodic induction implies that a change in GnRH-I synthesis could be a component in the photoinduction pathway.

With these results in mind the lack of an increase in cFos-lir in GnRH-I cell bodies after 18 hours of photostimulation that Meddle and Follett (1997) observed could be interpreted in several ways.

It is possible that cFos is not involved in GnRH-I transcription and that other transcriptional markers are more suitable for studies of GnRH-I neurone activation. Though during ovulation in rats an increase in Fos expression and GnRH mRNA is seen in GnRH neurones (Lee *et al.,* 1992; Park *et al.,* 1990; Zoeller and Young, 1988). Therefore, some genes, within the GnRH neurones, are linked to the cFos transcriptional marker. It is possible that the glial cells with cFos-lir in photostimulated quail could project to the GnRH-I neuronal cells bodies. However, it is also possible that within the two hours between hour 18 when cFos first increases

and 20 from dawn when GnRH-I mRNA increased, that GnRH-I peptide is released from the nerve terminals. This would be consistent with two main hypotheses: that either a store of readily releasable GnRH-I peptide exists in the GnRH-I neurones or the release of GnRH-I peptide from the network of GnRH-I neurones becomes co-ordinated, increasing the pulse amplitude of GnRH-I peptide release. There is some evidence from chapter 8 that the GnRH-I mRNA increase is dependent on the degree of photostimulation as birds that had been held on 13 hours of light per day for 6 days exhibited no detectable increase in GnRH-I message, despite the increase in plasma LH. It is possible that 13 hours of photostimulation was sub-maximal because it did not illuminate all of ϕi.

9.4. THE EFFECT OF ONE LONG DAY OF PHOTOSTIMULATION ON GnRH-I GENE EXPRESSION

GnRH-I mRNA concentrations remain elevated until the morning of the day after the first day of photostimulation but then falls rapidly back to basal levels by the third day. LH secretion remains high for three days before gradually returning to basal levels by the 14th day (my data and Nicholls *et al.,* 1983, Perera and Follett, 1992; Follett and Meddle and Follett, 1997). This can be explained in two main ways. Firstly the carry over effect of LH could be due to the translation of mRNA transcripts into proGnRH peptide which persists in the GnRH-I neurone and are further processed after the GnRH-I gene transcription has returned to basal levels. Secondly there could be a store of processed GnRH-I which is released progressively after the GnRH-I mRNA transcription has returned to basal levels.

In conclusion the carry over effect of one long day of photostimulation persists more for plasma LH than for hypothalamic GnRH-I mRNA and this may be due to an exhaustion of pro-GnRH-I transcribed from GnRH-I mRNA produced by transcription of the GnRH gene after exposure to one long day. The first day photoinduced transcript of the GnRH-I gene appears to persist for no more than 48 hours.

9.5. HYPOTHALAMIC GnRH-I mRNA CONTENT AND THE DEVELOPMENT PHOTOREFRACTORINESS

This study provides the first tantalising evidence that a decrease in GnRH-I gene expression may be correlated with the development of relative photorefractoriness. It provides a physiological means of distinguishing between relative photorefractory and photosensitive quail held on long days. This is the first report of GnRH-I mRNA concentrations not being directly proportional to photoperiod. Even though GnRH-I mRNA concentrations were at sub maximal values, plasma LH levels were high and the quail were in breeding condition. This would suggest that photosensitive birds on long days have GnRH-I mRNA concentrations in excess of that necessary to maintain plasma LH and breeding. Interestingly the short day breeding birds GnRH-I mRNA levels were also at this intermediate level, though the difference was not significant. This possible 'excess' of GnRH-I message adds weight to the hypothesis that the carry over effect is due to the progressive processing of mRNA. If there are supernumerary transcripts of GnRH-I mRNA then it is possible that there is a 'back log' of pro-GnRH-I awaiting processing, this could account for the carry over effect of LH.

Whether the depression of GnRH-I gene expression occurs at the onset of relative photorefractoriness is not known. The birds used in this research had been exposed to long days for one year and the decrease in GnRH-I message may not be causally related to the development of relative photorefractoriness. Caution should also be taken with these results because of the lack of significant difference when using post hoc Tukey's tests. However, Tukey's tests are conservative and the Least Squares Means and Two Sample T-test comparisons are in agreement.

9.6. PRL INJECTION DEPRESSES HYPOTHALAMIC GnRH-I mRNA IN FEMALE JAPANESE QUAIL

Prolactin levels are always high in birds that are relatively photorefractory, including the relatively photorefractory quail described in chapter 7. The aim of chapter 8 was to determine whether a causal link exists between increased levels of prolactin and relative photorefractoriness. The work presented in this chapter is the first evidence that hypothalamic GnRH-I mRNA is suppressed by PRL-injection. PRL-injection significantly depressed GnRH-I mRNA in photosensitive quail stimulated with 20 hours of light and also in laying quail held on long days, even when there was no significant decrease in plasma LH or gonadal size. This observation supports the hypothesis that the anti-gonadal properties of PRL are mediated in part through decreased GnRH-I gene expression. PRL-injection did not induce relative photorefractoriness in quail, as 13L:11D should be non-stimulatory for relatively photorefractory quail, but resulted in an increase in plasma LH. Birds injected with PRL on 20L:4D were not tested for the development of relative photorefractoriness

by being placed on 13L, but this would be an interesting experiment for future research.

