POTENTIAL FUNCTIONS OF GONADOTROPIN INHBITORY HORMONE AND DYNORPHIN IN BROILER BREEDER REPRODUCTION

by

ASHLEY GERA-ANN STEPHENS

(Under the Direction of ADAM DAVIS)

ABSTRACT

Broiler breeders given free access to feed can reach their ideal bodyweight for reproduction by 5 weeks of age, but functional sexual maturation of the brain does not occur until about 20 weeks of age. To match the ideal bodyweight for reproduction with brain-based sexual maturation, broiler breeders are severely feed restricted, and this optimizes their reproduction relative to that achieved if they are fed *ad libitum*. However, feed restriction management programs for broiler breeders result in fasting periods during which caloric insufficiency signals can negatively impact reproductive development and efficiency. The hormonal signal pathways activated by fasting are not well delineated in avian species. However, based on mammalian research and preliminary avian research, gonadotropin inhibitory hormone (GnIH), the gonadotropin inhibitory hormone receptor (GnIH-R), dynorphin, translated from the preprodynorphin (Pdyn) mRNA transcript, and dynorpin's receptor, the kappa opioid receptor (KOR), may be key hormonal participants. Therefore, the mRNA expression of GnIH, GnIH-R, Pdyn, and KOR was investigated in the pituitary and follicular tissues of broiler breeder hens that had been fed daily or fasted for 72 hours. Expression of the transcripts of interest was also investigated in F1, F3, and small yellow follicle granulosa cells cultured with gonadotropins

(LH and FSH) and steroid hormones (estrogen and testosterone). GnIH, GnIH-R, Pdyn, and KOR mRNA was detected in pituitary tissues, but only GnIH mRNA expression was significantly increased after 72 hours of fasting. GnIH mRNA was not detected in the follicular tissues of fed or fasted hens, but the mRNA transcripts for GnIH-R, Pdyn, and KOR were detected in fed and fasted follicular tissues. In freshly isolated granulosa cells from the F1 or F3 follicles, GnIH-R mRNA was not detected, but once the cells were cultured for 24 hours, expression of the mRNA for GnIH-R was induced, and LH and FSH do not prevent this induction. The results suggest that both the GnIH and dynorphin neuropeptide systems could play a role in follicular development both centrally and locally, and that increased production of pituitary GnIH during caloric insufficiency may be a key regulator in down regulating reproduction function during fasting.

INDEX WORDS: theca, granulosa, gonadotropin inhibitory hormone, gonadotropin inhibitory hormone receptor, dynorphin, preprodynorphin, kappa opioid receptor, broiler breeder hens

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DEDICATION

Without a moment's hesitation, I whole-heartedly dedicate this dissertation to the most incredible father and mother on this planet, Barry and Susan Stephens, who taught me all of the most important things in life, to a patient and kind husband, Anthony House, with whom I most gratefully and happily share my days, and to a wonderful aunt, Vicki Pilgrim, who has always been more like a second mother. Furthermore, it is written in honor of my life mentor and uncle, Bob Beck, who was far too good for this world and was taken too soon. Thankfully, God decided to share you all with me, and it is truly an honor. Your love is unending, your support is constant, your wisdom is abounding, and your presence is infinitely appreciated. Without you and the Lord, I would have never made it this far. This work is as much all of yours as it is mine, and there are no others with whom I would choose to share it.

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CHAPTER 1

REGULATION OF REPRODUCTIVE FUNCTION IN HENS

The Ovary of the Hen

In the laying hen, only the left ovary participates in reproduction, as the right ovary does not develop to a functional state. The left ovary of a laying hen at sexual maturity is comprised of a follicular hierarchy organized on the basis of size and time until ovulation. The largest follicles in the ovary, termed the hierarchical follicles, are the nearest to ovulation. Generally, the ovary contains four to six of these large, yolk-filled follicles, sized 12 to 40 mm in diameter. The smaller, less than 12 mm in diameter, follicles are more distant from ovulating and referred to as the prehierarchical follicles. Within the cluster of prehierarchical follicles, there are several size categories with many follicles in each category. The largest of the prehierarchical follicles are the small yellow follicles (SYF), sized 5-12 mm in diameter. These are followed by the large white follicles (LWF), which are 2-5 mm in diameter. All prehierarchical and primordial follicles that are less than 2 mm in diameter are referred to as the small white follicles (SWF).

The largest of the hierarchical follicles is referred to as the F1 follicle, and it will typically ovulate within 24 hours. Following the F1 follicle is the next largest of the hierarchy, the F2 follicle, which will ovulate in approximately 48 hours. This pattern in nomenclature and ovulation timing is retained for the remainder of the yellow, yolk-filled hierarchical follicles, with consecutive ovulations occurring 24-26 hours apart. Following the ovulation of the F1 follicle and the forward advancement in the hierarchy of the remaining hierarchical follicles, one

of the pool of small yellow prehierarchical follicles is selected and develops into the smallest hierarchical follicle. In succession, several small white follicles will begin the uptake of yellow yolk and proceed into the pool of small yellow follicles. Only 5% of the developing prehierarchical follicles will grow to reach a size of 6-8 mm in diameter (Gilbert et al., 1983a). The fate for the vast majority of the prehierarchical follicles is follicular atresia, during which the individual cells of the follicle undergo apoptosis (Johnson et al., 1996a).

Surrounding each yolk-filled oocyte of the avian ovary are distinct tissue layers. The yolk-filled oocyte of each hierarchical follicle is enveloped by its plasma membrane, followed in succession by the inner perivitelline layer (IPVL), the granulosa cell layer, a basement membrane, and, finally, the theca cell layer. The granulosa cells form a single layer in hierarchical follicles, but in prehierarchical follicles, the granulosa cells are arranged in multiple layers. Further, the theca tissue can be subdivided into two tissue layers, the theca interna and externa. Unlike the avascular granulosa cell layer, the theca tissue is highly vascularized, which facilitates the transfer of yolk precursors from the plasma to the developing follicles in the ovary (Etches and Cheng, 1981). The follicular acquisition of yolk precursors and development of endocrine capabilities are indicative of the maturation of the ovarian follicles (Huang and Nalbandov, 1979). This follicular maturation is regulated primarily by two anterior pituitary glycoprotein hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), and is mediated partly by the expression of LH and FSH receptors in granulosa tissue.

For Leghorn laying hens, the development and maintenance of the ovary and follicular hierarchy, as a part of general reproductive function, tends to progress without issue. However, this is not the case for the broiler breeder hen. Improvements in genetic selection and bird management practices created a broiler with a voracious appetite and a rapid growth rate that are,

unfortunately, also shared by their genetically similar parent stock. Overeating and rapid growth are not conducive to reproductive function, leaving broiler breeder hens to suffer various ovarian dysfunctions including but not limited to follicular atresia, internal ovulations, multiple ovulations, and double-yolked eggs (Leeson and Summer, 2000; reviewed by Walzem and Chen, 2014). Improvement in broiler breeder reproductive function has been observed only with the implementation of feed restriction programs designed to limit feed intake and maintain optimal body weight.

Effects of Feed Restriction in Broiler Breeder Hens

In broiler breeders, optimum reproductive efficiency is largely controlled by photostimulation, consuming a nutritionally adequate diet, and attaining an ideal body weight to support reproduction. The ideal body weight for reproduction is about 2.5 kilograms, which can be achieved in less than six weeks in broiler breeder hens that have free access to food (Leeson and Summer, 2000). However, hypothalamic maturation for optimum reproduction in broilers does not occur until roughly 20 weeks of age (Leeson and Summer, 2000).

To prevent broiler breeders from growing too quickly and becoming obese prior to the photosensitivity-based sexual maturity that occurs at 20 weeks of age, the dietary intake of the broiler breeders is highly restricted. Feed restriction of broiler breeder hens is a management tool that has been successful in increasing the productive efficiency of these birds. Utilizing feed restriction in broiler breeder hen husbandry delays sexual maturation of the flock (Robbins et al., 1986; Yu et al., 1992a; Heck et al., 2004; Bruggeman et al., 2005; Hocking and Robertson, 2005; Onagbesan et al., 2006), promotes body weight uniformity across the flock (Bennett and Leeson, 1989; de Beer and Coon, 2007), and decreases the mortality within the flock (Robbins et al., 1986; Katanbaf et al., 1989; Heck et al., 2004; Bruggeman et al., 2005). In addition, feed

restriction implementation during the rearing and the laying periods reduces the development of an abnormally high number of large hierarchical follicles on the ovary of the broiler breeder hens at the same time (Hocking, 1987; Hocking et al., 1989; Heck et al., 2004; Hocking and Robertson, 2005). However, of most importance, broiler breeder hens that are managed on feed restriction regimens produce more eggs over their lifetimes (Leeson and Summers, 1985; McDaniel et al., 1981; Yu et al., 1992a; Heck et al., 2004; Bruggeman et al., 2005; Onagbesan et al., 2006) because they lay longer sequences (Robinson et al., 1991a), persist in lay for a longer period of time (Fattori et al., 1991), lay fewer abnormal eggs, and have fewer multiple ovulations in a single day (Fattori et al., 1991; Yu et al., 1992a; Heck et al., 2004) compared to full-fed broiler breeder hens. Broiler breeders that grow too large or become overweight display compromised fertility, resulting from reduced locomotive capabilities, as well as physical difficulty in successful copulation (Duff and Hocking, 1986). Even when artificial insemination is employed, fertility in overweight broiler breeder hens is reduced (Brake and McDaniel, 1981), which may be a consequence of their fat or hormones derived from the fat making successful insemination more difficult, blocking the sperm storage tubules, or inhibiting sperm movement (Hocking, 1987).

Despite the success of feed restriction in broiler breeder hens, these hens still produce over 100 eggs less, annually, than their Leghorn laying hen counterparts. Furthermore, ovarian function of the broiler breeder hen continues to be plagued by an unacceptable incidence of atresia of follicles and internal ovulations. Presently, it is not understood why broiler breeders fed *ad libitum* produce excessive numbers of follicles simultaneously or what role feed restriction plays in controlling this process and in improving egg production. Because there is a lot to be learned about feed restriction and its impacts on reproductive function, it is not

surprising that the degree, the timing, and the duration of feed restriction varies greatly among commercial broiler breeder management programs. In typical broiler breeder management scenarios, feed allocations are 60-80 percent less during the rearing period and 25-50 percent less during the laying period than *ad libitum* consumption of the breeder pullets/hens (Renema and Robinson, 2004). Not only is there variation across broiler breeder management programs, there are also conflicting research reports on the optimum timing and duration of feed restriction. Pym and Dillon (1974) reported that the best feeding regimen for broiler breeder hens could be implementing severe restriction during the rearing period followed by *ad libitum* feeding during the laying period. McDaniel et al. (1981) and Yu et al. (1992b) suggested that, for optimum performance, feed restriction should occur during both the rearing and the breeding periods. Robbins et al. (1986, 1988) reported increased egg production in birds whose feed intake was restricted during the rearing period followed by *ad libitum* feeding during part or all of the laying period compared to birds that were feed restricted during both periods. However, Robinson et al. (1991b) reported that *ad libitum* feeding during the breeding period resulted in lower egg production. Subsequently, Bruggeman et al. (1999) indicated that *ad libitum* feeding from 1 to 7 weeks of age, followed by restriction of feeding from 7 to 15 weeks of age and *ad libitum* feeding again from week 15 until the first egg, resulted in improved reproductive performance in broiler breeders compared to any other combination of *ad libitum* or restricted feeding during the rearing period.

In the United States, broiler breeder pullets, during rearing, are typically provided feed once every other day, according to the skip-a-day feed regimen. Then, at photostimulation for reproduction, the flock is provided feed once every day. This provided feed is rapidly consumed by the birds, and as a result, broiler breeder pullets and hens will have significant fasting periods.

Morris and Nalbandov (1961) suggested that fasted hens have a decline in egg production as a result of a lack of gonadotropin secretion from the pituitary gland. Scanes et al. (1976) then found that plasma LH concentrations in 6-week-old male chicks fasted for 12 hours were significantly decreased when compared to control-fed cockerels. Additionally, when compared to *ad libitum* control-fed hens, laying hens have lower plasma concentrations of LH after 48 hours of fasting and reduced estradiol and progesterone concentrations after 24 hours of fasting (Tanabe et al., 1981).

Research endorses the idea that the fasting periods created as a result of feed restriction practices employed by the poultry industry depress total egg production in broiler breeder hens. Previously, in commercial settings, a skip-a-day feeding program was often continued until the broiler breeder flock reached 5 percent egg production in order to control flock body weight uniformity and to help control body weight gain, as even a slight excess in body weight prior to peak production results in a significant decrease in total egg production (as reviewed by Robinson et al., 1991b). Subsequently, Gibson et al. (2008) reported that implementing an every day feeding regimen upon photostimulation for reproduction, at 21 weeks of age, increased total egg production by approximately 19 eggs per broiler breeder hen by the end of 65 weeks of age when compared to continuing the skip-a-day feeding regimen until 5 percent egg production was reached. The research reported by Gibson et al. (2008) also suggested that the significant fasting period experienced by the broiler breeder pullets between meals on a skip-a-day feeding program after photostimulation for reproduction may be detrimental to normal ovarian development, as plasma estrogen levels were increased and plasma progesterone levels were decreased for the entire breeding period in the hens that had been on a skip-a-day feeding regimen until 5 percent

egg production when compared to the hens that were on an every day feeding regimen after being photostimulated.

Similar to the work of Gibson et al. (2008), Spradley et al. (2008) conducted research that differed only in that, when the pullets were photostimulated for reproduction, they were fed either once a day (equivalent to the every day treatment) or twice a day. In each of the feeding treatment groups, the pullets received an equivalent total amount of feed daily, but the fasting duration between meals was reduced for the pullets fed twice a day. Feeding the hens according to the twice a day program improved the total number of eggs produced per hen through 41 weeks of age by 5 eggs and increased the overall percent hen-day egg production through 59 weeks of age by 2 percent. However, due to a higher mortality associated with feeding broiler breeder hens twice a day, these gains in egg production were lost on a hen-housed basis at 59 weeks of age. For the hens fed once a day and twice a day, cumulative mortality from 23 to 59 weeks of age was 12 and 18 percent, respectively. Necropsy results showed that only 25 percent of the hens that died from the once a day feeding program treatment were in lay, as indicated by a normal ovarian hierarchy and/or an egg in the oviduct, while 63 percent of the hens that died from the twice a day feeding program treatment were in lay. Twice a day feeding of the broiler breeder hens improved flock body weight uniformity, increased egg weight without compromising shell quality, increased hatching egg production by decreasing the production of dirty eggs, and, lastly, had no effect on fertility of the eggs produced.

Similarly, as the degree of feed restriction is increased during the rearing phase of broiler breeder roosters, the onset of testosterone production in these males is delayed (Stevens, 2010). Research conducted by Stevens (2010) also indicated that the severity of current feed restriction programs could be lessened in male broiler breeders without hurting fertility. Previous research

supports this stance as well, suggesting that broiler breeder males are excessively feed restricted during the end of the production cycle (Buckner et al., 1986; Sexton et al., 1989a, 1989b; Cerolini et al., 1995; Bramwell et al., 1996; Romero-Sanchez et al., 2008).

As evidenced by the afore mentioned research, feed restriction programs are valuable as a part of broiler breeder management. However, there are some undeniable drawbacks associated with utilizing the programs, as their impact on broiler breeders is not fully characterized or understood at this time. While feed restriction programs have improved egg production and fertility, these programs also allow for periods of fasting during which caloric insufficiency signals could be negatively impacting reproductive function and, thus, egg production. Thus, one aim of research in this area is to understand, at the endocrine level, the finite details of the impact of feed restriction, and the associated periods of fasting, on reproduction.

Central and Peripheral Endocrine Regulation of Reproduction

Gonadotropin-releasing hormone (GnRH) is a 10-amino acid neuropeptide first discovered in the hypothalamus of mammalian species (Matsuo et al., 1971; Burgus et al., 1972). Expression of this neuropeptide was subsequently detected in the hypothalamus of non-mammals, and it was determined that more than one form of GnRH existed (King and Millar, 1982; Miyamoto et al., 1982, 1984; Sherwood et al., 1983, 1986). Presently, researchers have identified three forms of the GnRH (GnRH-I, GnRH-II, and GnRH-III) neuropeptide, and in avian species, GnRH-I appears to be the only form responsible for stimulating gonadotropin secretion from the anterior pituitary gland (Katz et al., 1990; Sharp et al., 1990; Wilson et al., 1990a, 1990b, 1991; Millam et al., 1998). This form of GnRH is produced by neurons located in the preoptic area of the hypothalamus. The neuronal fibers extend from the perikarya in the preoptic area to the median eminence, where GnRH is released into hypothalamic-hypophyseal

portal circulation. Once in the hypothalamic-hypophyseal portal circulation, GnRH travels to the gonadotrope cells of the anterior pituitary gland to stimulate the production and release of FSH and LH, which enter systemic circulation destined for the gonads.

At the avian ovary, FSH and LH regulate follicular maturation and ovulation through the expression of FSH and LH receptors in the granulosa tissue of the follicles. The FSH receptor (FSH-R) is most highly expressed in the granulosa cells of the SYFs, and as a follicle matures, this level of expression decreases (Calvo and Bahr, 1983; Ritzhaupt and Bahr, 1987; You et al., 1996; Woods and Johnson, 2005). The theca cells express less FSH-R than the granulosa cells, and FSH-R expression in the theca cells does not change significantly with follicle maturation (Etches and Cheng, 1981; Gilbert et al., 1985; You et al., 1996). FSH promotes granulosa cell proliferation and maturation in the prehierarchical follicles (Davis et al., 2000, 2001) and aids in maintenance of the follicular hierarchy through prevention of atresia (Palmer and Bahr, 1992; Johnson et al., 1996a, 1999). Additionally, FSH induces the expression of LH receptor, steroidogenic acute regulatory protein, and P450 cholesterol side chain cleavage enzyme in granulosa cells for subsequent steroid production (Li and Johnson, 1993; Johnson and Bridgham, 2001; Johnson et al., 2004), and FSH stimulates progesterone production (Calvo and Bahr, 1983; Robinson et al., 1988; Davis et al., 1999, 2001; Johnson et al., 2004). As a whole, these results indicate that the prehierarchical follicle that is most responsive to FSH will avoid cell death by apoptosis and be recruited into the avian follicular hierarchy.

A newly selected follicle transitions from being largely FSH dependent to being predominantly LH dependent (Calvo and Bahr, 1983). LH receptor (LH-R) expression is highest in the granulosa cells of the hierarchical follicles, particularly the F1 through F3 follicles (Calvo et al., 1981; Calvo and Bahr, 1983; Gilbert et al., 1983b, 1985; Johnson et al., 1996b). In the

theca tissue, LH-R mRNA expression varies little with follicular development (Johnson et al., 1996b), and the interaction of LH with these receptors promotes steroidogenesis within the theca cells of the prehierarchical and hierarchical follicles (Robinson et al., 1988; Kowalski et al., 1991). In prehierarchical follicles sized 3-5 mm in diameter, decreased expression of the LH-R mRNA is associated with atresia (Johnson et al., 1996b). Furthermore, LH promotes granulosa cell proliferation (Davis et al., 2000) and progesterone production (Davis et al., 1999; Johnson et al., 2004). In response to progesterone production from the granulosa cells of the F1 follicle, plasma LH concentrations peak 4-6 hours prior to ovulation (Etches, 1990). The peak in plasma progesterone level and LH concentration stimulates ovulation of the F1 follicle.

Kisspeptin

GnRH is produced and released from the hypothalamus in response to environmental and physiological cues (Contijoch et al., 1992; Advis and Contijoch, 1993). Adequate photostimulation (greater than 12 hours), attaining a body weight sufficient to support reproduction, and adequate nutrition are the most important cues to initiate GnRH production in the domestic hen. In mammalian species, these cues are recognized and translated to the reproductive axis by a gatekeeper system consisting of the neuropeptide, kisspeptin, and its G-protein coupled receptor, GPR54 (Dhillo et al., 2005; Luque et al., 2007; Brown et al., 2008; reviewed by Pinilla et al., 2012).

The kisspeptin neuropeptide is encoded by the *Kiss1* gene and was first identified as an anti-metastasis factor in human carcinoma and designated metastin (Lee et al., 1996). Kisspeptin was not implicated in regulating reproduction until 2003, when a loss of function mutation in GPR54 was found to have initiated hypogonadotropic hypogonadism in a group of humans (de Roux et al., 2003; Seminara et al., 2003). Subsequently, this finding was supported by studies in

mice and humans, which indicated loss of function mutations in both GPR54 and kisspeptin to be detrimental to reproductive function and overexpression of GPR54 and kisspeptin to initiate precocious puberty (Funes et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007; Teles et al., 2008; Silveira et al., 2010).

Following the discovery of its role in reproductive regulation, mRNA transcripts for kisspeptin and GPR54 were detected within the brain and the gonadal tissues of mammals (Kotani et al., 2001; Ohtaki et al., 2001; Terao et al., 2004; Castellano et al., 2006). In the hypothalamus, kisspeptin expression was localized to neuronal perikarya in the arcuate and anteroventral periventricular nuclei of the hypothalamus, from whence neuronal fibers extend to GnRH perikarya and neuronal fibers (Clarkson and Herbison, 2006; Decourt et al., 2008; Hahn and Coen, 2006; Ramaswamy et al., 2008; Smith et al., 2009). Furthermore, GnRH neurons were found to express GPR54 mRNA and to be activated by kisspeptin, resulting in *in vivo* secretion of GnRH and, therefore, LH (Gottsch et al., 2004; Irwig et al., 2004; Navarro et al., 2004; Han et al., 2005; Messager et al., 2005; Shahab et al., 2005). Given the stimulatory action of kisspeptin on GnRH production and secretion, it seems fitting that the kisspeptin system functions as the gatekeeper of mammalian reproduction, gathering all physiological and environmental cues and translating them to GnRH neurons. Two of the hormones that act through the kisspeptin system to mediate nutritional cues to the reproductive axis are leptin and ghrelin (reviewed by Pinilla et al., 2012).

Leptin is an adipocyte-derived peptide hormone that regulates food intake, body weight, and energy balance (Zieba et al., 2005). Regarding reproduction, leptin receptors, which are responsible for signal transduction, are localized to the gonads, the pituitary gland, and the hypothalamus (Cioffi et al., 1997; Karlsson et al., 1997; Spicer et al., 1997, 1998; Ruiz-Cortés et

al., 2000; Donato et al., 2011). Leptin signals that adipose stores are sufficient to begin puberty, maintains normal sexual maturation, and initiates fertility (Friedman and Halaas, 1998; Baldelli et al., 2002). However, when plasma leptin concentrations become excessive, reflective of obesity or a similar metabolic disorder, this adipokine functions to inhibit normal reproductive processes (reviewed by Hausman et al., 2012; Pérez-Pérez et al., 2015). In obese mammals, increased levels of circulating leptin activate the leptin receptor that is present within the gonadal tissues, which depresses steroidogenesis and inhibits overall reproductive function within the gonads (Spicer et al., 1997, 1998, 2000; Zachow et al., 1997; Agarwal et al., 1999; Brannian et al., 1999; Ruiz-Cortés et al., 2003; Hausman et al., 2012; Pérez-Pérez et al., 2015).