9.7. COMPARISON OF ABSOLUTE AND RELATIVE PHOTOREFRACTORINESS

Thesis supports the hypothesis that absolute and relative photorefractoriness are both caused by decreased hypothalamic drive and suggests that the differences between relative and absolute photorefractoriness are quantitative and not qualitative. A comparison of photorefractoriness in quail and starlings is shown in table 9.7.

Table 9.7. A summary of the similarities and differences in GnRH neural function between relatively photorefractory Japanese quail and absolutely photorefractory starlings held on long days.

	Relatively Photorefractory Quail	**Absolutely Photorefractory Starlings**
Breeding on fixed day lengths	Yes (Gibson *et al.*, 1975)	No (see Goldsmith and Nicholls, 1984)
Plasma LH levels on fixed days	High - no change (see Robinson and Follett, 1982).	Low - spontaneous decrease (Dawson and Goldsmith, 1984; Goldsmith and Nicholls, 1984).
Hypothalamic GnRH-I peptide content (RIA)	No change (Foster *et al.*, 1988)	Increases at the onset (Goldsmith *et al.*, 1988) Decreases in chronic (Dawson *et al.*, 1985)
Hypothalamic GnRH-I immuno-cytochemistry	Intensity and density of immunostaining greater (Foster *et al.*, 1988, Teryuma and Beck, 2000).	No decrease in GnRH neuronal numbers. Decrease in intensity (Goldsmith *et al.*, 1988).
GnRH-I gene expression	Decrease (this thesis)	
PRL levels	High (Boswell *et al.*, 1995; this thesis)	High at onset (Ebling *et al.*, 1982; Dawson and Goldsmith, 1983; Goldsmith and Nicholls, 1984)
PRL-injection	Decreases GnRH-I mRNA May not initiate relative photorefractoriness (this thesis)	
VIP-immunisation		Delays but does not prevent absolute photorefractoriness (Dawson and Sharp, 1998).