Within the hypothalamus, the kisspeptin neurons express the leptin receptor (Smith et al., 2006). Furthermore, during feed restriction and starvation, there is a decrease in plasma leptin concentration and an associated decrease in GnRH and LH secretion from the hypothalamus and anterior pituitary gland, respectively. Additionally, GnRH and LH levels can be restored even during fasting by both intracerebroventricular and intraperitoneal injections of leptin (Henry et al., 2001; Farooqi et al., 2002), indicating the role of leptin as a positive regulator of reproductive function.

In the late 1990s, leptin was successfully cloned in avian species, and its expression was localized to adipose and hepatic tissues (Taouis et al., 1998; Ashwell et al., 1999). However, the biology of the chicken leptin receptor has been well characterized (Horev et al., 2000; Ohkubo et al., 2000). Additionally, the avian leptin receptor is expressed in the ovary of the hen (Ohkubo et al., 2000; Paczoska- Eliasiewicz et al., 2003).

While the role of leptin in avian reproduction is not well investigated, preliminary reports in hens indicate that, as in mammals, it may provide an endocrine mechanism that allows

nutritional status to influence reproduction (Ohkubo et al., 2000; Paczoska-Eliasiewicz et al., 2003). When hens were injected with mammalian leptin twice daily during a five-day fast, Paczoska-Eliasiewicz et al. (2003) reported a delay in the cessation of egg laying, less hierarchical follicle regression, and lower rates of fasting-induced follicular apoptosis when compared to non-injected, fasted birds. In the broiler breeder hen, leptin receptor mRNA was detected in the theca and granulosa tissues of the F1 through F4 hierarchical follicles, and its expression increased during *ad libitum* feeding (Cassy et al., 2004).

Ghrelin, a 28-amino acid hormone originally isolated from rat stomach, is the endogenous ligand of the growth hormone (GH) secretogogue receptor (GHS-R) (Kojima et al., 1999) and is a potent stimulator of growth hormone secretion. Ghrelin signals energy insufficiency to central and peripheral tissues to regulate metabolism and reproduction in mammalian species (Elmquist and Zigman, 2003; van der Lely et al., 2004). Intracerebroventricular or peripheral injection of ghrelin decreases the pulse frequency of LH release from the pituitary and, thus, plasma concentrations of LH in female and male rats, female monkeys, ewes, and human males, regardless of gonadal intactness (Furuta et al., 2001; Vulliemoz et al., 2004; Fernandez-Fernandez et al., 2005; Iqbal et al., 2006; Garcia et al., 2007; Kluge et al., 2007). Additionally, GnRH secretion by cultured hypothalamic fragments from ovariectomized female rats is significantly inhibited by ghrelin (Fernandez-Fernandez et al., 2004, 2005). Given recent findings that ghrelin inhibits mRNA expression of kisspeptin in the hypothalamus, it is likely that ghrelin is acting through the kisspeptin system to negatively regulate GnRH and, thus, downstream reproductive function (Forbes et al., 2009).

In chickens, ghrelin mRNA expression is highest in the proventriculus (Kaiya et al., 2002; Richards et al., 2006; Dimova, 2012). Similar to mammals, plasma ghrelin levels increase

when chicks are fasted and, after refeeding, return to baseline levels (Kaiya et al., 2007). Plasma ghrelin levels also increase in broiler breeder hens that are fasted (Freeman and Davis, 2008). The mRNA for the ghrelin receptor has been detected in the theca and granulosa cells from hierarchical and nonhierarchical follicles, and the mRNA expression of the ghrelin receptor is down-regulated by FSH and LH in cultured granulosa cells (Freeman and Davis, 2008).

Research results indicate that leptin and ghrelin have an impact both centrally and peripherally in regulating reproductive function on the basis of nutrition. In mammals, central regulation of these hormones is mediated to the reproductive axis by the kisspeptin system (Figure 1.1). However, in 2014, Pasquier et al. conclusively determined that the kisspeptin gene and the GPR54 gene had been evolutionarily deleted from the avian genome and that the kisspeptin system is not functional in avian species.

The loss of the kisspeptin hormone system presents a roadblock in characterizing the nutritional regulation of reproduction in the central tissues of avian species and is stimulating the search for other hormone systems that would have replaced the role of kisspeptin. Recent research in mammalian species indicates co-localization of two other neuropeptides, along with kisspeptin, in the hypothalamic arcuate nucleus; one of the neuropeptides is a tachykinin peptide called neurokinin B (NKB), and the other, dynorphin (DYN), is an opioid peptide (Burke et al., 2006; Goodman et al., 2007; Navarro et al., 2009; Lehman et al., 2010; Wakabayashi et al., 2010). NKB and DYN are suggested to be produced by a subset of the neurons in the arcuate nucleus that also express kisspeptin, and for this reason, these cells are termed KNDy neurons (reviewed by Lehman et al., 2010; Joseph et al., 2013). NKB is suggested to be responsible for positive regulation of GnRH production, while DYN is associated with negative regulation of GnRH (Wakabayashi et al., 2010). Because these neurons respond to steroid hormone feedback

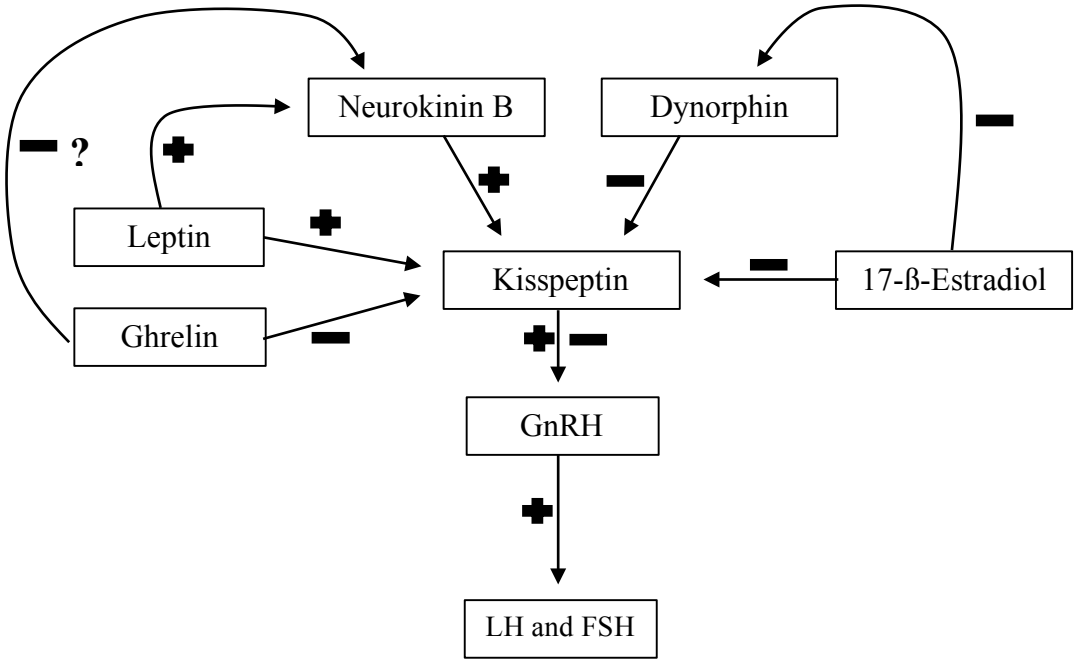

Figure 1.1. The current understanding of central regulation of mammalian reproduction.

and regulate hypothalamic GnRH release through the kisspeptin system in mammals (Figure 1.1), it is possible that, in avian species, NKB and DYN directly regulate GnRH production and secretion without kisspeptin.

In avian species, the RFamide neuropeptide, gonadotropin inhibitory hormone (GnIH), has been well-characterized as a regulator of reproductive function. The production of GnIH is largely localized to the paraventricular nucleus of the hypothalamus, from whence it functions to directly inhibit LH and FSH secretion from the gonadotrope cells of the anterior pituitary gland (Tsutsui et al., 2000). However, recent research indicates that GnIH may also be involved in direct inhibition of GnRH secretion from the hypothalamic preoptic area (Bentley et al., 2003; Ubuka et al., 2008). Thus, in avian species, GnIH has the potential to replace the functions where low levels of kisspeptin negatively impact reproduction in mammalian species.

Summary

Consequential to the intense genetic selection that created the present-day, fast growing broiler is the reproductive hardships of the broiler breeder. Relative to the Leghorn laying hen, follicular hierarchy maintenance, egg production, and fertility are poor in the *ad libitum*-fed broiler breeder hen. Fortunately, the implementation of feed restriction programs has improved the reproductive efforts of these hens. However, broiler breeders inevitably experience periods of fasting associated with these feed restriction programs, and it is well understood that caloric insufficiency is not positively correlated with reproductive function. In mammalian species, peripherally produced, nutrient intake-related hormones, like leptin and ghrelin, impact reproduction by acting through the kisspeptin system to alter expression of centrally-produced GnRH. However, the kisspeptin system is not functional in avian species. Thus, the hormones

NKB, DYN, and/or GnIH might function as a replacement for kisspeptin, as the key regulators of GnRH in avian species.

CHAPTER 2

THE GONADOTROPIN INHIBITORY HORMONE AND THE DYNORPHIN HORMONE SYSTEMS

Gonadotropin Inhibitory Hormone

Gonadotropin inhibitory hormone is a member of the RFamide peptide family of neuropeptides, which also includes the kisspeptin peptides, neuropeptide FF (NPFF), and prolactin-releasing peptide (PrRP). RFamide peptides are identified by the presence of a common Arg-Phe-NH_2 (RFamide) motif at their carboxyl-terminus. This family of neuropeptides first came to light when an FMRFamide (Phe-Met-Arg-Phe-NH_2) peptide with cardioexcitatory properties was identified in the ganglia of the venus clam (*Macrocallista nimbosa*; Price and Greenberg, 1977). Shortly thereafter, the first vertebrate RFamide pentapeptide (LPLRFamide) was isolated from the brain of the chicken, termed chicken LPLRFamide (Dockray et al., 1983). Subsequent studies using immunohistochemical techniques showed a strong RFamide presence within the vertebrate central nervous system. Furthermore, the indication that several of the RFamide neurons extended nearby the pituitary gland led researchers to believe that these peptides may have a functional role at the pituitary gland (Raffa, 1988; Tsutsui et al., 2000; Rastogi et al., 2001).

In 2000, a novel, 12-amino acid RFamide neuropeptide was isolated from the hypothalamus of the Japanese quail (*Coturnix japonica*), and the sequence of the peptide was determined to be SIKPSAYLPLRF-NH_2 (Tsutsui et al., 2000). Findings indicated that, based on

its location within the hypothalamus, the peptide may function as a part of the hypothalamo-hypophysial system. Furthermore, it was reported to inhibit the release of LH, but not prolactin (PRL), from the cultured anterior pituitary gland cells and, thus, was given the name gonadotropin-inhibitory hormone (GnIH; Tsutsui et al., 2000). Prior to the identification of GnIH, it was widely accepted that GnRH positively stimulated gonadotropin production and release from the anterior pituitary gland and that the removal of this positive stimulant (GnRH) resulted in negative regulation of gonadotropin production and release, as there had been no sign of a hypothalamic molecule whose role was inhibitory in nature. Therefore, the discovery that GnIH held potential to negatively regulate gonadotropin release from the anterior pituitary provided a significant advancement for the field of reproductive endocrinology.

Localization of GnIH

Following the discovery of GnIH, many studies attempted to specifically define its localization within the central nervous system. In the Japanese quail and the chicken, the peptide was most highly concentrated in the diencephalon, which comprises the thalamus, hypothalamus, subthalamus, and epithalamus, when compared to all other brain regions (Tsutsui et al., 2000; Satake et al., 2001; Ikemoto and Park, 2005; Bentley et al., 2008). Within the diencephalon of the Japanese quail, GnIH neuronal perikarya were most abundantly detected within the paraventricular nucleus (PVN) of the hypothalamus and were determined to be parvocellular neurons, distinct from vasotocin and mesotocin-producing magnocellular neurons (Tsutsui et al., 2000; Ubuka et al., 2003; Ukena et al., 2003). The neuronal fibers extending from these PVN-localized perikarya are distributed throughout the diencephalon and mesencephalon, which suggests multiple regulatory roles for GnIH within the central nervous system. However, many of these neuronal fibers extend toward and terminate in the median eminence (ME), where they

release GnIH into the hypothalamo-hypophyseal portal system for transport to the anterior pituitary gland (Ubuka et al., 2003; Ukena et al., 2003). Similar studies investigating localization of GnIH perikarya and neuronal fibers indicate the same results in the house sparrow (*Passer domesticus*), song sparrow (*Melospiza melodia*), Gambel's white crowned sparrow (*Zonotrichia leucophrys gambelii*), zebra finch (*Taeniopygia guttata*), and European starling (*Sturnus vulgaris*) as were seen in the Japanese quail (Bentley et al., 2003; Osugi et al., 2004; Ubuka et al., 2008; Tobari et al., 2010; Ubuka et al., 2012). Detection of GnIH mRNA has only been reported in the pituitary of the Japanese quail (Bentley et al., 2008). Overall, the localization of GnIH-producing neurons in the central nervous tissues is fairly conserved among avian species (reviewed by Tsutsui et al., 2005, 2006, 2007a, 2007b, 2013; Tsutsui, 2009; Ubuka et al., 2016).

In vertebrates, many of the neuropeptides found to be expressed within the brain are also expressed, along with their respective receptors, at the gonadal level. This indicates that, beyond their central role at the brain, these neuropeptides may function in an autocrine or paracrine manner within the gonads. The expression of GnIH and its receptor in the avian gonad was first reported in 2008 by Bentley et al., studying both Japanese quail and European starlings. In Japanese quail, GnIH precursor mRNA was found in the ovary, testis, epididymis, and vas deferens (Bentley et al., 2008). Furthermore, GnIH immunoreactivity was observed in the Leydig cells, germ cells, and seminiferous tubules of the Japanese quail testes as well as in the pseudostratified columnar epithelial cells of the epididymis (Bentley et al., 2008). In European starlings, the GnIH precursor mRNA was detected in the testis, ovary, and oviduct, while GnIH immunoreactivity was detected in both the theca and granulosa tissues of preovulatory follicles (Bentley et al., 2008). In house sparrows, expression of GnIH precursor mRNA was detected in

the interstitial tissue of the testes (McGuire and Bentley, 2010). Maddineni et al. (2008b) reported that, in contrast to the European starling and Japanese quail, GnIH precursor mRNA could not be detected in the ovarian tissue of the Leghorn hen.

Structure of the GnIH transcript and precursor polypeptide

Shortly after its discovery in 2000, a cDNA encoding the GnIH precursor polypeptide was identified in the brain of the Japanese quail using 3' and 5' rapid amplification of cDNA ends (3'/5' RACE), and the translated precursor polypeptide was found to be 173 amino acid residues in length (Satake et al., 2001). Furthermore, the sequence was found to encode for GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2), each of which contained the C-terminus sequence –LPXRFamide that distinguishes the RFamide family of peptides (Satake et al., 2001). Following the characteristic C-terminus sequence of each peptide, a glycine amidation signal was recognized, and the three peptides were flanked on each end by a single basic amino acid, representative of an endoproteolytic site (Satake et al., 2001). Further analysis of the three peptides using mass spectrometry indicated that both GnIH and GnIH-RP-2 were translated as mature peptides in the brain of the Japanese quail (Satake et al., 2001). Similarly, in Gambel's white-crowned sparrow, a cloned cDNA sequence encoding the GnIH precursor polypeptide was isolated, and the peptide sequence was found to be 173 amino acid residues in length and to contain one GnIH peptide and two GnIH-related peptide sequences (Osugi et al., 2004). The flanking amidation signals and endoproteolytic sites, as indicated by specific amino acids, were identical to that of the Japanese quail, and while the Japanese quail and white-crowned sparrow precursor peptide shared only 66% sequence homology, the C-terminus sequences of GnIH and its related peptides were identical in these avian species (Osugi et al., 2004).

In 2005, Ikemoto and Park isolated the cDNA encoding for the GnIH precursor polypeptide sequence from the brain of the White Leghorn chicken and sequenced it using 5'- and 3'-RACE. The cDNA sequence was 971-base pairs in length with a 5' untranslated region (UTR) containing 38 nucleotides and a 3'-UTR containing 411 nucleotides that flanked the 519-nucleotide long open reading frame, which encoded for the 173-amino acid chicken GnIH precursor polypeptide (Ikemoto and Park, 2005). As seen in the quail and sparrow, the chicken precursor polypeptide contained one GnIH peptide (cGnIH) and two GnIH-related peptides (cGnIH-RP-1 and cGnIH-RP-2) as well as the flanking amidation signals and endoproteolytic sites (Ikemoto and Park, 2005). Eventually, the presence of the mature chicken GnIH peptide was confirmed in brain tissue using mass spectrometry (McConn et al., 2014). Similar techniques to those used in the quail, white-crowned sparrow, and chicken were used to sequence the GnIH precursor polypeptide and, ultimately, to isolate the mature GnIH peptide in European starlings and zebra finches (Ubuka et al., 2008; Tobari et al., 2010).

Regulation of GnIH expression and release

Beyond sequencing and characterizing the GnIH precursor polypeptide, there was a desire to understand how its biosynthesis was regulated in avian species. Armed with the knowledge that GnIH has an inhibitory impact on gonadotropin expression in the anterior pituitary gland, researchers considered its potential connection to melatonin, given the implication of its involvement in inhibiting gonadotropin production and gonadal activity in avian species during seasonal changes (Ohta et al., 1989; Bentley et al., 1999; Bentley and Ball, 2000; Guyomarc'h et al., 2001; Rozenboim et al., 2002). The hormone, melatonin, is produced most abundantly in the pineal gland and displays a cyclic expression pattern, dependent upon the activity of the enzyme responsible for its production, N-acetyltransferase (Binkley, 1981;

Underwood et al., 1984). This enzyme functions at maximal capacity in the pineal gland when light energy is not being processed by the eyes and transmitted to the pineal gland. Thus, blood melatonin levels are higher during periods of darkness than in light (Binkley, 1981). Because the vast majority of birds are long day breeders, they reproduce when there is an abundance of light, and melatonin levels are low.

In house sparrows and song sparrows, Bentley et al. (2003) used immunohistochemical analysis to visualize GnIH neurons in the hypothalamus and found that these neurons were largest in sparrows at the end of the breeding season, a time when days would begin to shorten, darkness would be more abundant, and melatonin levels would rise.

In 2005, Ubuka et al. assessed the impact of melatonin on GnIH expression in the hypothalamus of the Japanese quail. Using pinealectomy (Px) and orbital enucleation (Ex) (Px plus Ex) procedures to remove the pineal gland and the eye, respectively, resulted in the anticipated decrease in melatonin levels, along with a decrease in hypothalamic expression of the GnIH precursor polypeptide mRNA and the GnIH peptide (Ubuka et al., 2005). Supplementing the Px plus Ex quail with melatonin resulted in an increase in the expression of the GnIH precursor polypeptide mRNA as well as the GnIH peptide (Ubuka et al., 2005). Furthermore, assessing the GnIH neurons of the PVN revealed that they expressed melatonin receptor, Mel_{1c}, mRNA and that melatonin exhibited binding activity within the PVN (Ubuka et al., 2005). These findings, along with the observation that GnIH expression fluctuated relative to melatonin production, indicated that melatonin stimulated GnIH production by binding directly to its receptors on the GnIH neurons (Ubuka et al., 2005). In another study using Px birds to assess the connection between melatonin and GnIH, immunohistochemical analysis detected an increase in the number of GnIH neurons in Px Indian weaver birds (*Ploceus philippinus*) given

exogenous melatonin for 16 hours compared to those given melatonin for 8 hours (Surbhi et al., 2014). In an *in vitro* experiment conducted by Chowdhury et al. (2010), hypothalamic PVN explants from the Japanese quail had an increase in GnIH release after melatonin exposure.

Given the presence of GnIH in the gonads of some avian species, McGuire et al. (2011) investigated the potential role of melatonin in the testes of European starlings. Testicular tissue expressed mRNA transcripts for GnIH, the GnIH receptor, and two of the melatonin receptors (Mel_{1b} and Mel_{1c}). Furthermore, prior to the onset of the breeding season, melatonin facilitated an increase in starling testes GnIH mRNA expression; however, in the breeding season, the expression of GnIH, GnIH receptor, Mel_{1b}, and Mel_{1c} were low.

Stress has a negative impact on reproductive function in avian species. Calisi et al. (2008) investigated the hypothalamic expression of GnIH in adult male and female house sparrows experiencing stress initiated by capture-handling. In comparing seasonally sampled hypothalamic tissues, GnIH neurons in adult sparrows were more numerous in the fall than in the spring, and within the spring-sampled sparrows, those exposed to capture-handling stress had more GnIH neurons than the control non-stressed sparrows (Calisi et al., 2008). Conversely, adult male and female zebra finches exposed to capture handling stress for one hour had fewer GnIH perikarya when compared to control finches in a study utilizing immunohistochemical analysis (Ernst et al., 2016). However, despite detecting fewer GnIH cell bodies, the stressed zebra finch females displayed lower mRNA expression of the FSH-ß subunit in the pituitary gland, which is characteristic of the action of GnIH and caused authors to imply that the immunohistochemical results observed in the hypothalamus could have been false based on the neurons releasing their GnIH (Ernst et al., 2016).

Despite the contradictory results, other findings do indicate the likelihood that stress acts through the GnIH system to inhibit reproduction in avian species. The avian glucocorticoid hormone, corticosterone (CORT), is responsible for facilitating the stress response as a part of the hypothalamic-pituitary-adrenal (HPA) axis. The action of CORT is mediated in part by its binding to the glucocorticoid (GC) receptor. In Japanese quail, Son et al. (2014) found GnIH neurons in the hypothalamic PVN to express GC receptor mRNA and determined that, when treated with CORT for 24 hours, these diencephalic tissues displayed an increase in GnIH mRNA expression. Furthermore, in an experiment utilizing *in ovo* CORT injections, researchers found high doses of CORT to elicited a decrease in GnRH-I expression and an increase in GnIH expression in the hypothalamic tissues of post-hatch chickens (Ahmed et al., 2014). At sexual maturity, the chickens that had been exposed to high CORT *in ovo* were found to exhibit reduced ovary and oviduct weight along with poor egg production and quality (Ahmed et al., 2014).

Because the GC receptor is also expressed in the gonadal tissues, CORT may regulate reproductive function at the gonadal level, apart from its influence on reproduction at the brain. When exposed to physiological doses of CORT or metabolic stress (induced by glucose utilization inhibitor, 2-deoxy-D-glucose, and the fatty acid oxidation inhibitor, ethyl 2-mercaptoacetate), McGuire et al. (2013) found that ovarian and testicular tissues collected from photostimulated European starlings and cultured with LH and FSH displayed decreased estrogen and testosterone secretion. Furthermore, metabolic stress stimulated GnIH expression in the ovarian and testicular tissue, and in testicular tissue, GnIH expression was stimulated by CORT exposure (McGuire et al., 2013). Comparably, following one hour of restrain-induced stress in adult male and female zebra finches, testicular tissue, but not ovarian tissue, displayed an increase in GnIH mRNA expression (Ernst et al., 2016). Following a fasting stress-induced

increase in CORT levels, the testicular tissue of the male zebra finch has increased GnIH expression and decreased testosterone production (Lynn et al., 2015).

Additionally, the link between metabolic stress and GnIH stimulation has been solidified by two other research reports. Following exposure to high ambient temperatures, chicks exhibited a decrease in feed intake and an increase in GnIH mRNA expression in the hypothalamus (Chowdhury et al., 2012). In adult Pekin drakes, Fraley et al. (2013) reported that, after a 48 hour fast, GnIH neurons were activated and LH levels were decreased.