9.8. DIAGRAMATIC SUMMARY

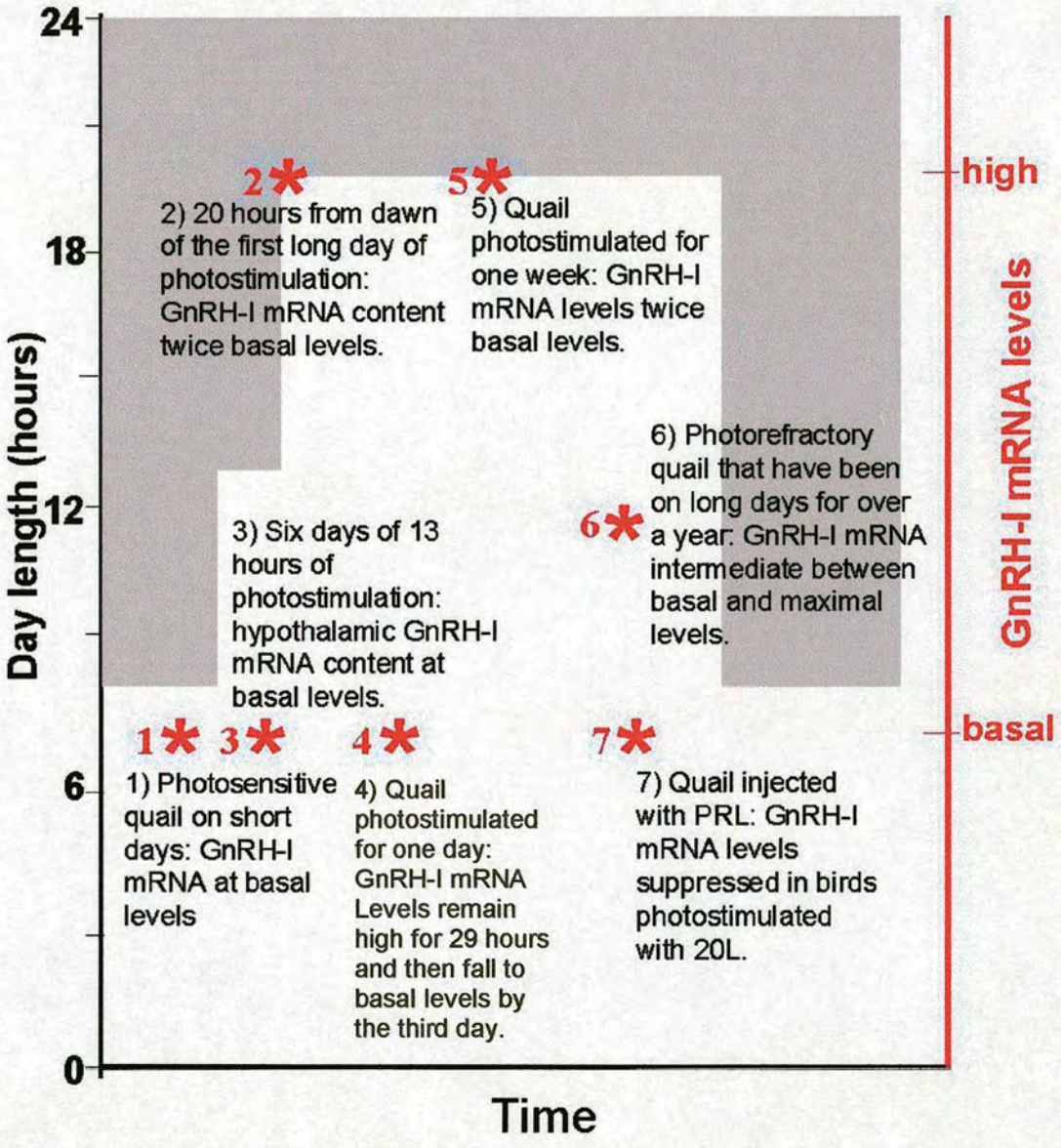


Figure 9.8. A diagrammatic representation of the results presented in this thesis. The grey area represents the dark/night photoperiod and corresponds to the left y axis. The red stars correspond to the right y axis and give an approximate level of hypothalamic GnRH-I mRNA in relation to the photoperiod and conditions described on the diagram.

9.9. FURTHER WORK

Confirmation of all the results presented in this thesis in castrates would be desirable, to remove differences in steroid feedback, but further tuned time course experiments are also needed. These could possibly determine whether the control of GnRH-I release on the first long day of photostimulation involves a store of GnRH-I being released prior to a change in GnRH-I gene expression or whether an increase in GnRH-I gene expression alone followed by new synthesis of GnRH-I accounts for the rapid release of the decapeptide. The simplest experiment to investigate this would be a continuation of the experiments reported here. Starting with hour 18 from dawn, and determining whether the timing of the first rise of plasma LH can be dissected from an increase in hypothalamic GnRH-I mRNA. However, there is no guarantee that the assay has sufficient sensitivity to detect such changes, and bird processing time also has to be considered in the experimental design. An alternative approach would be to use pulse-labelling of pro-GnRH. This could be used to determine whether newly synthesised GnRH is stored and released later or whether newly synthesised GnRH is released immediately. It would also be useful to develop a Q RT-PCR for cFos, and other gene transcription factors, such as zenc. This would allow more sensitive measurements of photoperiodic changes than immunocytochemical techniques. A key study would be to halt GnRH translation prior to the illumination of ϕi. This could be achieved with GnRH-I mRNA anti-sense or transcription inhibitors actinomycin D or 5,6-dichlorobenzimidazole riboside, or the translation inhibitors anisomycin or puromycin as have been used in immortalized GT1–1 GnRH neurons (Pitts *et al.,* 2001). This would reveal whether the first day release of LH is independent or dependent of the increase in

hypothalamic GnRH-I mRNA. It would also of interest to study the time-course of GnRH-I mRNA after one week of photostimulation and also to measure GnRH-I mRNA levels in quail exposed to natural photoperiods. It is possible that as well as there being increases in GnRH-I mRNA associated with photostimulation that there are changes in message stability, rates of translation and also changes in protein stability all of which could be explored.

The control of the GnRH-I neurones is just one part of the pathway of photoperiodic induction, further to understanding the control of GnRH-I release from these neurones, the elements further up the photoinduction pathway could be explored by looking for interactions with the GnRH-I neurones. For example, which cells project to the GnRH-I neurones and what transmitters might be acting on them? Of particular interest are the population of neurones in the infundibular nucleus which exhibit an increase in cFos-lir after 18 hours of photostimulation (Meddle and Follett, 1997).

The photorefractoriness experiment was unfortunately inconclusive. Further repeats of this experiment is necessary. The experimental design should be altered to include a photosensitive short day group transferred from 8L:16D to 13L:11D to compare plasma LH levels with the photorefractory group moved from 18L:6D to 13L:11D. Measurable differences in LH would likely only be feasible in castrates. This would reduce the large amount of variation but would also be a more sound design as the steroid background would be the same in all groups.

Although prolactin injection does not induce relative photorefractoriness in birds that have recently been photostimulated, it is possible that prolactin is essential in developing relative photorefractoriness. VIP-immunisation would be able to determine whether the development of photorefractoriness is independent or dependent on prolactin.

9.10. CONCLUSION

In conclusion quail hypothalamic GnRH-I mRNA changes in relation to the photoperiodic control of gonadotrophin secretion. This thesis suggests that changes in the expression of the GnRH-I gene are involved in the mediation of the photoinduced gonadotrophin release and the development of photorefractoriness.

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