Gonadotropin Inhibitory Hormone Receptor

At its target tissues, the GnIH neuropeptide facilitates its functions by binding to and activating the GnIH receptor (GnIH-R). The GnIH-R is a member of the large family of G-protein coupled receptors (GPCR). A GPCR is a large protein that weaves through and embeds in the cellular membrane creating seven transmembrane domains, with the N-terminal, extracellular domain and the C-terminal, cytoplasmic domain. The transmembrane domains of the GPCR, along with the extracellular domain, create a binding pocket for ligand binding, and the C-terminal, cytoplasmic region interacts with the G-protein. Upon ligand binding, the interaction between the amino acids of the ligand and those in the binding pocket of the GPCR causes the receptor structure to undergo a conformational change that activates the intracellular G-protein, which is responsible for initiating the intracellular downstream effects of the ligand (reviewed by Ostrowski et al., 1992).

First identified in rats and humans, Hinuma et al. (2000) determined that the receptor for the mammalian RFRP, orthologous to avian GnIH, was identical to a GPCR that had previously been identified and named GPR147. Additionally, GPR147 had been identified as one of two receptors, the other being named GPR74, for neuropeptide FF (NPFF), an RFamide neuropeptide

involved in pain modulation (Bonini et al., 2000). Despite both being RFamide neuropeptides, NPFF and RFRP (GnIH) differ structurally in the portion of their C-terminal motif just upstream of the terminal RFamide, as NPFF terminates in PQRFamide and RFRP terminates in LPXRFamide (reviewed by Roumy and Zajac, 1998). Binding affinity studies determined that NPFF was the primary agonist for GPR74, while RFRP had highest affinity for GPR147 (Bonini et al., 2000; Liu et al., 2001).

Identification and Characterization of Avian GnIH-R

Among avian species, the GnIH-R was first identified in the Japanese quail utilizing the 5'/3' RACE technique along with the previously obtained mammalian GPR147 sequence (Yin et al., 2005). Northern blot analysis detected expression of the quail GnIH-R mRNA in the pituitary, diencephalon, cerebrum, mesencephalon, and spinal cord (Yin et al., 2005). Furthermore, transfection of cultured cells with the identified quail GnIH-R sequence followed by exposure of the cells to GnIH and its related peptides showed specific binding to the GnIH-R in a concentration dependent manner, which indicated that, as in mammals, GPR147 is the receptor for GnIH and the GnIH-RPs (Yin et al., 2005). Yin et al. (2005) also showed the importance of the C-terminal LPXRFamide motif shared by GnIH and its related peptides in binding to the receptor when it was determined that non-amidated GnIH was unable to bind to the receptor.

Ikemoto and Park (2005) utilized 5'-and 3'-RACE to sequence the cDNA of both GPR147 and GPR74 in the brain of the Leghorn chicken. The cDNA for chicken GPR147 was 1442 bp in length, with a 32 nucleotide-long 5'-UTR and a 210 nucleotide-long 3'-UTR flanking a 1197 nucleotide-long open reading frame, which encodes for a 399-amino acid GPR147 (Ikemoto and Park, 2005). In the Leghorn chicken, brain expression of GPR147 mRNA was

highest in the pituitary gland followed by the diencephalon and other brain regions (Ikemoto and Park, 2005). After assessing the interaction of avian GnIH and GnIH-RPs with both GPR147 and GPR74, it was determined that, while GnIH and its related peptides can bind to both receptors, they induce a significantly greater response upon binding to GPR147 (Ikemoto and Park, 2005). Additionally, the observed binding of GnIH, GnIH-RP, and chicken LPLRFamide with both GPR147 and GPR74 further indicates an importance for the shared C-terminal RFamide motif in binding to the receptor (Ikemoto and Park, 2005). Subsequently, the mRNA expression of GPR147 was confirmed in the diencephalon and pituitary in Leghorn hens (Maddineni et al., 2008a).

With the subsequent discovery of GnIH expression in the gonadal tissues of some avian species, researchers investigated gonadal expression of the avian GnIH-R. In the Japanese quail, European starling, and house sparrow, the expression of GnIH-R mRNA was detected in testicular and ovarian tissues (Bentley et al., 2008; McGuire and Bentley, 2010). In the ovarian tissue of the Leghorn hen, where the mRNA for the GnIH precursor was not detected, the GnIH-R mRNA was detected, and additionally, GnIH-R mRNA was found in the testicular tissue of the male Cobb broiler chicken (Maddineni et al., 2008b).

Cell signaling of GnIH

Because the GnIH-R is a GPCR, binding of GnIH to the extracellular domain elicits the activation of the G-protein associated with the intracellular domain of the receptor, and this G-protein facilitates downstream effects within the cell. According to Hinuma et al. (2000), a decrease in production of cyclic adenosine monophosphate (cAMP) was observed in cultured Chinese hamster ovarian cells transfected with GPR147 upon exposure to GnIH. Similarly, in cultured rat pituitary cells (GH_3 cells) transfected with chicken GnIH-R, it was determined that

exposure to GnIH decreases cAMP production and, more specifically, that this reduction in cAMP production is mediated by the inhibitory action of the $G_{\alpha i}$ protein on adenylate cyclase (AC) activity (Shimizu and Bedecarrats, 2010). In contrast, the binding of GnRH to its receptor stimulates AC and increases cAMP production, which is necessary for downstream intracellular signaling; however, the reduction in cAMP observed as a consequence of GnIH exposure is sufficient to significantly hinder GnRH intracellular signaling in target cells, highlighting at the intracellular signaling level the negative impact that GnIH has on a cell's response to GnRH (Shimizu and Bedecarrats, 2006, 2010). Subsequently, the intracellular signaling pathway activated by mouse GnIH was investigated in a mouse gonadotrope cell line that naturally expresses mouse GnIH-R (Son et al., 2012). Similar to the findings reported by Shimizu and Bedecarrats (2010), it was observed that the presence of mouse GnIH inhibited the cAMP signaling initiated by GnRH by inhibiting AC and reducing cAMP production (Son et al., 2012). Additionally, reduction of cAMP was found to prevent phosphorylation of ERK, a protein required for transcription of the genes encoding the gonadotropin subunits, resulting in decreased LH and FSH production (Son et al., 2012).

Physiological Impacts of Gonadotropin Inhibitory Hormone

Impact of GnIH at the brain

Given the branching of GnIH neuronal fibers from the hypothalamic PVN to various portions of the brain and the expression of the GnIH-R throughout the diencephalon, GnIH could have regulatory roles within the brain of avian species. Using double-label immunocytochemistry, Bentley et al. (2003) investigated the positioning of GnIH neurons relative to GnRH-I and GnRH-II neurons within the diencephalon of song sparrows and house sparrows. GnRH-I perikarya are housed within the hypothalamic preoptic area, from which

many of the neuronal fibers extend to the median eminence. Within the median eminence, GnRH-I enters portal circulation, travels to the anterior pituitary gonadotrope cells, and stimulates LH and FSH release into systemic circulation (King and Millar, 1982; Miyamoto et al., 1982; Sharp et al., 1990; Ubuka and Bentley, 2009, 2010; Ubuka et al., 2009). GnRH-II is an initiator of reproductive behavior in avian and mammalian species, and the neurons responsible for producing this version of GnRH are housed in the midbrain (Maney et al., 1997; Temple et al., 2003; Barnett et al., 2006). GnIH neuronal fibers were observed to be positioned nearby GnRH-I perikarya within the preoptic area, GnRH-I neuronal fibers in the median eminence, and GnRH-II perikarya within the midbrain (Bentley et al., 2003). In a subsequent study using double-label immunocytochemistry, Ubuka et al. (2008) observed a similar proximity of GnIH and GnRH neurons in European starlings to what had been observed in song and house sparrows (Bentley et al., 2003). Additionally, Ubuka et al. (2008) observed the terminal portions of GnIH neuronal fibers to be in contact with GnRH-I and GnRH-II neurons and detected the presence of GnIH-R mRNA within the hypothalamic preoptic area-localized GnRH-I perikarya as well as the midbrain-localized GnRH-II perikarya. These findings suggest that GnIH could have an inhibitory impact on GnRH-I and GnRH-II secretion.

Bentley et al. (2006) investigated the impact of GnIH at the brain by inserting cannulas into the third ventricle of the female white-crowned sparrow brain and infusing physiologically-relevant doses of GnIH. Plasma LH levels in the females were reduced following the central injection of GnIH (Bentley et al., 2006). Furthermore, female sparrows exposed to GnIH were observed to solicit copulation less frequently when compared to saline-exposed females (Bentley et al., 2006). Six years later, the central role of GnIH in white-crowned sparrows was revisited by Ubuka et al. (2012) using RNA interference (RNAi). This was accomplished by injecting

small interfering RNA (siRNA) molecules into the cannulated third ventricle of the white-crowned sparrow brain that specifically blocked the expression of the GnIH precursor mRNA (Ubuka et al., 2012). Following injection of the siRNAs, there was an expected reduction in GnIH precursor mRNA along with increased male and female sexual arousal, as indicated by heightened activity level and song production, responsiveness to songs, and aggressiveness in response to territorial challenges (Ubuka et al., 2012).

Impact of GnIH at the anterior pituitary

The identification of numerous GnIH neurons extending to and terminating within the median eminence of many avian species suggested a potential role for GnIH at the avian pituitary gland (Bentley et al., 2003; Ubuka et al., 2003; Ukena et al., 2003; Osugi et al., 2004; Ubuka et al., 2008; Tobari et al., 2010; Ubuka et al., 2012). In cultured Japanese quail anterior pituitary cells, administration of GnIH resulted in a significant decrease in LH secretion and a non-significant but marked decrease in FSH secretion (Tsutsui et al., 2000). Subsequently, pituitary gland tissue was collected from adult ISA Brown cockerels for cell culture, and when exposed to GnIH, the pituitary cells showed a significant reduction in mRNA expression of the α-subunit shared by LH and FSH as well as the FSH ß-subunit (Ciccone et al., 2004). Therefore, the presence of GnIH significantly reduced LH and FSH production and release from the cultured cockerel pituitary cells (Ciccone et al., 2004). In an *in vivo* experiment using song sparrows, intravenous injection of a saline cocktail including both GnRH-I and GnIH resulted in a significant decrease in plasma LH concentration when compared to sparrows injected with a saline cocktail including only GnRH-I (Osugi et al., 2004). Furthermore, intravenous injection of saline containing GnIH into castrated, photostimulated white crowned sparrows resulted in a significant decrease in the plasma LH concentration, while injection of saline alone had no effect

on plasma LH levels (Osugi et al., 2004). In the mature male Japanese quail, administration of GnIH via an osmotic pump for a two-week period resulted in a significant, dose-dependent decrease in mRNA expression of the LH ß-subunit and a decrease, though not significant, in mRNA expression of the FSH ß-subunit (Ubuka et al., 2006). The mRNA expression of the α-subunit shared by LH and FSH was significantly reduced following GnIH injection as well (Ubuka et al., 2006). Additionally, a dose-dependent decrease in plasma LH concentration was noted following GnIH administration (Ubuka et al., 2006). In a subsequent experiment, the addition of GnIH to cultured pituitary tissues collected from sexually immature or sexually mature Leghorn hens significantly decreased LH secretion in the sexually immature Leghorn hens, but surprisingly, the GnIH addition had no impact on LH secretion in pituitary cells obtained from the sexually mature hens (Maddineni et al., 2008a). Additionally, GnIH exposure did not alter FSH secretion in cultured pituitary cells from sexually immature or mature Leghorn hens (Maddineni et al., 2008a). Despite the results from the Maddineni et al. (2008a) research, the general consensus of researchers within the avian GnIH field is that GnIH reduces the synthesis and secretion of LH and FSH in the anterior pituitary.

Impact of GnIH at the gonads

Based on mRNA and/or protein expression data, GnIH, GnIH-R, or both have been detected in the testes and ovary of European starlings, Japanese quail (Bentley et al., 2008), house sparrows (McGuire and Bentley, 2010), and chickens (Maddineni et al., 2008b). Despite the apparent ability of centrally or locally produced (some species) GnIH to have impacts on testicular and ovarian function, very little research has investigated the potential roles of GnIH at the gonad. Cultured, gonadotropin-stimulated sparrow testis cells exposed to physiological doses of GnIH have a reduction in testosterone production (McGuire and Bentley, 2010).

Additionally, granulosa cells isolated from prehierarchical follicles from the laying hen cultured in the presence of exogenous GnIH have decreased viability compared to control cells or granulosa cells cultured with both GnIH and FSH (Maddineni et al., 2008b). Based on these two reports, it would appear that GnIH has a negative effect on gonad function.

Impact of GnIH on feeding behavior

Beyond the behaviors associated with reproduction that GnIH has been reported to regulate, research additionally suggests that GnIH may be involved in the regulation of feeding behavior in some animals. Tachibana et al. (2005) designated GnIH an orexigenic peptide after finding that it stimulates feed intake in chicks. Tachibana et al. (2008) also determined that the orexigenic effects displayed by GnIH are likely being carried out by activation of the opioid mu receptor. In adult Pekin drakes, Fraley et al. (2013) observed that intracerebroventricular GnIH injections stimulated feed intake and reduced plasma LH levels, indicating a simultaneous role for GnIH in regulating reproductive function and feeding behavior. McConn et al. (2014) reported that central brain injections of GnIH into chicks stimulated feeding behavior, increased hypothalamic mRNA expression of Neuropeptide Y (NPY), an endogenous appetite stimulant, and decreased proopiomelanocortin (POMC) mRNA expression, an endogenous appetite suppressant. Therefore, the results suggest that GnIH may indirectly initiate feeding via modulation of appetite regulators, like NPY, through activation of the opioid mu receptor.

Dynorphin

From the Greek word *dynamis*, meaning power, and the suffix –orphin, suggesting opioid, comes the name dynorphin, which was given to a potent endogenous opioid neuropeptide upon its discovery in 1979 (Schwarzer, 2009). The opioid dynorphin neuropeptides are

expressed throughout the central nervous system and are involved in regulation of pain, stress response, learning, memory, and emotional control (Schwarzer, 2009). Furthermore, the role of dynorphin and its receptor in addiction, epilepsy, chronic pain, depression, and schizophrenia have been extensively investigated (reviewed by Wagner and Chavkin, 1995; Lai et al., 2001; Laughlin et al., 2001; Schwarzer, 2009; Chavkin, 2013). Recently, dynorphin expression within the brain has also been implicated in regulating GnRH secretion in response to gonadal steroid hormone feedback (Goodman et al., 2004; Foradori et al., 2005).

Dynorphin was first isolated from porcine pituitary extracts and determined to be 13-amino acids in length (Goldstein et al., 1979). Subsequently, it was determined that this sequence was only a portion of an active, 17-amino acid dynorphin neuropeptide, later given the name dynorphin A (Goldstein et al., 1981). Dynorphin A, along with several other bioactive dynorphin neuropeptides, are translated from the preprodynorphin (Pdyn) mRNA transcript, which is encoded by the prodynorphin gene (Kakidani et al., 1982). The dynorphin neuropeptides are derived from the prodynorphin precursor polypeptide and are members of the endogenous opioid peptide (EOP) family, which also includes the neuropeptides derived from the proopiomelanocortin and proenkephalin precursor polypeptides (Weber et al., 1982; Whitnall et al., 1983; Zamir et al., 1984).

Structure of the Pdyn transcript and precursor polypeptide

Neurons throughout the central nervous system, as well as pituitary and gonadal cells, express the preprodynorphin mRNA transcript, from which the prodynorphin precursor polypeptide is derived. In mammals, there are two well-characterized splice variants of the preprodynorphin mRNA transcript—FL1 and FL2. The FL1 transcript variant is more predominant and is spread throughout the brain; whereas, the FL2 variant is localized to the

hypothalamus (Nikoshkov et al., 2005). The splice variants differ only in the 5' non-coding region and in the initiation site for transcription (Douglass et al., 1994; Telkov et al., 1998).

After translation from the preprodynorphin mRNA transcript, the prodynorphin precursor polypeptide is inactive and must be enzymatically cleaved into biologically active neuropeptides. Prohormone convertase (PC) enzymes, PC1 and PC2, along with carboxypeptidase E enzyme, are responsible for liberating the biologically active dynorphin neuropeptides from the precursor polypeptide (Seizinger et al., 1984; Day and Akil, 1989; Day et al., 1998). PC1 breaks the initial polypeptide into two large segments, which are acted on by PC1 and carboxypeptidase E to generate big dynorphin (Big-DYN), dynorphin A (DYN A) 1-17, dynorphin A (DYN A) 1-8, dynorphin B (DYN B) 1-13, α-neoendorphin, ß-neoendorphin, and leumorphin, also designated dynorphin B (DYN B) 1-29 (Seizinger et al., 1984; Day and Akil, 1989; Day et al., 1998). Big-DYN is a 32-amino acid peptide that comprises DYN A 1-17 and DYN B 1-13 linked together by the basic amino acid pair, Lysine-Arginine, and from the DYN A 1-17 peptide, the DYN A 1-8 neuropeptide is derived (Minamino et al., 1980; Fischli et al., 1982a, 1982b; reviewed by Schwarzer, 2009; Chavkin, 2013). Additionally, leumorphin, or DYN B 1-29, is comprised of DYN B 1-13 along with a 16-amino acid C-terminal C-peptide (Kakidani et al., 1982; Watson et al., 1983; reviewed by Schwarzer, 2009; Chavkin, 2013).

The dynorphin neuropeptides share an identical N-terminal amino acid sequence, Tyr-Gly-Gly-Phe-Leu, which is referred to as Leu-enkephalin, and the five-amino acid Leu-enkephalin sequence is important for binding of the neuropeptides to the opioid receptor (Chavkin and Goldstein, 1981). Given that the dynorphin neuropeptides share this sequence, it is reasonable that all are considered bioactive. However, despite the shared Leu-enkephalin binding sequence, a hierarchy of activity amongst the dynorphin neuropeptides exists. Based on

an *in vitro* experiment in which the ability of each neuropeptide to activate the opioid receptor was investigated, James et al. (1984) proposed a range of potency or activity for the dynorphin neuropeptides. According to this hierarchy, DYN A 1-17 is ten to twenty times more potent than Big-DYN, which is equal in potency to DYN B 1-13, DYN B 1-29 (leumorphin), and α-neoendorphin; the later four dynorphin neuropeptides are, in turn, ten to twenty times more potent than DYN A 1-8 and ß-neoendorphin, which are equal in potency (James et al., 1984). Subsequent studies assessing the affinity of each dynorphin neuropeptide for its opioid receptor indicated that DYN A 1-17 had a higher receptor affinity than that of DYN A 1-8 and DYN B 1-13 (Yoshino et al., 1990; Li et al., 1993; Meng et al., 1993; Simonin et al., 1995; Zhu et al., 1995, 1997; Toll et al., 1998). The presence of basic amino acid residues, Arginine and Lysine, at the C-terminus of the neuropeptides is thought to indicate their potency or selectivity for their opioid receptor. Thus, the presence of Arginine and Lysine at the C-terminus of DYN A 1-17, DYN B 1-13, and α-neoendorphin makes these neuropeptides more potent than DYN A 1-8 and ß-neoendorphin, which lack the C-terminal basic amino acids that are important for opioid receptor selection (Chavkin and Goldstein, 1981; James et al., 1984). Thus, overall, DYN A 1-17 is designated the most biologically active or potent dynorphin neuropeptide.

Localization of Dynorphin

Given that dynorphin was first isolated from pituitary tissue, the localization of this opioid neuropeptide to the pituitary gland is well characterized (Cox et al., 1975; Goldstein et al., 1979; Lowney et al., 1979). In the rat, dynorphin neuropeptides were observed to be produced within and secreted from the anterior pituitary gland (Spampinato and Goldstein, 1983; Khachaturian et al., 1985). Furthermore, within the anterior pituitary gland,

immunocytochemical analysis localizes the dynorphin neuropeptides to a group of gonadotrope cells (Khachaturian et al., 1986).

Beyond the pituitary gland, the neurons that produce the bioactive dynorphin neuropeptides are found in a variety of locations within the central nervous system. Early studies using immunocytochemical and radioimmunoassay techniques in rats and primates found dynorphin perikarya and neuronal fibers localized to the hippocampus, amygdala, cerebral cortex, striatum, spinal cord, and to various nuclei within the hypothalamus (Khachaturian et al., 1982; Khachaturian et al., 1985; Abe et al., 1988). Subsequently, Foradori et al. (2005) assessed the specific location of Pdyn mRNA expression as well as the DYN A 1-17 peptide within the sheep hypothalamus. Expression of both the mRNA and protein were found within the magnocellular neurons of the PVN and supraoptic nucleus and within the parvocellular neurons of the preoptic area, the bed nucleus of the stria terminalis, the lateral hypothalamus, the dorsomedial nucleus of the hypothalamus, and the arcuate nucleus (Foradori et al., 2005). In 2007, dynorphin mRNA and protein expression within the arcuate nucleus of the hypothalamus was determined to be co-localized with kisspeptin and neurokinin B expression in a subset of neurons later termed KNDy neurons (Goodman et al., 2007; Lehman et al., 2010). Given the role of kisspeptin in regulating GnRH secretion in mammals, the presence of dynorphin in these neurons implicated the opioid neuropeptide in the central regulation of reproductive function.

Often, centrally-produced neuropeptides that are associated with reproductive function can also be found within the gonadal tissues, along with their respective receptor. In female rats, Lolait et al. (1986) detected Pdyn mRNA in follicular and luteal cells. Douglass et al. (1987) reported detection of Pdyn mRNA in rat testicular, ovarian, and uterine tissue. Furthermore, immunohistochemical analysis showed localization of the dynorphin peptides within the

testicular tissues of the rat, rabbit, and guinea pig and the uterine and ovarian tissues of the rat (Douglass et al., 1987). More specifically, immunohistochemistry of rat testicular tissues indicated that the prodynorphin neuropeptides are localized to the leydig cells (Douglass et al., 1987). While somewhat contradictory to the results of Douglass et al. (1987), Collard et al. (1990) reported that Pdyn mRNA was primarily expressed in the sertoli cells of the rat testes. In the female rat, *in situ* hybridization detected Pdyn mRNA in the granulosa cells and the luteal cells of the ovary (Kaynard et al., 1992b). In female pigs, Slomczynska et al. (1997) reported detection of one of the dynorphin neuropeptides produced from the prodynorphin precursor protein in the ovarian follicular fluid. Subsequently, preprodynorphin mRNA was detected in cultured porcine theca cells only when exposed to LH and granulosa cells only when exposed to FSH (Staszkiewicz et al., 2007).

Regulation of Dynorphin expression and release

Matthews et al. (1993) observed that psychological stress increased Pdyn mRNA expression in the hypothalamic PVN, and dehydration stress increased Pdyn mRNA expression in both the PVN and supraoptic nucleus of the hypothalamus. Additionally, because dynorphin neuropeptides were observed to regulate food intake in sheep (Baile et al., 1987), the impact of feed restriction on Pdyn mRNA expression has been investigated. Berman et al. (1994) reported feed-restricted rats had an increase in DYN A 1-17 levels in dorsal medial, ventral medial, and medial preoptic regions of the hypothalamus. Subsequently, in the brain of the male rat, five regions were assayed for alterations in Pdyn mRNA expression following feed restriction (Berman et al., 1997). A significant increase in Pdyn mRNA was observed in the central amygdala and lateral hypothalamus of feed-restricted rats (Berman et al., 1997). Iqbal et al. (2003) reported that feed restriction did not significantly alter Pdyn mRNA expression in any

assayed region of the sheep hypothalamus. Therefore, feed restriction appears to regulate Pdyn mRNA expression and dynorphin neuropeptide production in the hypothalamic tissues of some but possibly not all mammalian species.

The impact of sex steroid hormones on mRNA expression of Pdyn in various tissues has also been investigated. In female rats, Kaynard et al. (1992a) investigated anterior pituitary expression of Pdyn mRNA following subcutaneous administration of estradiol, estradiol plus progesterone, and dihydrotestosterone (DHT). They reported that DHT was the only hormone to significantly alter expression of Pdyn mRNA in the anterior pituitary gland, and it was observed to reduce Pdyn mRNA expression (Kaynard et al., 1992a). Scott et al. (2008) utilized *in situ* hybridization to assay alterations in Pdyn mRNA expression in the hypothalamus based on testosterone exposure and season. Regardless of season, testosterone was observed to increase hypothalamic Pdyn mRNA expression (Scott et al., 2008). When compared to rams in the non-breeding season, Pdyn mRNA expression was higher in the hypothalamic PVN, supraoptic nucleus, and caudal preoptic area of rams in the breeding season, and in the breeding season, Pdyn mRNA expression was increased by the presence of testosterone in the hypothalamic supraoptic nucleus and bed nucleus of the stria terminalis (Scott et al., 2008). Foradori et al. (2005) observed a significant increase in DYN A 1-17 neuropeptide levels in the cerebrospinal fluid of ovariectomized (OVX) ewes administered progesterone subcutaneously when compared to OVX ewes not administered progesterone. Furthermore, the Pdyn mRNA expression in the preoptic area, anterior hypothalamic area, and the arcuate nucleus were significantly reduced in ewes following OVX (Foradori et al., 2005). However, in these OVX ewes, administration of progesterone increased the Pdyn mRNA expression in the preoptic and anterior hypothalamic areas to that of an intact ewe (Foradori et al., 2005).

There is some evidence indicating that Pdyn mRNA expression is also regulated in the gonadal tissues of mammals. Following *in vivo* gonadotropin stimulation of female rats, Kaynard et al. (1992b) reported an increase in Pdyn mRNA expression in the ovary. Furthermore, *in vitro* assessment of Pdyn promoter activity in follicular granulosa cells collected from rats showed heightened promoter activity in response to gonadotropin hormones (Kaynard et al., 1992b).

Kappa Opioid Receptor

The Kappa Opioid Receptor (KOR), Delta Opioid Receptor (DOR), and Mu Opioid Receptor (MOP) are encoded by the *OPRK1*, *OPRD1*, and *OPRM1* genes, respectively, and each receptor is responsive to exogenous and endogenous opioid ligands (Martin et al., 1976; Lord et al., 1977; Fernandez Robles et al., 2012). MOP, DOR, and KOR respond to the endogenous opioid neuropeptides derived from the proopiomelanocortin, proenkephalin, and prodynorphin precursor polypeptides, respectively (Chavkin et al., 1982; Wagner et al., 1991).

Identification and Characterization of KOR

Cloning of the opioid receptors in the early 1990s enabled researchers to investigate sequence, structure, and distribution details of the receptor. Similar to the GnIH-R described previously, KOR is a member of the superfamily of G-protein coupled receptors (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Yasuda et al., 1993). The KOR shares 65 to 70% homology with MOR and DOR, with the majority of the similarities residing in the transmembrane domains and intracellular loops, and the divergence among the receptors is found in the N- and C-terminal tails as well as the second and third extracellular loops, which are involved in opioid ligand binding (reviewed by Mansour et al., 1995; Jordan and Devi, 1998). A unique second extracellular loop and fourth transmembrane domain of KOR, along with the

presence of C-terminal basic amino acid resides on the bioactive dynorphin neuropeptides, dictate the selective binding of the dynorphin neuropeptides to KOR (Kong et al., 1994; Xue et al., 1994; Meng et al., 1995). Additionally, the mRNA transcript for mammalian KOR has recently been found to encode for 2 isoforms of the KOR protein, KOR1 and KOR1x. The KOR1x isoform has a longer amino acid sequence than the KOR1 isoform and is created as a result of stop codon readthrough during translation of the KOR mRNA transcript in mammals (Loughran et al., 2014).

Results indicating that the dynorphin neuropeptides showed specificity in binding to the KOR lead researchers to investigate the distribution of these receptors within the central and peripheral tissues of mammalian species. Expression of the KOR has been detected throughout the brain of the rat, with highest density detected in the caudate putamen, nucleus accumbens, amygdala, median eminence, pituitary gland, and hypothalamus, as reviewed by Mansour et al. (1988, 1995). Within the rat hypothalamus, highest density of KOR expression has been observed in the preoptic area, paraventricular nucleus, arcuate nucleus, dorsomedial hypothalamus, and ventromedial hypothalamus (reviewed by Mansour et al., 1988, 1995). Furthermore, differential opioid receptor abundances were observed when expression was assayed across species. Ten percent of the total opioid receptors in the rat brain were KOR, while in the brains of humans, guinea pigs, and pigeons, the KOR accounted for 37%, 50%, and 76%, respectively, of the total opioid receptors expressed therein (reviewed by Mansour et al., 1988).

More recently, absolute quantitative real-time PCR was utilized to quantify central and peripheral tissue expression of KOR mRNA in humans (Peng et al., 2012). Similar to previous mammalian results generated using techniques like *in situ* hybridization and Northern blotting,

KOR mRNA expression was detected largely in the caudate nucleus, putamen, and nucleus accumbens, though the hypothalamic tissue was not assayed, and beyond its expression in the brain, KOR mRNA was detected in a variety of peripheral tissues, including but not limited to the spleen, heart, lung, thymus, intestine, and adrenal gland (Peng et al., 2012). While Peng et al. (2012) did not assay human gonadal tissues in this experiment, expression of KOR has been investigated in mammalian reproductive tissues.

Utilizing immunocytochemical analysis, Slomczynska et al. (1997) detected KOR in the granulosa cells of maturing porcine follicles. The granulosa cells of the small, prehierarchical follicles exhibited greater staining for KOR than that of the hierarchical follicle granulosa cells (Slomczynska et al., 1997). Additionally, Slomczynska et al. (1997) observed KOR staining in prehierarchical granulosa cells to be localized to the cytoplasm but to shift to the plasma membrane of the granulosa cell as the follicle matured into the hierarchy. When KOR was visualized in developing human oocytes using the immunocytochemical technique, KOR localization within the oocyte was observed to change with development (Agirregoitia et al., 2012). In the germinal vesicle stage, KOR was localized to the periphery of the oocyte, and as the oocyte progressed to the metaphase-II stage, KOR was observed to become evenly spread throughout the oocyte (Agirregoitia et al., 2012). Furthermore, studies analyzing testicular expression of KOR, MOR, and DOR in male rats indicate that the receptors are localized to the sertoli cells (Gerendai et al., 1986; Bilinska and Slomczynska, 1996; reviewed by Subiran et al., 2011). Recently, the KOR, along with its sister opioid receptors, have been detected in the plasma membrane of human sperm, indicating the potential for opioid regulation of sperm function (Agirregoitia et al., 2006; Albrizio et al., 2006; reviewed by Subiran et al., 2011). The localization of KOR and its ligands, the dynorphin neuropeptides, to the mammalian gonadal

tissues implies the opportunity for a local autocrine or paracrine system of reproductive regulation within the ovarian and testicular tissues.

Cell signaling of Dynorphin

Given that KOR is a G-protein coupled receptor, the interaction of the dynorphin neuropeptides with the extracellular surface of KOR initiates activation of the intracellular receptor-associated heterotrimeric G-protein. The KOR typically associates with G_i and G_o α-subunits, but occasionally couples to the G_z α-subunit (Reisine et al., 1996; reviewed by Connor and Christie, 1999). Upon binding of the dynorphin neuropeptide agonist to the KOR, the heterotrimeric G-protein dissociates into the α- and ßγ-subunit component parts, which each have roles in downstream intracellular signaling.

The KOR-associated Gα-subunit inhibits adenylate cyclase, which results in decreased cAMP production within the cell (Sharma et al., 1977; Xie et al., 1994; reviewed by Jordan and Devi, 1998; Law et al., 2000; Schwarzer, 2009; Sobczak et al., 2014). The α-subunit of the G-protein also interacts with and activates G-protein-gated inwardly rectifying potassium channels (Henry et al., 1995; Darlinson et al., 1997; reviewed by Jordan and Devi, 1998; Law et al., 2000). The ßγ-subunit functions to inhibit voltage-gated calcium ion channels and to activate potassium channels in the plasma membrane of the cell (Hescheler et al., 1987; North et al., 1987; Surprenant et al., 1990; Rusin et al., 1997; reviewed by Law et al., 2000). Thus, activation of the cell-surface KOR allows for potassium efflux but halts calcium influx. However, there is some contradictory evidence stating that the KOR G-protein ßγ-subunit increases calcium levels within the cell by stimulating phospholipase Cß (PLCß) to generate the second messenger, IP_3, which liberates intracellular calcium stores (Misawa et al., 1990; Periyasamy et al., 1990; Jin et al., 1992; reviewed by Law et al., 2000). The ßγ-subunit also has been shown to stimulate ERK1

and ERK2 kinases, which are involved in the mitogen-activated protein kinase (MAPK) intracellular signaling cascade (Fukuda et al., 1996; Belcheva et al., 1998; Bruchas et al., 2006; reviewed by Law et al., 2000; Bruchas and Chavkin, 2010). The intracellular effects of the KOR-associated α- and ßγ-subunits mediate the impact of the dynorphin neuropeptides to the target cells.

Physiological Impacts of Dynorphin

Though the dynorphin neuropeptides regulate many biological functions through activation of the KOR, a specific interest is in the regulatory role of the dynorphin-KOR system in reproductive function at the central and peripheral levels.

One of the first observed functions of hypothalamic-produced dynorphin neuropeptides was in regulation of prolactin secretion from the pituitary gland. In rats, injection of dynorphin neuropeptides into the ventricles of the brain resulted in a significant increase in prolactin production (Kato et al., 1981; van Vugt et al., 1981; Leadem and Kalra, 1985; Kapoor and Willoughby, 1990). Subsequently, Youngren et al. (1993) reported that infusion of dynorphin neuropeptides, Big-DYN, DYN A 1-17, and DYN B 1-13, into the third ventricle of the brain of female turkeys resulted in increased circulating prolactin. Thus, dynorphin appears to impact prolactin secretion from the pituitary gland in both mammalian and avian species.

Since the early 1990s, it has been widely accepted that opioid peptides also play an influential role in regulating the reproductive axis by inhibiting hypothalamic secretion of GnRH and, in turn, anterior pituitary secretion of LH (Quigley et al., 1980; Masotto et al., 1990; Barb et al., 1991; Kalra, 1993; Okrasa et al., 1995). Furthermore, evidence in rats, primates, and sheep indicated that endogenous opioid peptides may mediate the negative feedback effects of progesterone (Ferin et al., 1984; Horton et al., 1987; Gindoff et al., 1988; Whisnant and

Goodman, 1988; Yang et al., 1988; Kalra, 1993; Goodman, 1994; Goodman et al., 1995). However, the specific opioid peptide-receptor system responsible for mediating the negative feedback effects of progesterone was not uncovered until 2004.

Goodman et al. (2004) determined, by administering opioid receptor antagonists to both the medial basal hypothalamus and hypothalamic preoptic area of the luteal phase ewe, that the LH pulse frequency was being inhibited by opioid peptide action on the KOR in both hypothalamic locations. Furthermore, neurons producing dynorphin neuropeptides were experimentally visualized to be in close contact to GnRH perikarya, and more importantly, the neuronal fibers of dynorphin-producing neurons were observed to synapse on the GnRH-producing neurons (Goodman et al., 2004). Subsequently, Foradori et al. (2005) showed progesterone to stimulate Pdyn mRNA production and DYN A 1-17 secretion in the preoptic area, anterior hypothalamic area, and arcuate nucleus of the ewe hypothalamus. These findings from Goodman et al. (2004) and Foradori et al. (2005), supplemented by a report from Foradori et al. (2002) indicating that dynorphin neurons express the progesterone receptor, imply that dynorphin neurons are responsive to progesterone from the gonads and mediate that response from progesterone by inhibiting hypothalamic GnRH neuron secretion of GnRH.

In 2007, Goodman et al. determined that a subset of neurons in the ewe hypothalamic arcuate nucleus that were producing dynorphin neuropeptides were also producing kisspeptin and neurokinin B. As reviewed by Lehman et al. (2010) and Joseph et al. (2013), given (1) the well-characterized role of kisspeptin in stimulating GnRH production and secretion in mammals, (2) the role of dynorphin in mediating the negative feedback progesterone response to the GnRH neurons, and (3) the expression of both progesterone and estrogen receptors by these KNDy neurons, this subset of neurons within the arcuate nucleus likely regulate downstream

reproductive function in response to gonadal steroid hormone feedback by controlling hypothalamic production and secretion of GnRH. More recently, Mostari et al. (2013) have implicated the dynorphin-KOR system within the arcuate nucleus population of KNDy neurons in taking part in mediating the negative feedback response of estrogen on GnRH and, thus, LH secretion in female rats.

Despite localization of the dynorphin neuropeptides and KOR to the reproductive tissues of several mammals, there is limited research investigating direct regulation of reproductive function by dynorphin at the gonads. The majority of research investigating endogenous opioid regulation of reproductive function in the reproductive tissues is specific to opioid neuropeptides derived from proopiomelanocortin and proenkephalin precursor polyepeptides. However, Kaminski et al. (2003, 2004) assessed the impact of dynorphin neuropeptide exposure on steroidogenesis in cultured porcine theca and granulosa cells. Kaminski et al. (2003, 2004) reported that the KOR agonists, DYN A 1-17 and DYN B 1-13, showed a generally inhibitory impact on steroid hormone production and secretion from the cultured theca and granulosa cells of the porcine ovarian follicles (Kaminski et al., 2003, 2004). Therefore, there is evidence that dynorphin could have a regulatory impact at the ovarian tissues. Furthermore, in a review by Subiran et al. (2011), they imply the potential for the dynorphin-KOR system to regulate male fertility at the level of the testicular tissues and even the sperm.

Though research assessing the dynorphin-KOR system has exponentially increased in recent years, there is yet much to learn about its role in regulation of reproduction function within the central and peripheral tissues of mammals and, particularly, in other non-mammalian vertebrate species where research in this area is scant. Generally speaking, mammalian species have been the research model for characterizing the dynorphin neuropeptide, the KOR, and the

impact that the dynorphin-KOR system on reproductive regulation. After having thought the gene had been evolutionarily deleted in avian species, as was the case for the kisspeptin gene, the prodynorphin gene was recently located in the chicken genome (Sundstrom et al., 2010; Joseph et al., 2013; Pasquier et al., 2014). Furthermore, the *OPRK1* gene, encoding for KOR, has also been detected in the avian genome (Dreborg et al., 2008). Given the presence of both the prodynorphin and KOR genes in the chicken genome, along with the knowledge that avian species lack kisspeptin, there is an interest in assessing avian dynorphin and KOR expression patterns in an effort to determine if the dynorphin-KOR system (1) is acting in lieu of kisspeptin as a regulator of GnRH production in the central tissues and (2) is acting as a direct regulator of reproduction within the gonadal tissues.

Summary

Evidence suggests that both the GnIH and dynorphin hormone systems negatively regulate reproductive function. GnIH and the GnIH-R were discovered in avian species, and thus, have been fairly well characterized in birds. However, the majority of the research has been conducted in wild bird species and quail. Though the majority of dynorphin and KOR research has been conducted in mammals, the genes encoding for dynorphin and KOR have been identified in the genome of the chicken. However, no research has been published characterizing the expression patterns of dynorphin or the KOR in the tissues of the chicken. The results available for dynorphin and KOR in mammalian species, along with what is known about GnIH and GnIH-R expression in avian and mammalian species, indicate that both the GnIH and dynorphin systems have the propensity to function at the brain and at the gonadal tissues.

CHAPTER 3

STATEMENT OF PURPOSE

Broiler breeders given free access to feed can reach their ideal bodyweight for reproduction by 5 weeks of age, but functional sexual maturation of the brain does not occur until about 20 weeks of age. To match the ideal bodyweight for reproduction with brain-based sexual maturation, broiler breeders are severely feed restricted, and this practice optimizes their reproduction relative to that achieved if they are fed *ad libitum.* However, feed restriction management programs for broiler breeders result in fasting periods during which caloric insufficiency signals can negatively impact reproductive development and efficiency. After fasting, when the broiler breeders are given access to feed, they quickly consume their entire allotment of feed and have a period of caloric insufficiency which can negatively impact metabolic status. In mammalian species, two key hormones that transmit metabolic status to the hypothalamus to influence reproductive function are leptin and ghrelin. The production of leptin increases after feeding, and the amount of circulating leptin increases as fat stores increase. In contrast, the production of ghrelin by the stomach is maximal during times of inadequate caloric intake. Leptin positively and ghrelin negatively regulate the production of kisspeptin in the hypothalamus, and kisspeptin is the key regulator of GnRH production. Kisspeptin also regulates gonadal function in mammalian species. While ghrelin and leptin are present in avian species and appear to function as they do in mammalian species, the kisspeptin gene has been evolutionarily deleted from the avian genome. With the deletion of the kisspeptin regulatory

system in avian species, another or other hormone signaling systems must be involved in translating metabolic hormonal inputs to the reproductive hormonal axis at both the central and gonadal level. Based on mammalian research and preliminary avian research, GnIH and dynorphin, are two likely candidate hormone systems that appear to be responsive to metabolic status and to potentially regulate reproduction both at the central and gonad level.

Therefore, the overall purpose for this research is to characterize the expression of gonadotropin inhibitory hormone, gonadotropin inhibitory hormone receptor, preprodynorphin, and the kappa opioid receptor in the pituitary and gonadal tissues of broiler breeders in order to determine if these two neuropeptides and their respective receptors are involved in regulating reproductive function on the basis of nutritional status in avian species. Specifically, the primary objectives of the current research are (1) to determine if GnIH, GnIH-R, Pdyn, and KOR mRNA are expressed in the pituitary tissue or the ovarian follicular theca and granulosa tissues of the broiler breeder hen, (2) to investigate whether fasting alters the expression of GnIH, GnIH-R, Pdyn, and KOR mRNA in the pituitary tissues or the ovarian follicular tissues of the broiler breeder hen, (3) to determine whether exposure of cultured broiler breeder follicular granulosa cells to LH, FSH, estrogen, or testosterone influences the granulosa cell expression of GnIH, GnIH-R, Pdyn, and KOR mRNA, and (4) to determine whether sexual maturity alters GnIH mRNA expression in testicular tissues. Characterization of GnIH, dynorphin, and their respective receptors in the pituitary and ovarian follicular tissues of the broiler breeder hen could aid in determining the reasons for the abnormal follicular development and egg production observed in the broiler breeder relative to the laying hen.

CHAPTER 4

MATERIALS AND METHODS

Experiment 1

The purpose of the first experiment was to examine the expression of GnIH and GnIH-R mRNA in the pituitary tissue of fed and fasted broiler breeder hens.

Animals

Cobb 500 fast feathering broiler breeder pullets were reared in floor pens from day 1 of age at the University of Georgia Poultry Research Center. They were provided a standard broiler breeder pullet diet on a skip a day feed restriction program. Ten percent of the pullets were randomly selected and weighed once per week in the rearing phase to determine feed allocation. This method was used to ensure that the body weight gain of the pullets matched the recommended guidelines of the primary breeder (Cobb-Vantress 2005a). From placement on day 1 until 21 weeks of age, the pullets received 8 hours of light.

The lighting program was adjusted to provide 14 hours of light per day for photostimulation at 21 weeks of age. At time of photostimulation, the hens were provided a standard broiler breeder layer diet on an every day basis. The amount of feed provided to the hens every day was determined using the guidelines of the primary breeder (Cobb-Vantress 2005a, Cobb-Vantress 2005b), which are based on the weekly body weight measurements and egg production rates of the hens. At 40 weeks of age, a group of hens were removed from floor pens and placed into individual cages, where egg laying was monitored and feed continued to be

provided on an every day basis, until tissues were collected roughly two weeks later. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue Collection

Twenty-eight caged hens were selected for tissue collection based on records indicating consistent egg laying patterns. The hens were divided into two treatment groups. The fourteen hens placed in the fed treatment group continued to receive their daily feed allotment, and in the fasted treatment group, the remaining fourteen hens did not receive any feed. After 72 hours, the hens were killed by cervical dislocation 2 to 4 hours prior to ovulation, as indicated by the presence of a hard-shelled egg in the shell gland. At the time of tissue collection, the broiler breeder hens were 42 weeks of age. In each hen, the cranial portion of the skull was carefully removed to display the area beneath the hypothalamus, which allowed for collection of the pituitary gland. Within each treatment, the pituitary gland tissue collected from one hen was combined with the pituitary gland tissue from another hen, meaning that each replicate tissue sample contained the pituitary gland tissues from two hens in the same treatment. Each replicate sample of pituitary gland tissue was placed into 1.5mL of guanidinium isothiocyanate solution (Chomczynski and Sacchi, 1987) and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The tissue solutions were stored at -80ºC for subsequent RNA extraction. A total of seven replicate samples of pituitary gland tissue was collected for each treatment (n=7).

Experiment 2

The aim of the second experiment was to investigate the expression patterns of GnIH and GnIH-R mRNA in the hierarchical and prehierarchical follicular tissues of fed and fasted broiler breeder hens.

Animals

Management of the Cobb 500 fast feathering broiler breeder pullets and hens was as described in Experiment 1. At 42 weeks of age, 40 hens were placed into individual cages to monitor individual egg production.

Tissue Collection

Tissue was collected from the hens when they were between 45 and 52 weeks of age. At each collection, 4 hens were utilized that had been fed either 5 or 72 hours earlier. Hens were killed by cervical dislocation 2 to 4 hours prior to ovulation, as evidenced by the presence of a hard-shelled egg in the shell gland. From each hen, the ovary was removed. The four largest hierarchical follicles, F1, F2, F3, and F4, the small yellow follicles (SYF, >5 to 12 mm in diameter), and the large white follicles (LWF, <2-5 mm in diameter) were gathered from each collected ovary.

In each hierarchical follicle (F1-F4), the granulosa cell layer was manually separated from the theca cell layer (Huang and Nolbandov, 1979). However, in the prehierarchical follicles (SYF and LWF), the theca and granulosa cell layers were separated enzymatically (Davis et al., 2000). Within each treatment, the individual theca and granulosa tissues for each follicle size collected from one hen were combined with the corresponding samples from another hen, meaning that each replicate tissue sample contains the tissues from two hens in the same treatment. The theca layers collected from the hierarchical follicles and the theca layers

enzymatically separated from the individual pools of prehierarchical follicles were placed into 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The single cell layer of granulosa tissue from each hierarchical follicle and the granulosa cells enzymatically separated from the individual pools of prehierarchical follicles were placed into 2 mL of guanidinium isothiocyanate solution and vortexed for 20 seconds. The tissue solutions were stored at -80ºC for subsequent RNA extraction. This collection procedure was repeated until a total of four replicate follicular tissue sets were obtained for each treatment (n=4).

Experiment 3

The purpose of the third experiment was to examine the expression of Pdyn and KOR mRNA in the pituitary tissue of fed and fasted broiler breeder hens.

Animals and Tissue Collection

The tissues used in this experiment were the same tissues used in Experiment 1 (n=7).

Experiment 4

The objective of the fourth experiment was to investigate the expression patterns of Pdyn and KOR mRNA in the hierarchical and prehierarchical follicular tissues of fed and fasted broiler breeder hens.

Animals and Tissue Collection

The tissues used in this experiment were the same tissues used in Experiment 2 (n=4).

Experiment 5

The aim of the fifth experiment was to investigate GnIH mRNA expression in the testicular tissues of sexually immature male broilers and sexually mature broiler breeder roosters.

Animals

Cobb 500 male broilers were obtained from the Cleveland, Georgia hatchery on day of hatch and placed into floor pens in an environmentally controlled facility at the University of Georgia Poultry Research Center, where they were reared until 42 days of age. The broilers were fed a standard corn/soybean-based starter diet, grower diet, and finisher diet for the first, second, and third two-week phases of the study, respectively. The broilers were provided with *ad libitum* access to both water and feed and were maintained under typical temperature and lighting programs for the entirety of the 42-day experiment. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Ross broiler breeder roosters were obtained from a commercial broiler breeder farm in northeast Georgia at 62 weeks of age.

Tissue Collection

At 42 days of age, ten of the Cobb male broilers were selected for tissue collection and removed from the floor pens. The broilers were killed by cervical dislocation. From each sexually immature broiler, 300 mg of testicular tissue was collected (n = 10). Each replicate sample of immature testicular tissue was placed into 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The tissue solutions were stored at -80ºC for subsequent RNA extraction.

Ten of the 62 week old Ross broiler breeder roosters were killed by carbon dioxide asphyxiation. From each rooster, the left testicle was removed. The removed testicle was cut in half vertically, and a 300 mg sample of testicular tissue from the center of the testicle was collected (n = 10). Each replicate sample of mature testicular tissue was placed into 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The tissue solutions were stored at -80ºC for subsequent RNA extraction.

Experiment 6

The objective of the sixth experiment was to investigate whether GnIH-R, Pdyn, or KOR mRNA expression in cultured granulosa cells from F1, F3, and SY follicles is influenced by exposure to gonadotropin hormones, LH and FSH.

Animals

Cobb 500 slow feathering pullets were managed during rearing as previously described for Experiment 1. At the time of photostimulation, 100 of the pullets were moved to individual cages and fed daily with the amount of feed provided to the hens determined using the guidelines of the primary breeder (Cobb-Vantress 2005a, Cobb-Vantress 2005b), which are based on weekly body weight measurements and egg production rates of the hens.

Tissue Collection

At 45 weeks of age, three of the caged broiler breeder hens were selected for tissue collection based on an observed consistency in egg laying. The hens were killed by cervical dislocation 2 to 4 hours prior to ovulation, as evidenced by the presence of a hard-shelled egg in the shell gland. From each of the three hens, the ovary was removed, and the F1, F3, and SY

follicles were collected and pooled by size. The granulosa cells from each pool of F1, F3, and SY follicles were isolated, dispersed, and washed, as previously described (Davis et al., 2000). Using a hemocytometer with trypan blue exclusion, cell number and viability were estimated, and cell viability was greater than 95%.

Dispersed granulosa cells from each follicle size were cultured in 6-well tissue culture plates at a density of 2.5 x 10^6 cells/well with 4 mL of M199 culture media, as previously described (Davis et al., 2000) except in this experiment the lipoprotein supplement was not added to the M199 culture media. The granulosa cells were cultured for 24 hours with 0 or 50 ng/mL cell culture media of ovine LH (Lot AFP8468A) or human recombinant FSH (Lot AFP5551B). Both the LH and FSH were generously provided by Dr. A.F. Parlow of the National Hormone and Peptide Program, Torrance, CA. There were three wells per treatment for each follicle size. At the termination of the experiment, the granulosa cells from each of the three wells were collected in solution D (Chomczynski and Sacchi, 1987), combined, and placed in the -80ºC freezer for subsequent RNA extraction. Cell culture media from each treatment and follicle size was saved and stored at -80 ºC for subsequent analysis of progesterone content. This experiment was repeated 3 more times over the next 3 weeks to generate a total of four replicate experiments.

Experiment 7

The purpose of the seventh experiment was to investigate whether GnIH-R, Pdyn, or KOR mRNA expression in cultured granulosa cells from F1, F3, and SY follicles is influenced by exposure to sex steroid hormones, estrogen and testosterone.

Animals and Tissue Collection

The experimental procedures for this experiment were exactly the same as those utilized in Experiment 6, except that the hens utilized for replicate experiments were between 50 and 54 weeks of age, and the dispersed granulosa cells were cultured in the absence or presence of 1 x 10^{-6} testosterone (Steraloids, Newport, RI) or 17-β-estradiol (Sigma, St. Louis, MO).

RNA Extraction

Total RNA was extracted from the tissue samples and from the cultured granulosa cells using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA samples were stored at -80°C. The integrity of each RNA sample was assessed by the presence of intact bands for 28S and 18S rRNA on a 1.5% agarose gel stained with ethidium bromide.

Real Time RT-PCR

To remove any potential genomic DNA contamination, the extracted RNA samples were DNase treated using the TURBO-DNA-free kit (Ambion, Austin, TX). Taqman minor groove-binding (MGB) probes and primers designed to specifically detect chicken GnIH (GenBank accession # AB193126.1), GnIH-R (GenBank accession # AB193127.1), Pdyn (GenBank accession #NC_006107.4), KOR (GenBank accession # NM_001318774.1), and the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession #M11213) were generated using Primer Express software version 2.0 [Applied Biosystems, Foster City, CA (Table 4.1)]. The primers and Taqman MGB probes (Table 4.1) were synthesized by Applied Biosystems. The Pdyn assay was designed to amplify both of the Pdyn splice variants for the chicken, which are referred to as transcript variant x1 and transcript variant x2. The KOR assay

was designed to amplify both of the KOR1 and KOR1x protein isoforms. Each probe was labeled at the 5' end with FAM (6-carboxyfluorescein), the reporter dye, and at the 3' end with TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine), the quencher dye. Validation of the primer and MGB probe sets for real-time PCR was completed by determining the optimal amplification efficiency and primer/probe concentration, as described by the manufacturer (Applied Biosystems).

Synthesis of cDNA by reverse-transcription reactions was completed using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. Two-step real-time PCR, amplification of GnIH, GnIH-R, Pdyn, and KOR utilized 100 ng of cDNA generated from pituitary and testicular RNA samples and 450 ng of cDNA generated from ovarian follicular RNA samples. Amplification of GAPDH utilized 100 ng of cDNA from all tissue samples. All PCR reactions utilizing 100 ng of cDNA were performed in a 25 uL volume, while PCR reactions utilizing 450 ng were performed in a 50 uL volume. Both reaction volumes consisted of reaction buffer containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate primer pair, and 25 nM of the appropriate probe. An ABI 7500 Thermocycler (Applied Biosystems) was used to complete the real-time RT-PCR reactions. The thermocycler conditions were 10 minutes at 95°C and 40 cycles each of 15 seconds at 95°C and 1 minute at 60°C. The reactions for each sample were performed in duplicate for GnIH, GnIH-R, Pdyn, KOR, and GAPDH assays.

The C_T (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction using the Sequence Detection software (version 1.2.2, Applied Biosystems), and quantification was completed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The C_T values for GnIH, GnIH-R, Pdyn, and KOR were determined for each sample and,

subsequently, normalized to the GAPDH C_T value from the same sample (GnIH C_T - GAPDH C_T = ΔC_T). After the ΔC_T values for all reactions were obtained for an experimental replicate, the ΔC_T values for each individual GnIH, GnIH-R, Pdyn, or KOR reaction were compared to the sample within the replicate that had the highest mRNA expression for GnIH, GnIH-R, Pdyn, or KOR using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Therefore, all data for GnIH, GnIH-R, Pdyn, or KOR is expressed as the fold-difference relative to the sample with the highest expression.

Progesterone RIA

Cell culture media progesterone concentrations in Experiment 6 and Experiment 7 were determined by RIA using the Coat-A-Count Progesterone kit (Diagnostic Products Corporation, Los Angeles, CA, catalogue #TKTPG) following the manufacturer's protocol.

Statistics

In each experiment, the data were subjected to ANOVA using the General Linear Model (GLM) procedure. Tukey's multiple-comparison procedure (Neter et al., 1990) was used to detect significant differences among individual tissues, follicle sizes, and cell culture treatments. Differences were considered significant when $P<0.05$. All statistical procedures were completed with the Minitab statistical software package (Release17, State College, PA).

Table 4.1. Oligonucleotide primer pairs and probes for real time RT-PCR.

Product	Primer	Oligonucleotide Primer and Probe Sequence	Product Size (Base Pairs)
GnIH	Forward	5'-CTT TGG CTA CAG TGG CGT TTC T-3'	
	Reverse	5'-TCC AGG CTG GAT TTC ATT AGT TC-3'	70
	Probe	5'-ACC GCA TGG TAT GTG C-3'	
GnIH-R	Forward	5'-CCT GGT CAC CGG CTA TGT CT-3'	
	Reverse	5'-GGT TGG CAC TGC TGT TGA AGA-3'	65
	Probe	5'-CTT TGC CCA CTG GCT-3'	
Pdyn	Forward	5'-GCA TCC GGC CCA AGC T-3'	
	Reverse	5'-CGC GTG GTC ACC TTG AAC T-3'	79
	Probe	5'-AAG TGG GAC AAT CAG AAG-3'	
KOR	Forward	5'-ATC TCC CCC TCT ATT CCC ATC AT-3'	
	Reverse	5'-CCA CCA AGC CAA CAA CAA AGA-3'	67
	Probe	5'-TCA CCG CTG TCT ACT C-3'	
GAPDH	Forward	5'-CCT AGG ATA CAC AGA GGA CCA GGT T-3'	
	Reverse	5'-AGG TGG AGG AAT GGC TGT CA-3'	66
	Probe	5'-CTC CTG TGA CTT CAA TG-3'	

CHAPTER 5

RESULTS

Experiment 1

The mRNA transcripts encoding for GnIH and the GnIH-R were detected by real time RT-PCR in the pituitary tissue of the broiler breeder hen. The mRNA expression of GnIH in the pituitary tissue of broiler breeder hens fasted for 72 hours was significantly higher than that of the hens fed daily (Figure 5.1). In contrast, no significant difference existed between GnIH-R mRNA expression in the pituitary tissues collected from broiler breeder hens fed daily and fasted for 72 hours (Figure 5.2).

Experiment 2

While GnIH mRNA was detected in the pituitary tissue of the broiler breeder hen (Experiment 1), the mRNA for GnIH was not detected by real time RT-PCR in the theca or granulosa tissues of any follicle size (Figure 5.3). However, GnIH-R mRNA expression was detected in follicular tissue. In the normal, fed state, expression of the mRNA for GnIH-R in theca cells was highest in the LWF, while expression in the granulosa cells was highest in the SYF, and GnIH-R mRNA is not detected in the granulosa cells from the F1 or F2 follicles (Figure 5.4).

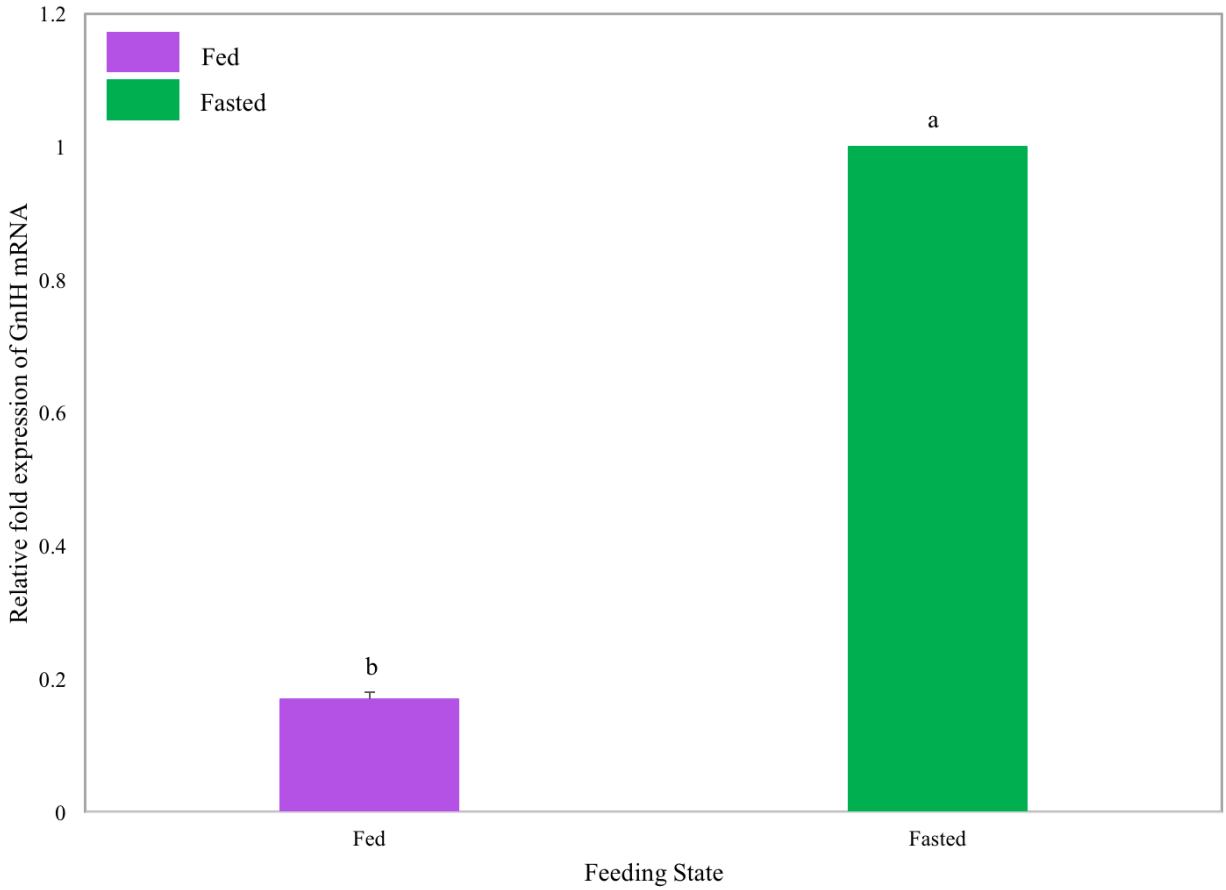

Figure 5.1. The relative fold expression of GnIH mRNA in the pituitary tissue collected from 42-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 1). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 7 for each feeding state. $^{a\text{-}b}$Means with different letters differ, $P < 0.05$.

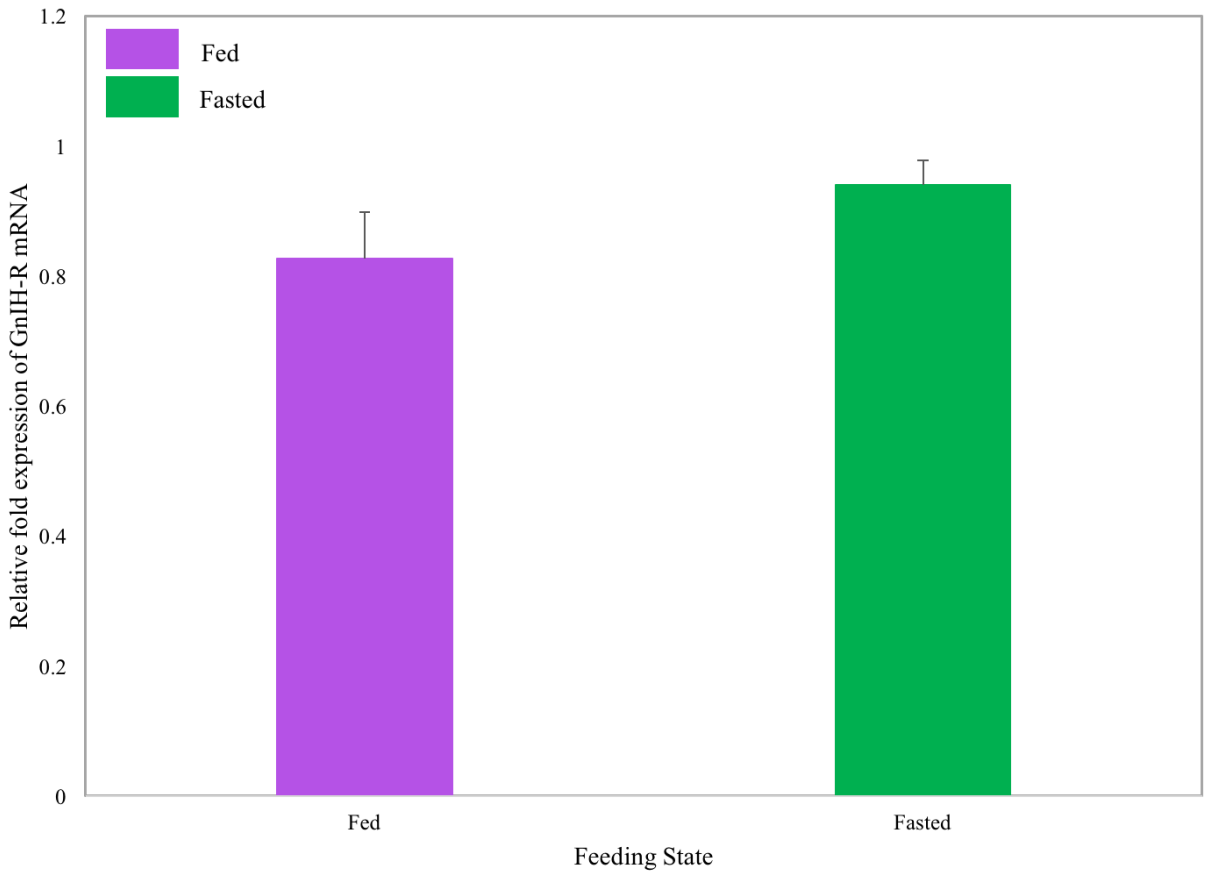

Figure 5.2. The relative fold expression of GnIH-R mRNA in the pituitary tissue collected from 42-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 1). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 7 for each feeding state.

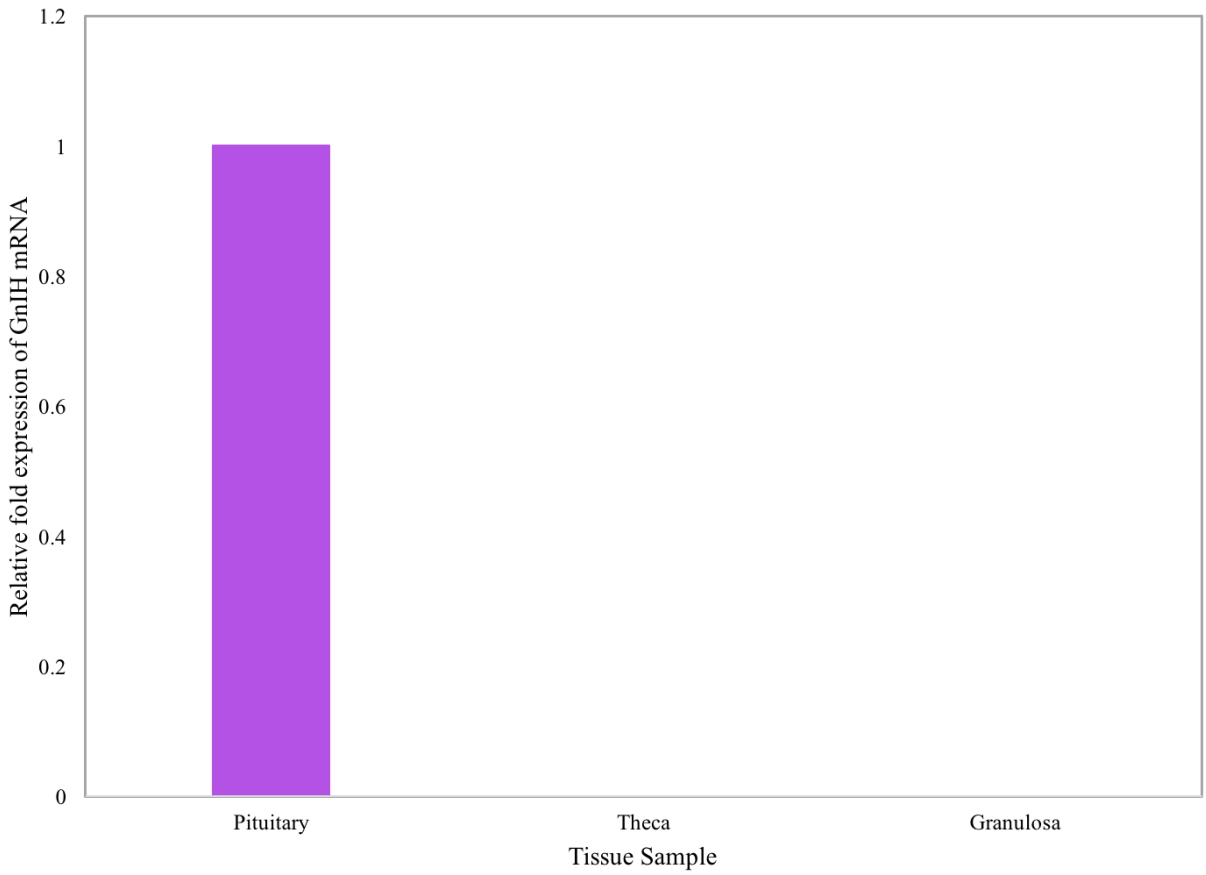

Figure 5.3. The relative fold expression of GnIH mRNA in the pituitary tissue collected from 42-week old broiler breeder hens compared to the theca and granulosa follicular tissues from 45 to 52-week old broiler breeder hens (Experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 7 for pituitary tissue and n = 4 for follicular tissues.

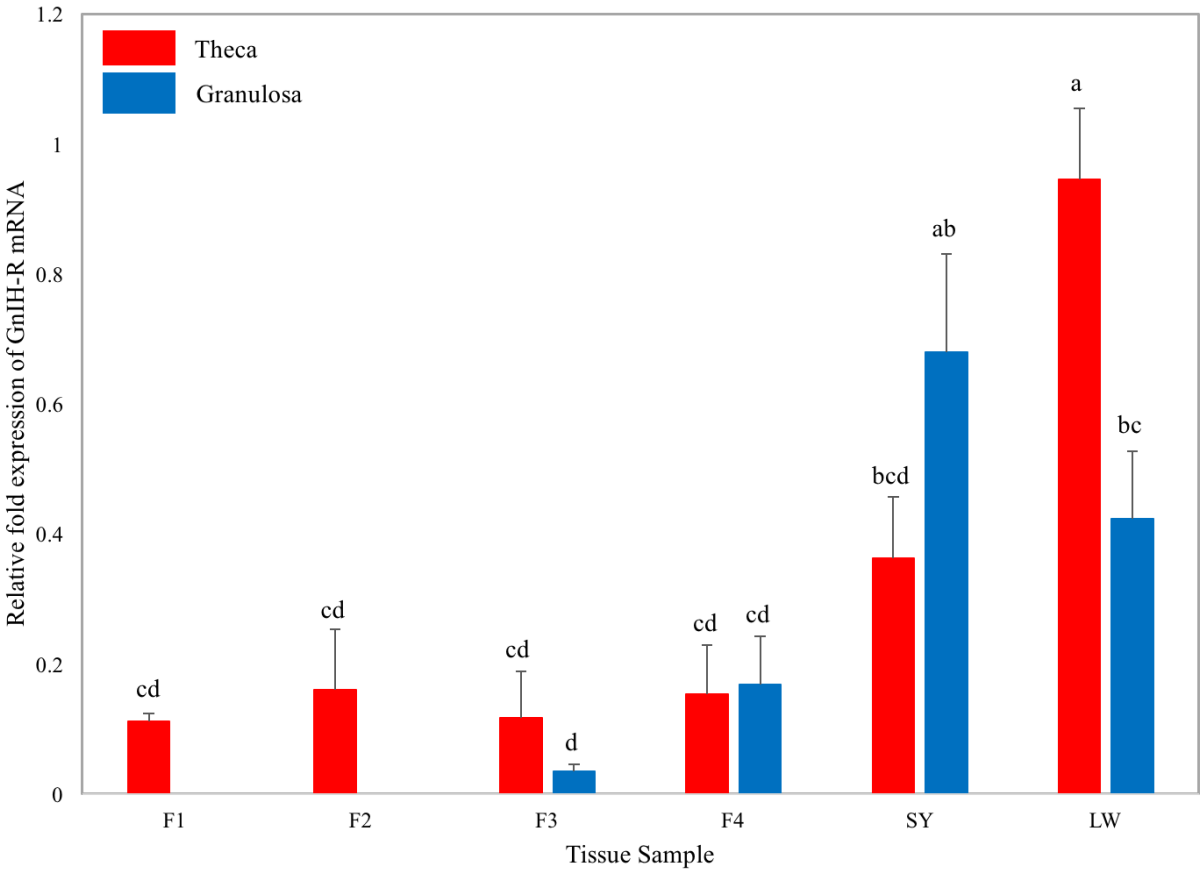

Figure 5.4. The relative fold expression of GnIH-R mRNA in the theca or granulosa tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles (SY), and the large white follicles (LW) from 45 to 52-week old broiler breeder hens fed daily (Experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

[a-d]Means with different letters differ, $P < 0.05$.

Overall, the mRNA expression of GnIH-R for all the theca and granulosa samples isolated from either broiler breeder hens that were fed or fasted for 72 hours did not differ significantly (Figure 5.5). In comparing theca and granulosa tissues from fed and fasted hens across the individual preovulatory follicles, the expression pattern of GnIH-R is very similar between the fasted and fed birds, except that GnIH-R mRNA is detected in the F2 granulosa cells of fasted birds and not detected in the equivalent sample from the fed birds (Figure 5.6).

Experiment 3

The mRNA expression of Pdyn in the pituitary tissue of broiler breeder hens that were fed daily or fasted for 72 hours did not differ significantly (Figure 5.7). Similarly, no significant difference existed between KOR mRNA expression in the pituitary tissues collected from broiler breeder hens fed daily and fasted for 72 hours (Figure 5.8).

Experiment 4

In the fed state, Pdyn mRNA was only detected in granulosa tissue and not theca tissue from all the preovulatory follicles (Figure 5.9). The mRNA expression of Pdyn in the granulosa cells of the F1 follicle was greater than the expression found in the granulosa cells of the F2, F3, or F4 follicles. The overall mRNA expression of Pdyn in all the theca and granulosa samples isolated from either broiler breeder hens that were fed or fasted for 72 hours did not differ significantly (Figure 5.10). In comparing Pdyn mRNA expression in theca and granulosa tissues from fed and fasted hens across the individual preovulatory follicles, the expression pattern of Pdyn is very similar between the fasted and fed birds except that the F4 theca cells of fasted hens express Pdyn (Figure 5.11), and there is no theca expression of Pdyn in fed birds.

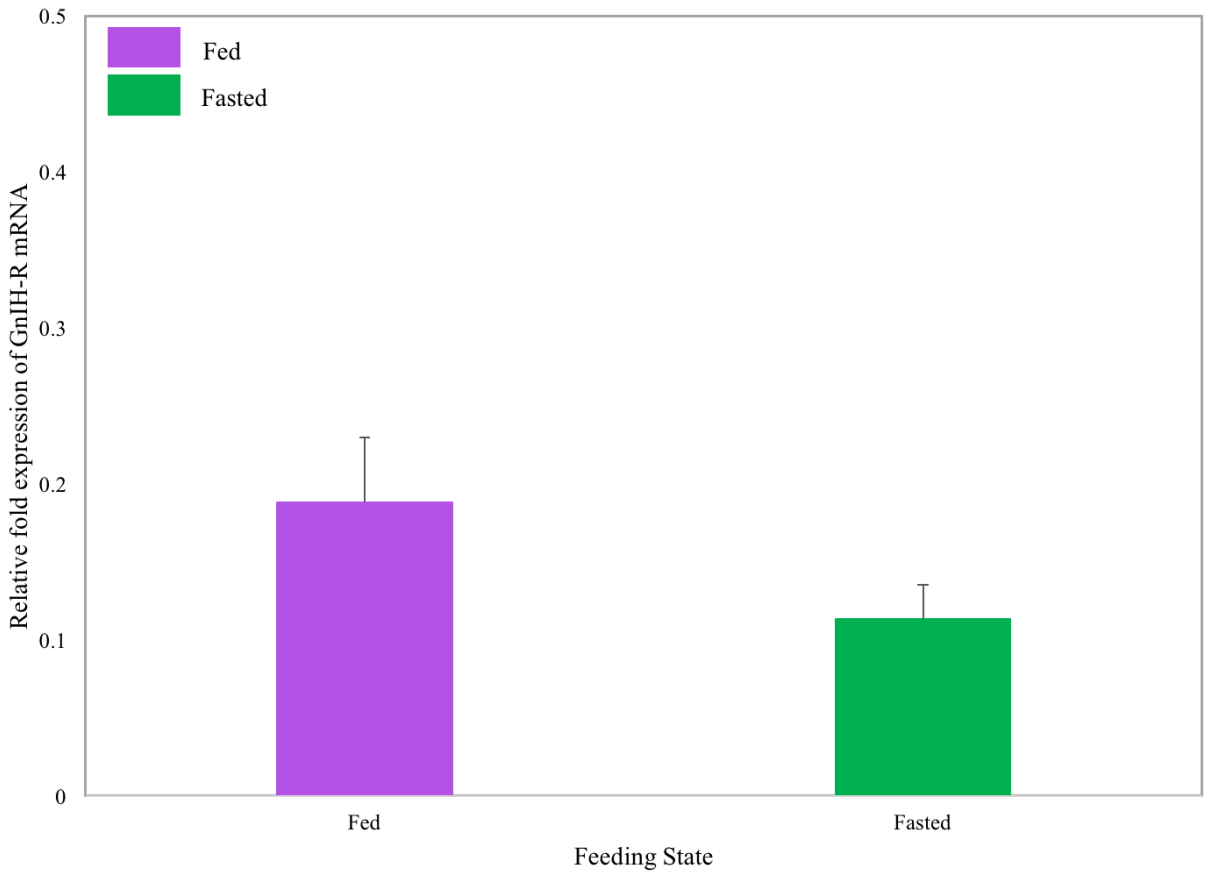

Figure 5.5. The overall relative fold expression of GnIH-R mRNA in theca and granulosa tissue collected from the four largest hierarchical follicles (F1 through F4) and the small yellow and large white follicles from 45 to 52-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 48 [8 samples (4 theca and 4 granulosa) from each of the 6 follicle sizes for each feeding state].

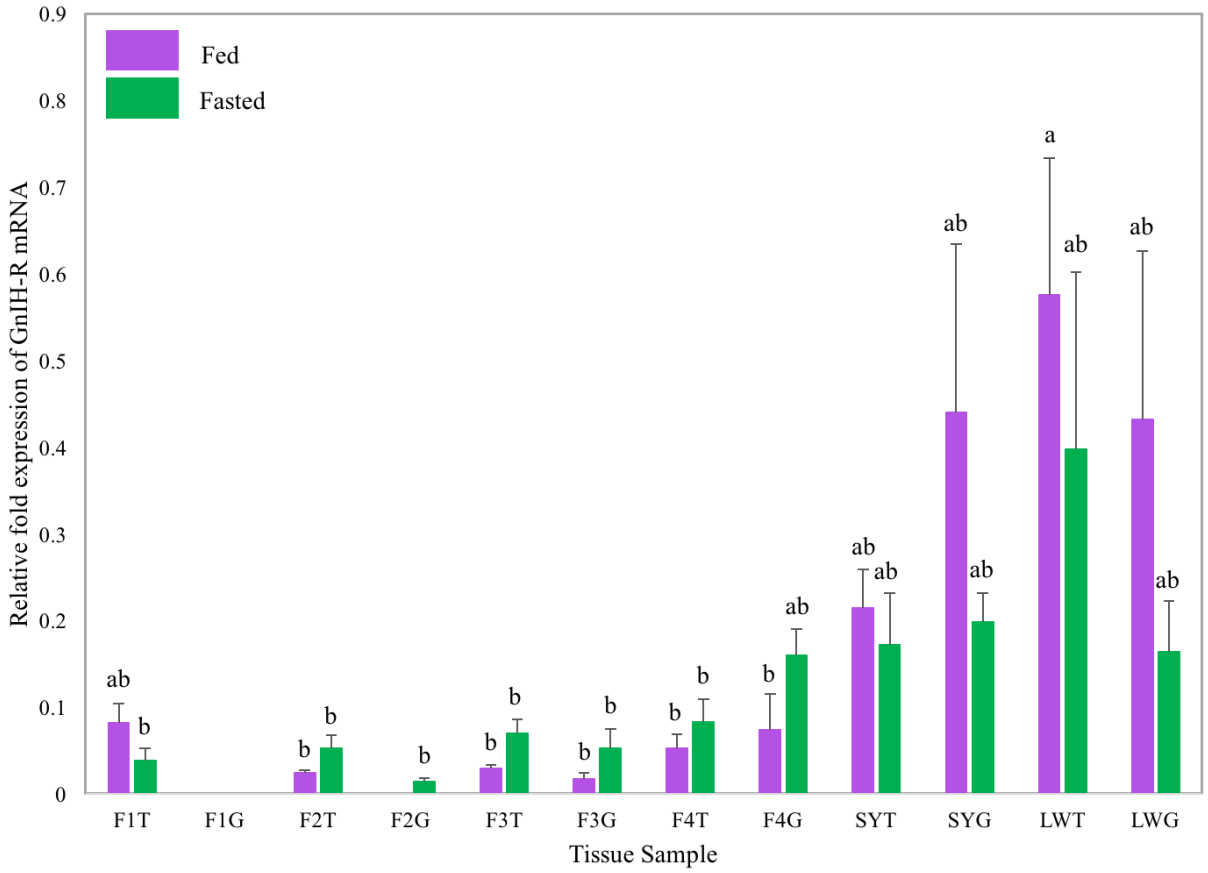

Figure 5.6. The relative fold expression of GnIH-R mRNA in the theca (T) or granulosa (G) tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles (SY), and the large white follicles (LW) from 45 to 52-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4 for each feeding state. $^{a-b}$Means with different letters differ, $P < 0.05$.

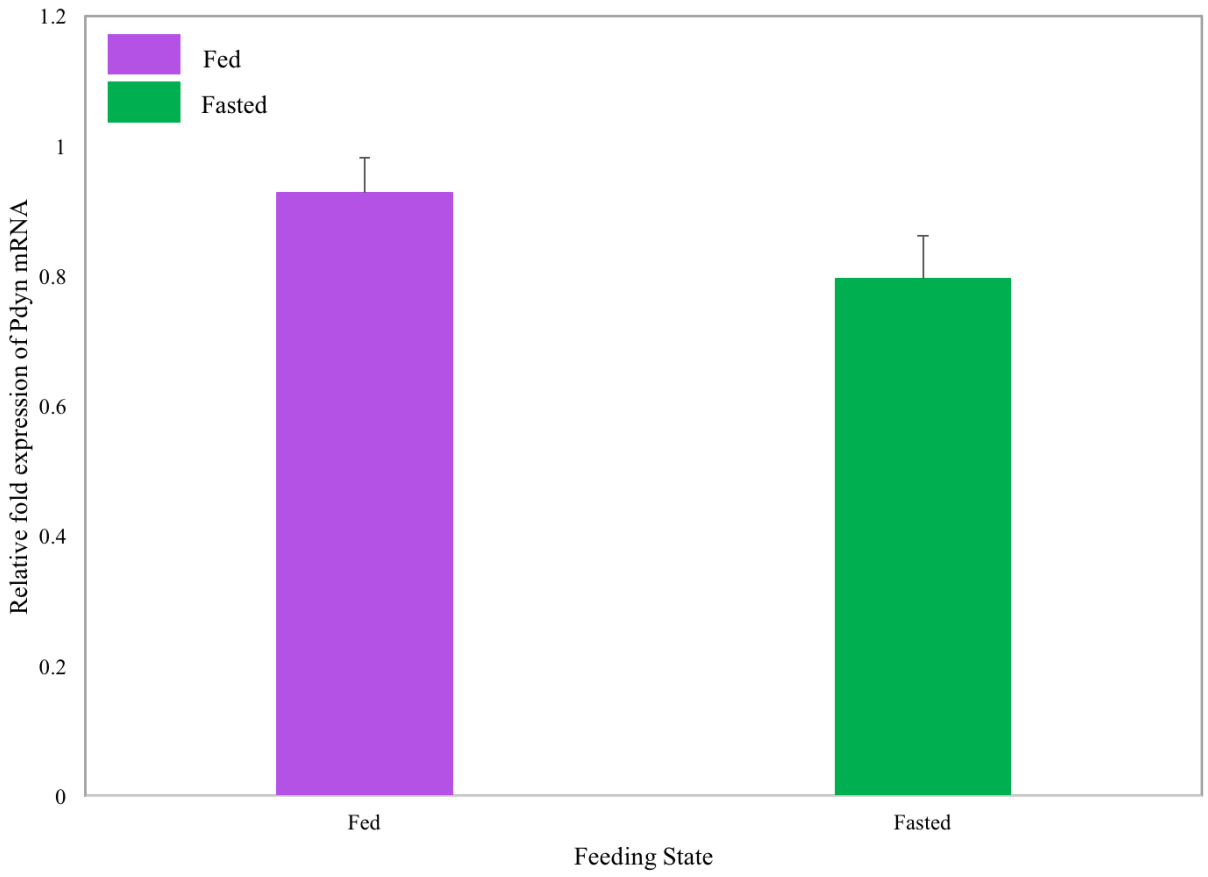

Figure 5.7. The relative fold expression of Pdyn mRNA in the pituitary tissue collected from 42-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 7 for each feeding state.

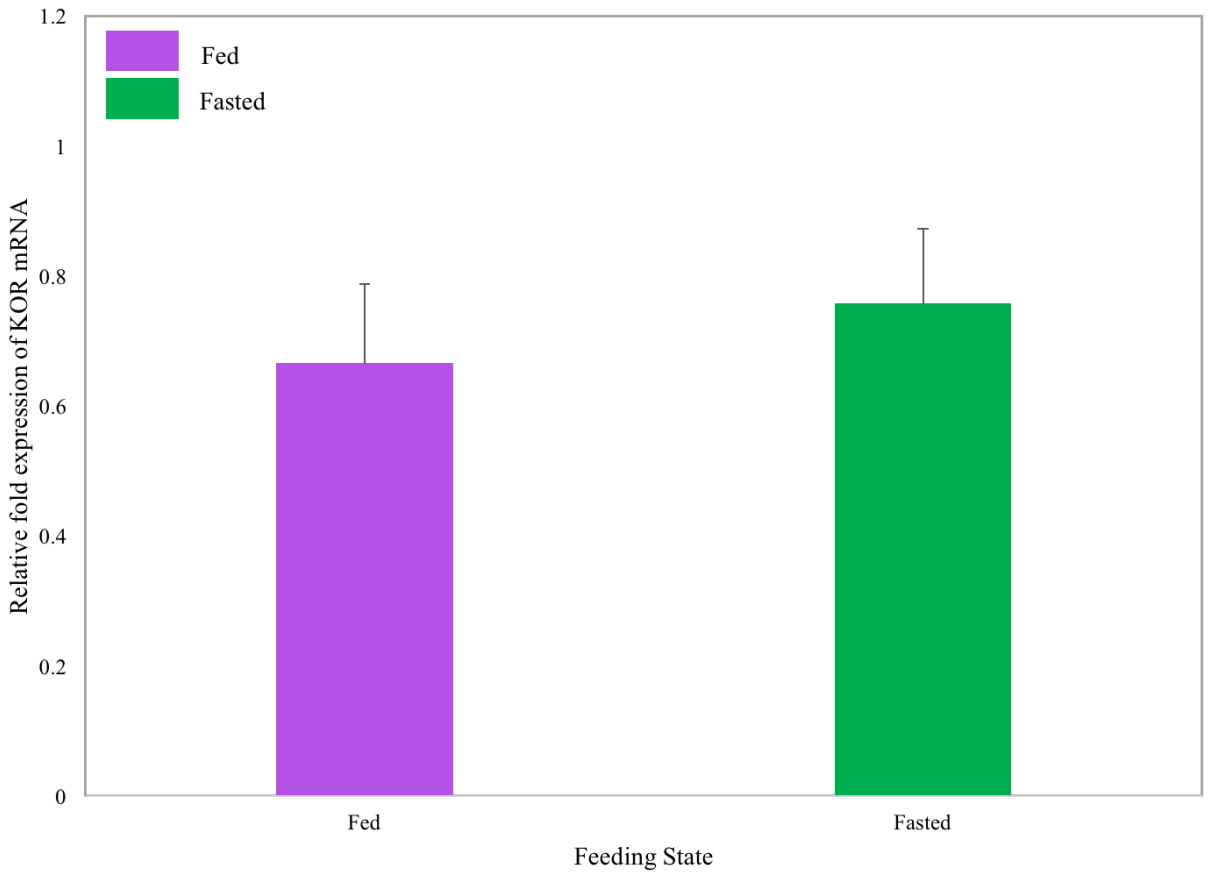

Figure 5.8. The relative fold expression of KOR mRNA in the pituitary tissue collected from 42-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 7 for each feeding state.

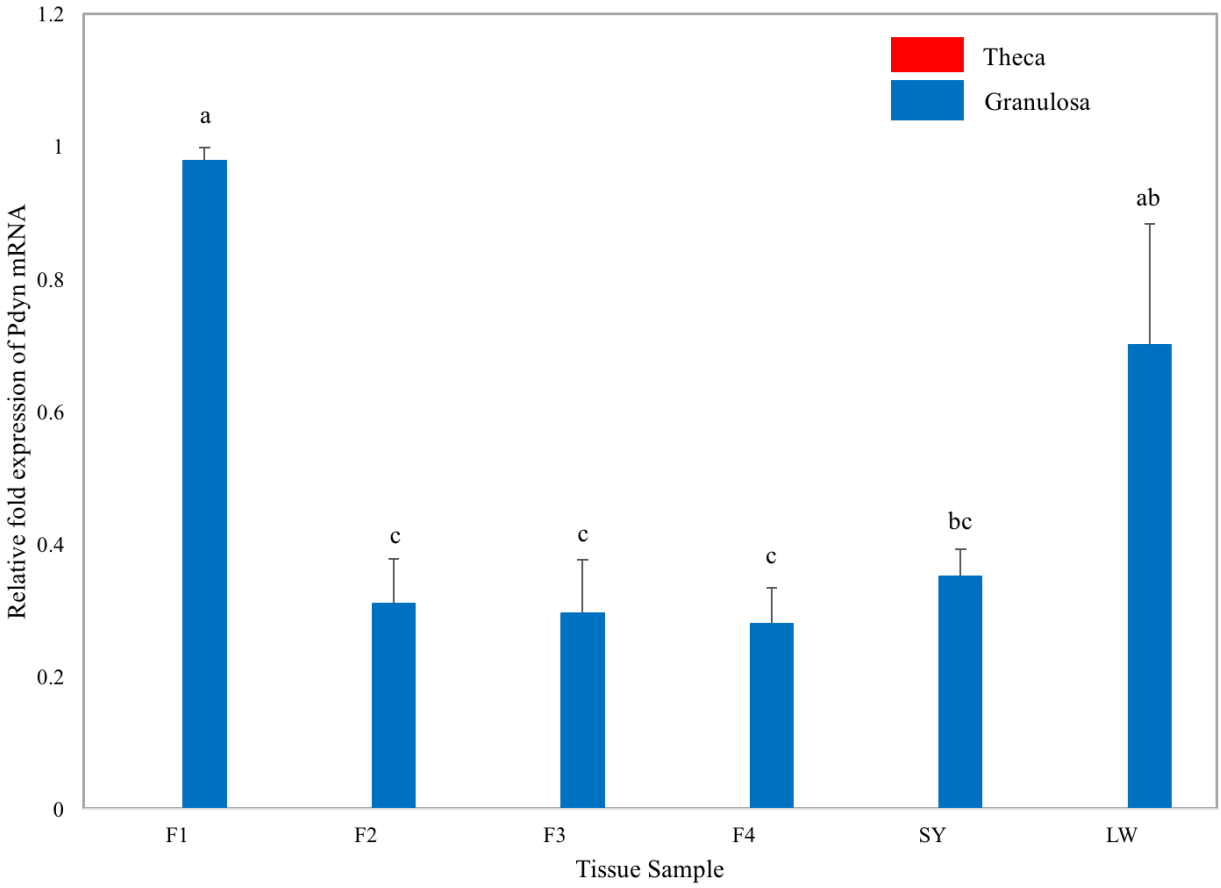

Figure 5.9. The relative fold expression of Pdyn mRNA in the theca or granulosa tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles (SY), and the large white follicles (LW) from 45 to 52-week old broiler breeder hens fed daily (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

[a-c]Means with different letters differ, $P < 0.05$.

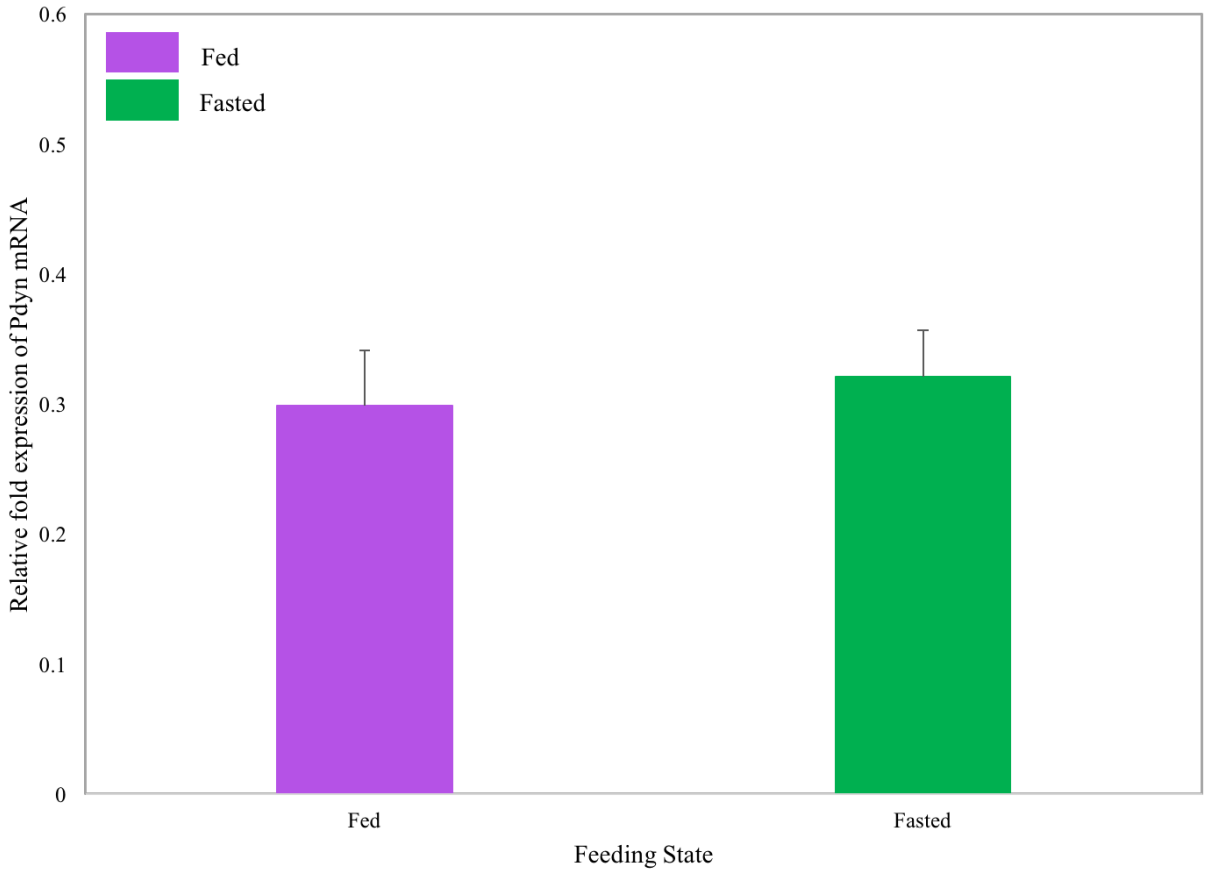

Figure 5.10. The overall relative fold expression of Pdyn mRNA in theca and granulosa tissue collected from the four largest hierarchical follicles (F1 through F4) and the small yellow and large white follicles from 45 to 52-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 48 [8 samples (4 theca and 4 granulosa) from each of the 6 follicle sizes for each feeding state].

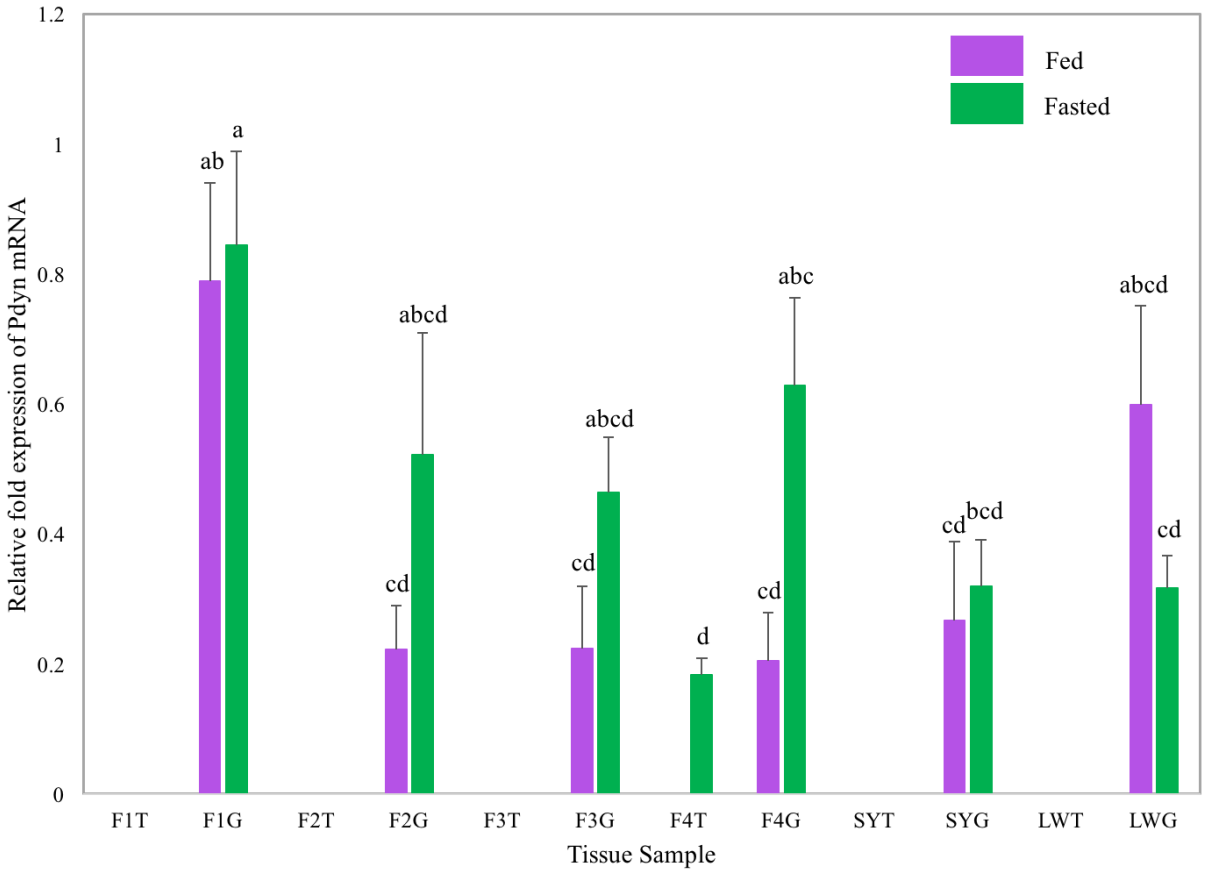

Figure 5.11. The relative fold expression of Pdyn mRNA in the theca (T) or granulosa (G) tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles (SY), and the large white follicles (LW) from 45 to 52-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4 for each feeding state. $^{a-d}$Means with different letters differ, $P < 0.05$.

The overall mRNA expression of KOR for all the theca and granulosa samples isolated from the fed hens was greater (P = 0.004) than the KOR expression in these samples from the hens fasted for 72 hours (Figure 5.12). Further investigation of this difference indicated that fasting for 72 hours significantly decreased KOR mRNA expression in theca tissue, but did not alter KOR expression in the granulosa tissue relative to fed broiler breeder hens (Figure 5.13). Individual follicle analysis indicates that, while significant KOR mRNA expression is detected in the theca tissues of the F1, F2, SY, and LW follicles from hens fed daily, KOR mRNA cannot be detected in the theca of these follicles after a 72 hour fast (Figure 5.14). Interestingly, in hens fasted for 72 hours, KOR mRNA increases to a detectable level in the granulosa cell of the F1, F2, and LW follicles (Figure 5.14).

Experiment 5

GnIH mRNA was detected in immature and mature testicle tissues (Figure 5.15). The expression of GnIH mRNA was significantly higher (P = 0.005) in testicular tissue collected from immature broiler chickens than in testicular tissue from mature broiler breeder roosters (Figure 5.15).

Experiment 6

GnIH-R mRNA was not detected in freshly dispersed granulosa cells from the F1 follicle (Figure 5.16). After 24 hours of culture with or without the gonadotropin hormones, the GnIH-R mRNA was detected in the granulosa cells of the F1 follicle (Figure 5.16). However, there was no significant difference in the GnIH-R mRNA expression level between untreated and gonadotropin-treated cells (Figure 5.16).

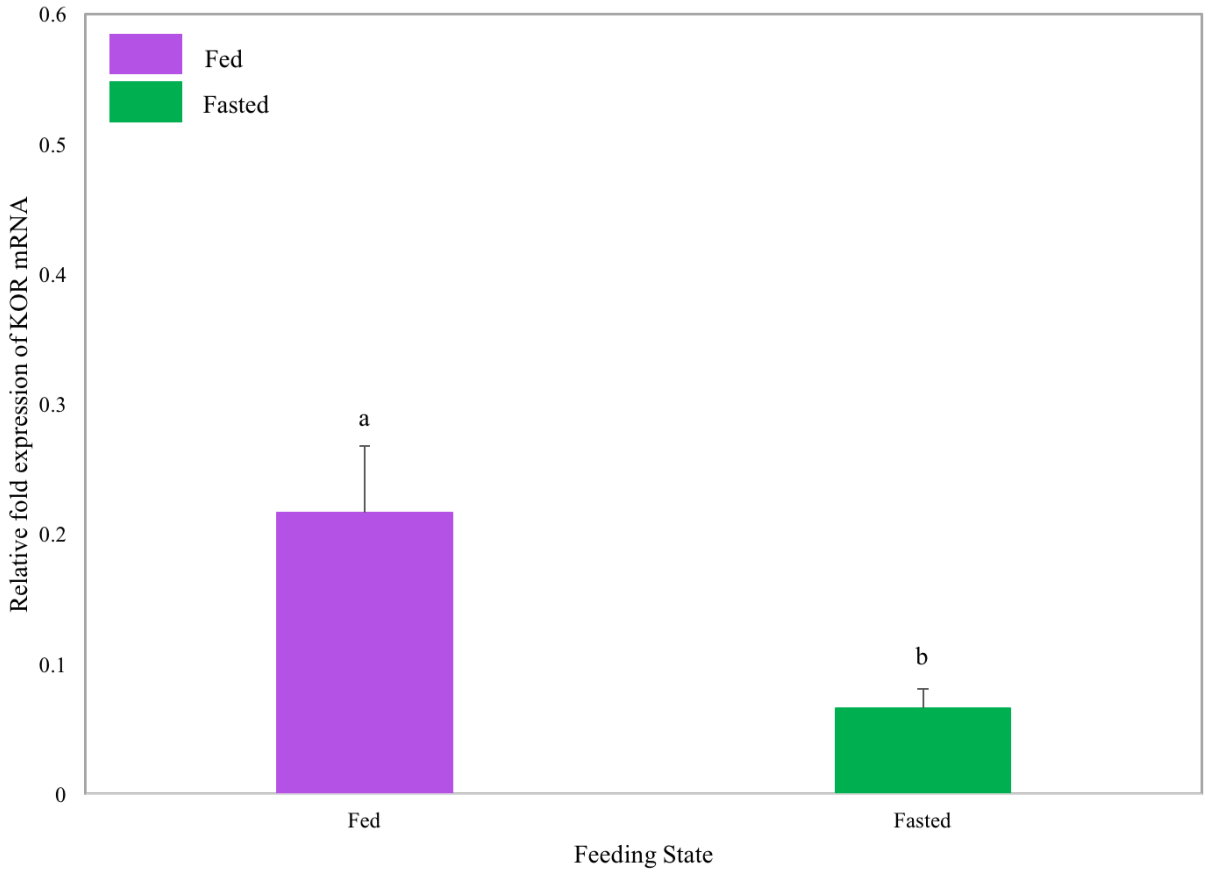

Figure 5.12. The overall relative fold expression of KOR mRNA in theca and granulosa tissue collected from the four largest hierarchical follicles (F1 through F4) and the small yellow and large white follicles from 45 to 52-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 48 [8 samples (4 theca and 4 granulosa) from each of the 6 follicle sizes]. $^{a\text{-}b}$Means with different letters differ, $P < 0.05$.

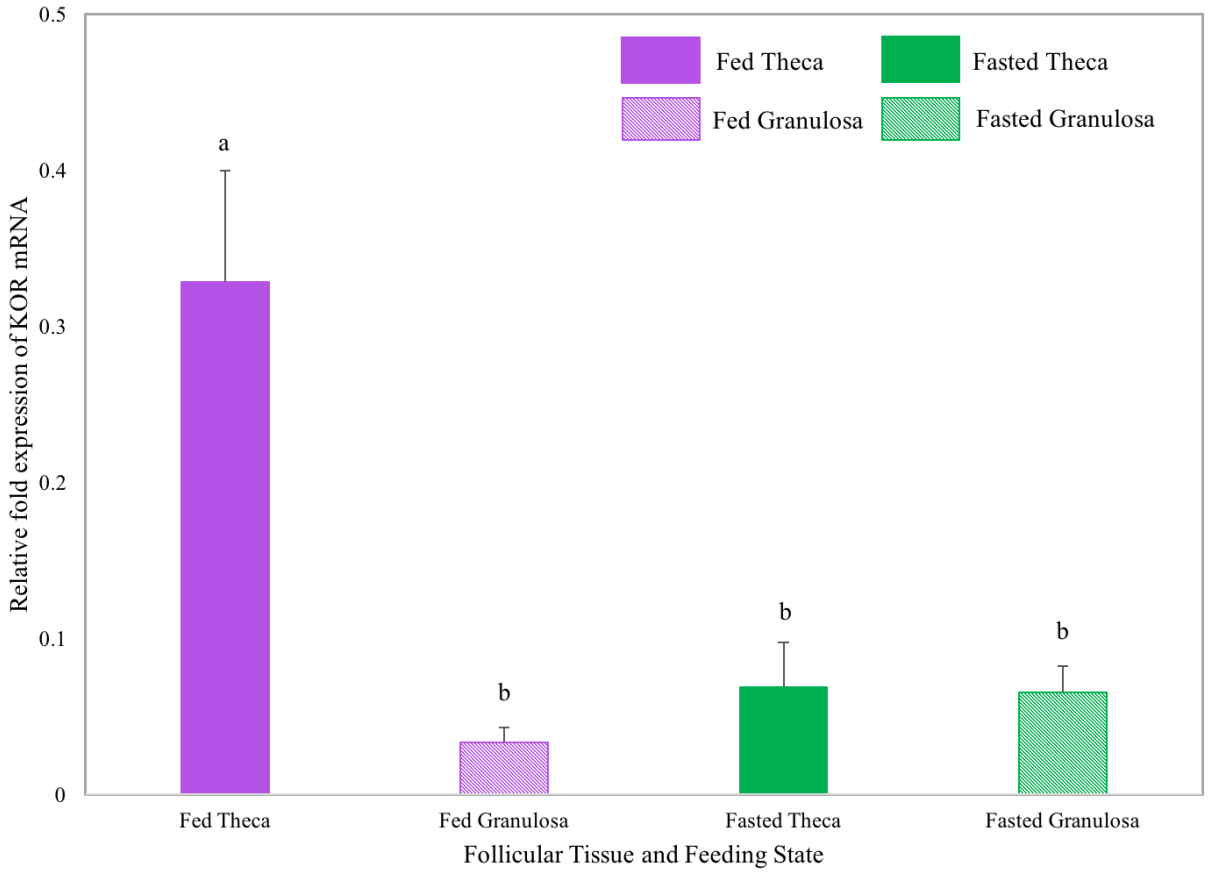

Figure 5.13. The overall relative fold expression of KOR mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles and the small yellow and large white follicles from 45 to 52-week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 24 (4 samples from each of the 6 follicle sizes]. $^{a-b}$Means with different letters differ, $P < 0.05$.

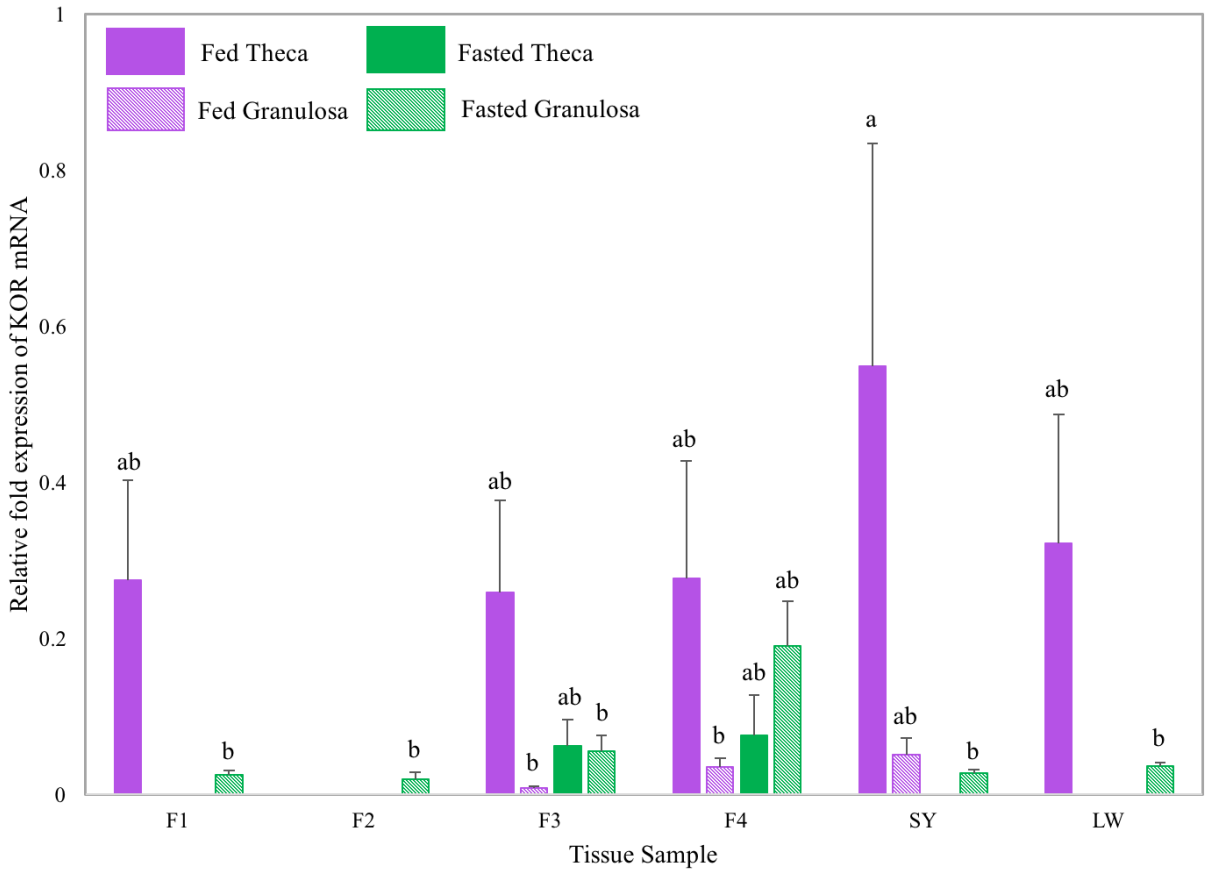

Figure 5.14. The relative fold expression of KOR mRNA in the theca or granulosa tissue collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles (SY), and the large white follicles (LW) from 45 to 52-week old broiler breeder hens fed daily (purple) or fasted (green) for 72 hours (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4 for each feeding state. $^{a\text{-}b}$Means with different letters differ, $P < 0.05$.

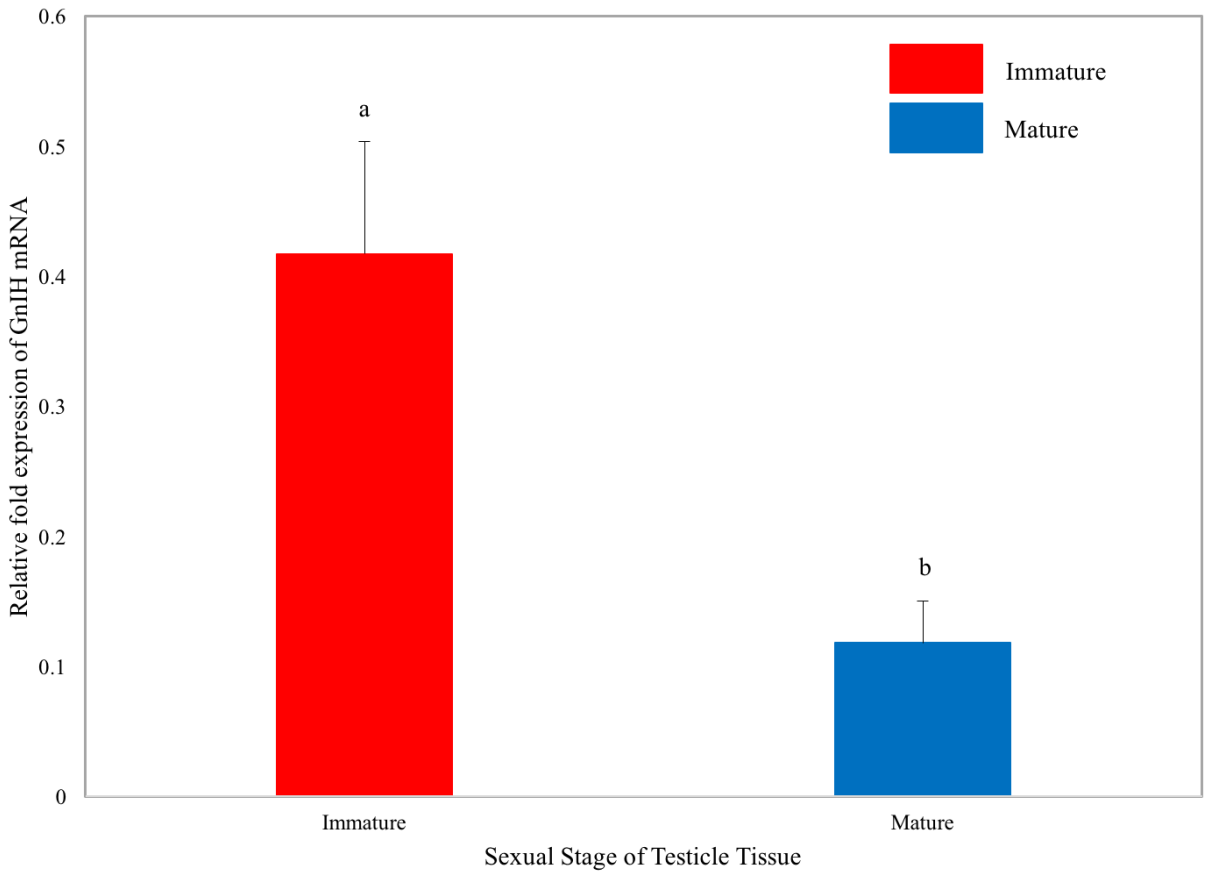

Figure 5.15. The relative fold expression of GnIH mRNA in the testicular tissue collected from 6-week old, immature broiler chickens and 62-week old, mature broiler breeder roosters (Experiment 5). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 10 for each sexual stage. $^{a-b}$Means with different letters differ, $P < 0.05$.

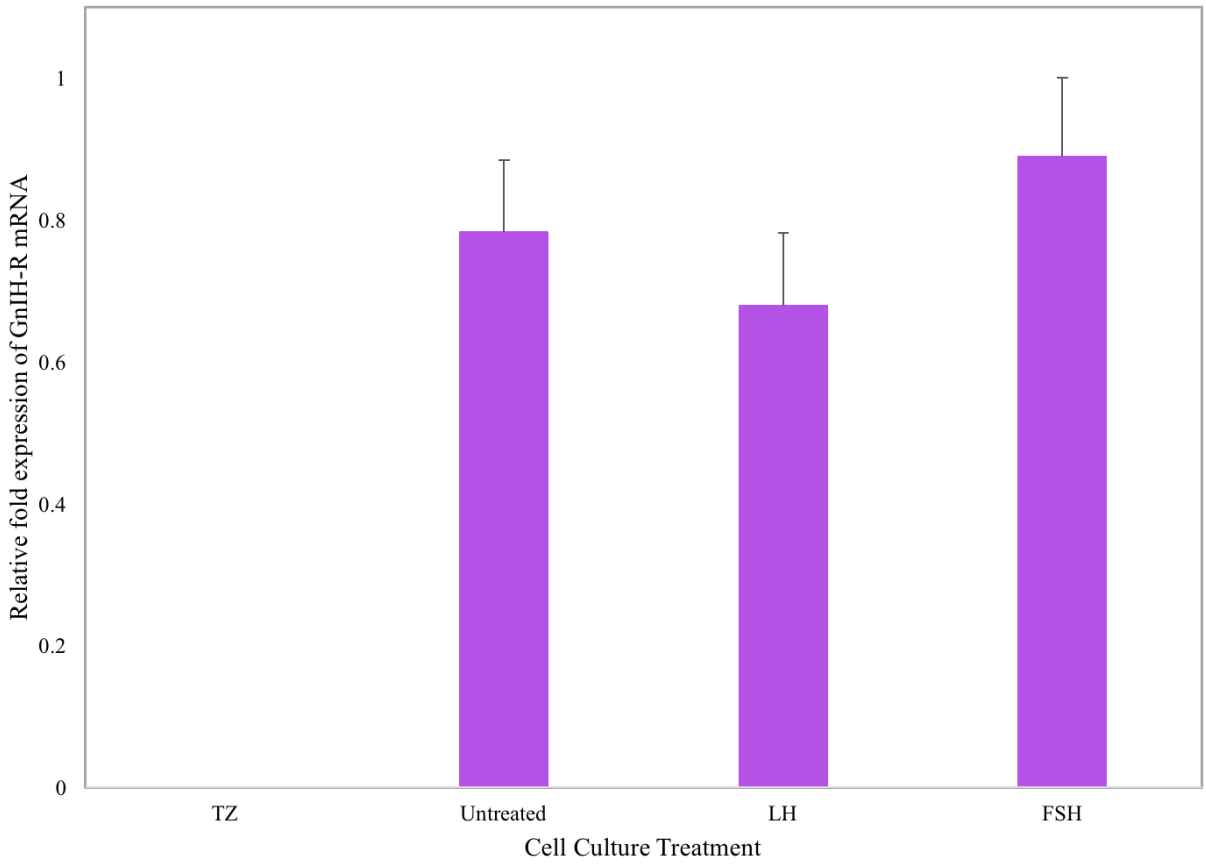

Figure 5.16. The relative fold expression of GnIH-R mRNA in granulosa cells from the F1 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). GnIH-R mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

In the F3 follicle, GnIH-R mRNA was also not detected in the freshly dispersed granulosa cells, but GnIH-R was detected in the F3 granulosa cells after 24 hours of culture with or without the gonadotropin hormones (Figure 5.17). There was no significant difference in GnIH-R mRNA expression level between untreated and gonadotropin-treated granulosa cells from the F3 follicle, however (Figure 5.17).

In contrast to the F1 and F3 granulosa cells, in the SY follicle, GnIH-R mRNA expression was significantly greater in freshly dispersed granulosa cells than in cells cultured for 24 hours (Figure 5.18). There were no differences in GnIH-R mRNA expression in cultured SY follicle granulosa cells treated with 0 or 50 ng/mL of LH or FSH (Figure 5.18).

Pdyn mRNA expression was significantly greater in freshly dispersed granulosa cells from the F1 follicle than in cells cultured for 24 hours (Figure 5.19). In comparing F1 granulosa cells cultured with 0 or 50 ng/mL of LH or FSH, there was no significant difference in Pdyn mRNA expression (Figure 5.19).

While Pdyn mRNA was not detected in the freshly dispersed granulosa cells from the F3 follicle, it was detected in F3 follicle granulosa cells after being cultured for 24 hours in the presence or absence of the gonadotropin hormones (Figure 5.20). In comparing the cultured F3 granulosa cells, the cells cultured with 50 ng/mL of LH or FSH had significantly reduced expression of Pdyn when compared to those cultured in the absence of the gonadotropin hormones (Figure 5.20).

In freshly dispersed granulosa cells from SY follicles, Pdyn mRNA was not detected (Figure 5.21). Pdyn mRNA was detected in the SY granulosa cells that were cultured for 24 hours, but the expression of Pdyn did not significantly differ between untreated and gonadotropin-treated cells (Figure 5.21).

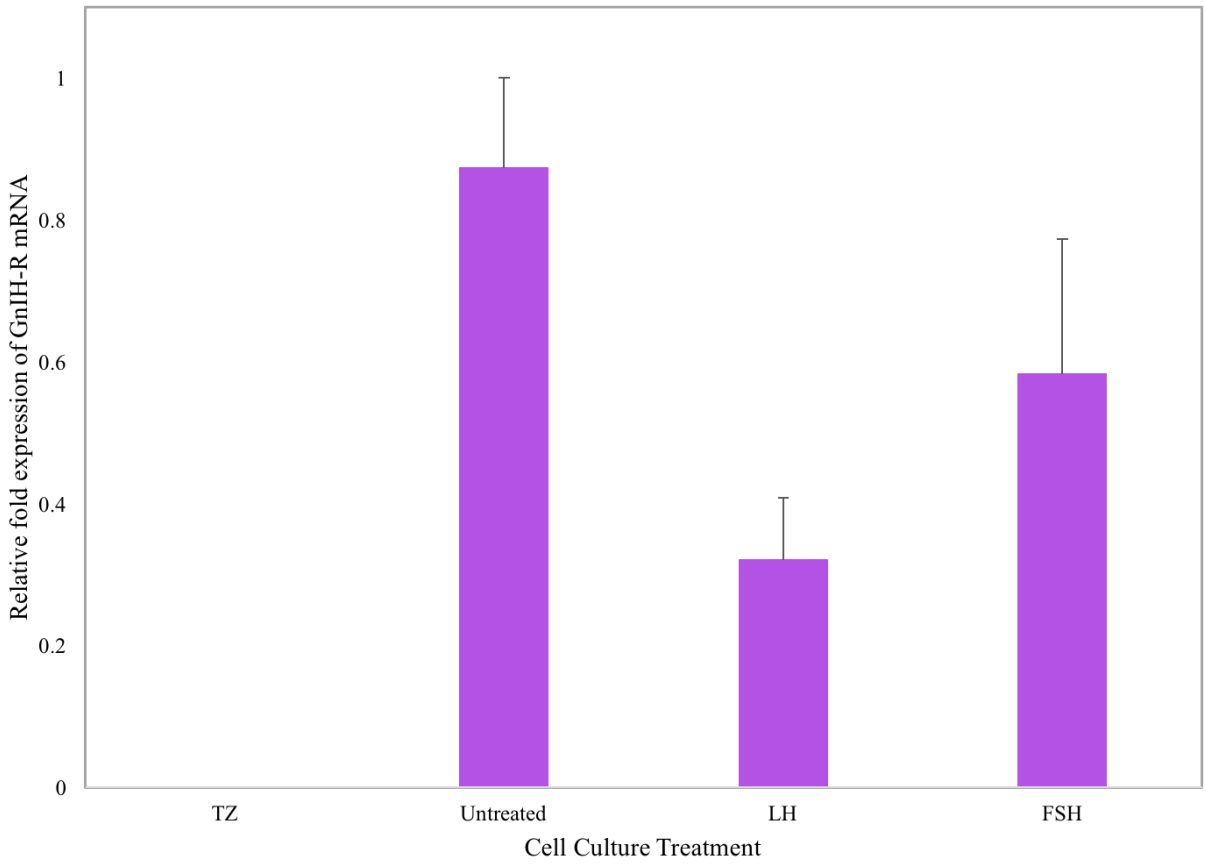

Figure 5.17. The relative fold expression of GnIH-R mRNA in granulosa cells from the F3 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). GnIH-R mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

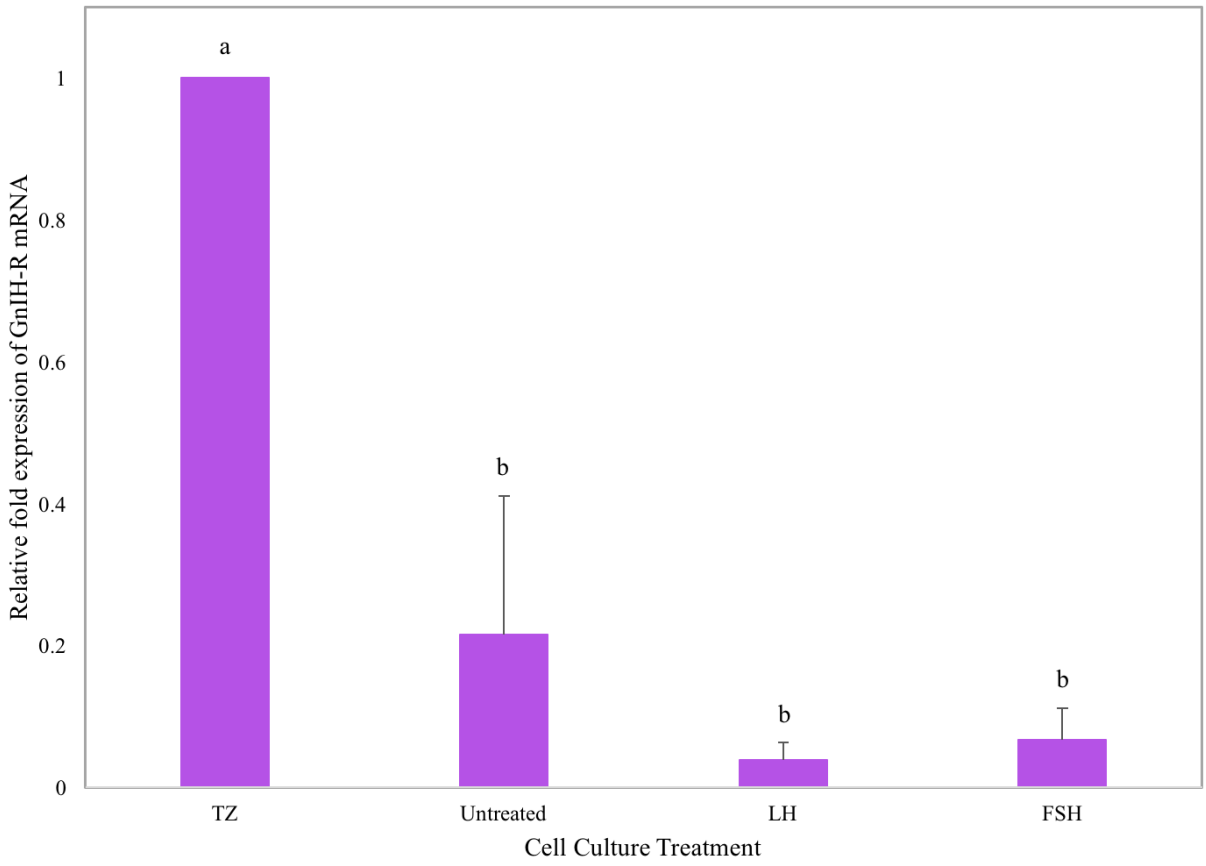

Figure 5.18. The relative fold expression of GnIH-R mRNA in granulosa cells from the SY follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). GnIH-R mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. $^{a-b}$Means with different letters differ, $P < 0.05$.

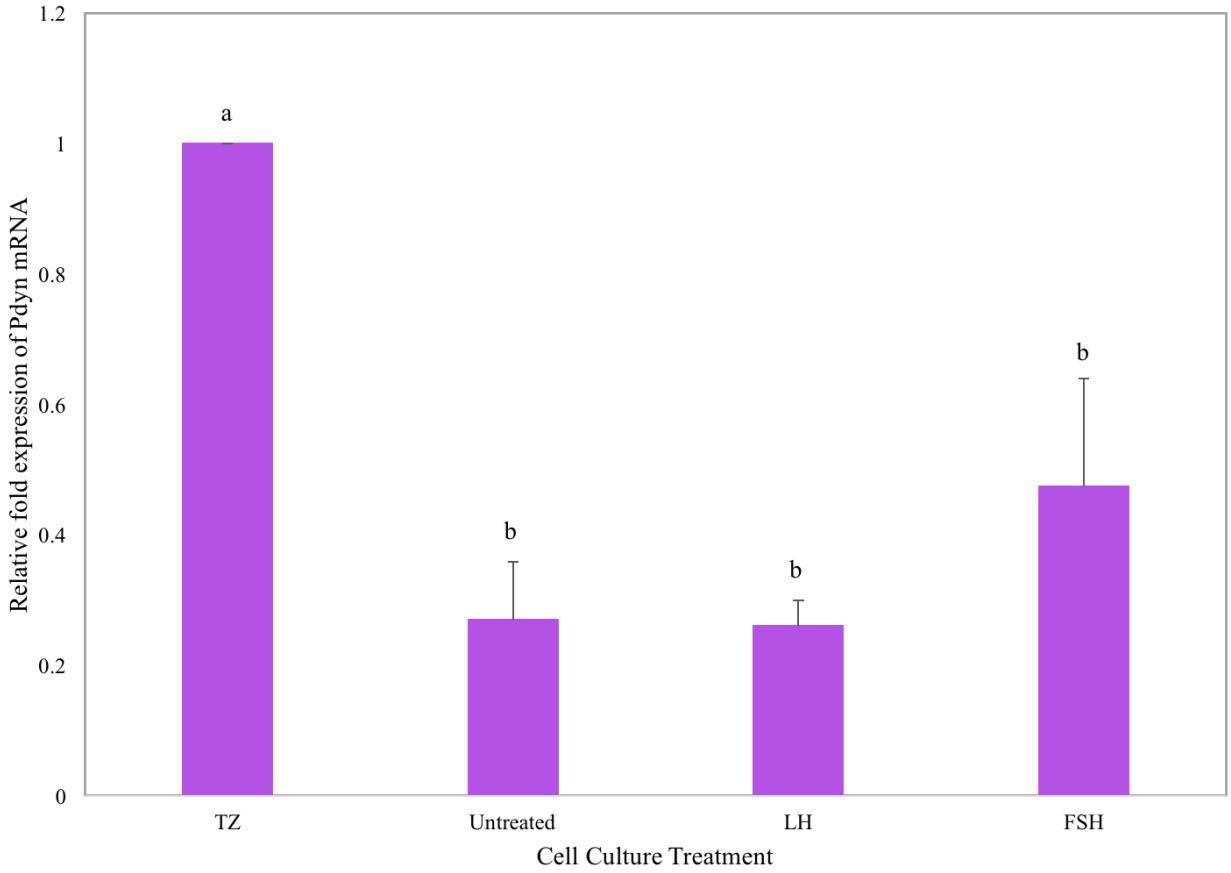

Figure 5.19. The relative fold expression of Pdyn mRNA in granulosa cells from the F1 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). Pdyn mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. $^{a\text{-}b}$Means with different letters differ, $P < 0.05$.

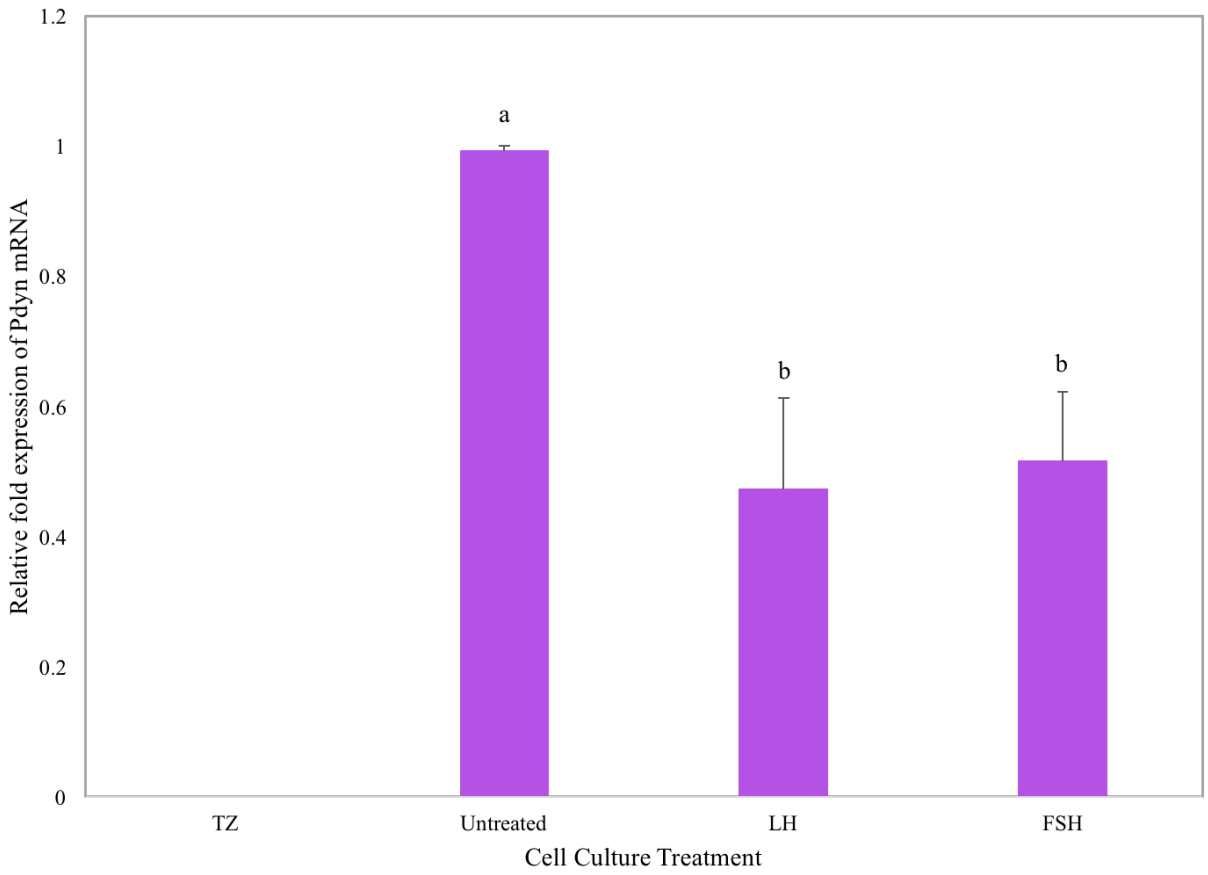

Figure 5.20. The relative fold expression of Pdyn mRNA in granulosa cells from the F3 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). Pdyn mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. $^{a\text{-}b}$Means with different letters differ, $P < 0.05$.

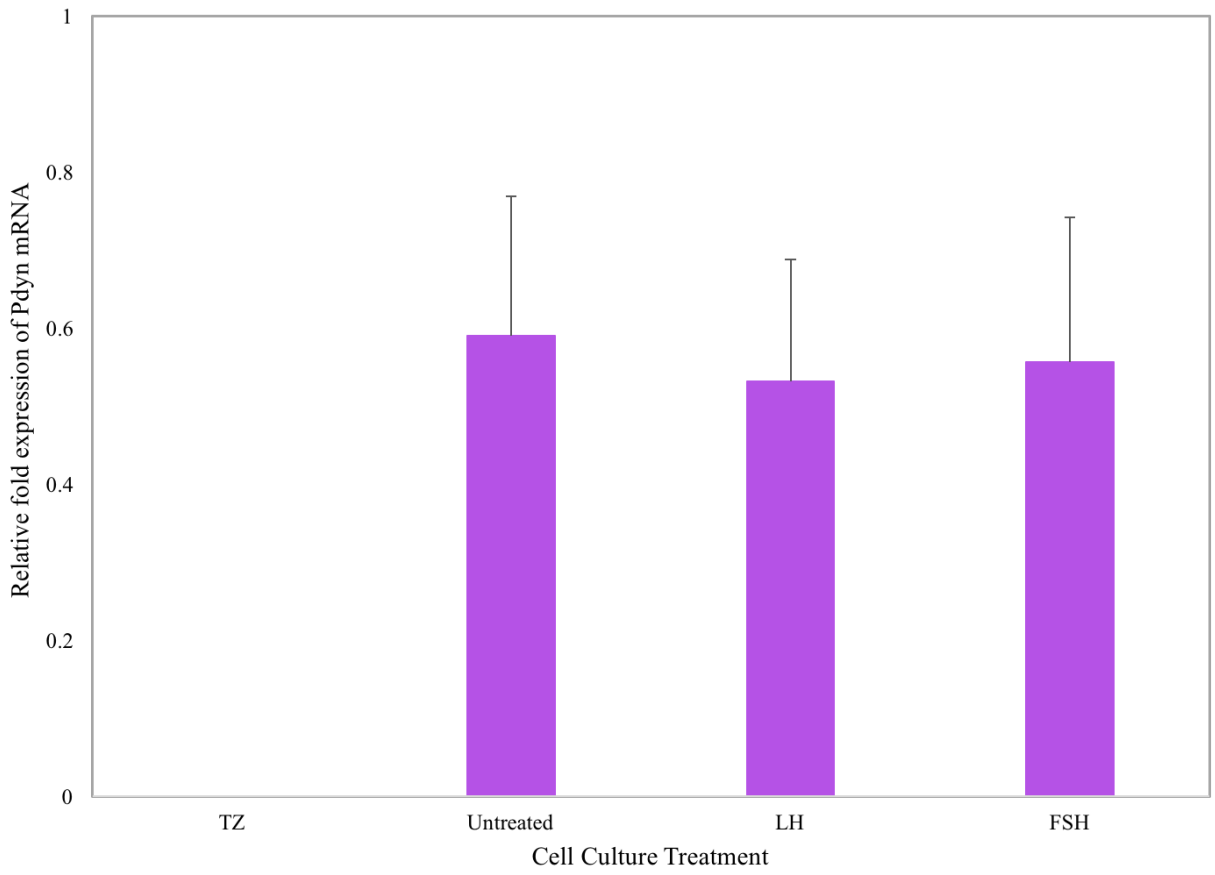

Figure 5.21. The relative fold expression of Pdyn mRNA in granulosa cells from the SY follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). Pdyn mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

In freshly dispersed granulosa cells from the F1 follicle, KOR mRNA was not detected (Figure 5.22). However, after being cultured with or without the gonadotropin hormones for 24 hours, KOR mRNA was detected in the granulosa cells of the F1 follicle, though expression in the granulosa cells did not differ between untreated and gonadotropin-treated cells (Figure 5.22).

KOR mRNA was not detected in the freshly dispersed or cultured granulosa cells from the F3 or SY follicles.

Progesterone concentration in the media of the granulosa cells cultured with LH was significantly greater than the progesterone accumulation in the untreated cell cultures for F1 and F3 follicles, but not for the SY follicle (Table 5.1). Addition of FSH to the granulosa cell culture media significantly increased the accumulation of progesterone for the granulosa cells from all three follicle sizes (Table 5.1).

Experiment 7

In the F1 follicle, GnIH-R mRNA was not detected in freshly dispersed granulosa cells, but GnIH-R was detected in the F1 granulosa cells after 24 hours of culture with or without 17-ß-estradiol (E2) or testosterone (T; Figure 5.23). Amongst the cultured F1 follicle granulosa cells, GnIH-R mRNA was significantly higher in the cells exposed to T than in the untreated cells (Figure 5.23).

GnIH-R mRNA was not detected in the freshly dispersed granulosa cells from the F3 follicle, but GnIH-R was detected in the F3 granulosa cells after 24 hours of culture in the presence or absence of E2 and T (Figure 5.24). There was no significant difference in GnIH-R mRNA expression level between untreated, E2-, and T-treated granulosa cells from the F3 follicle (Figure 5.24)

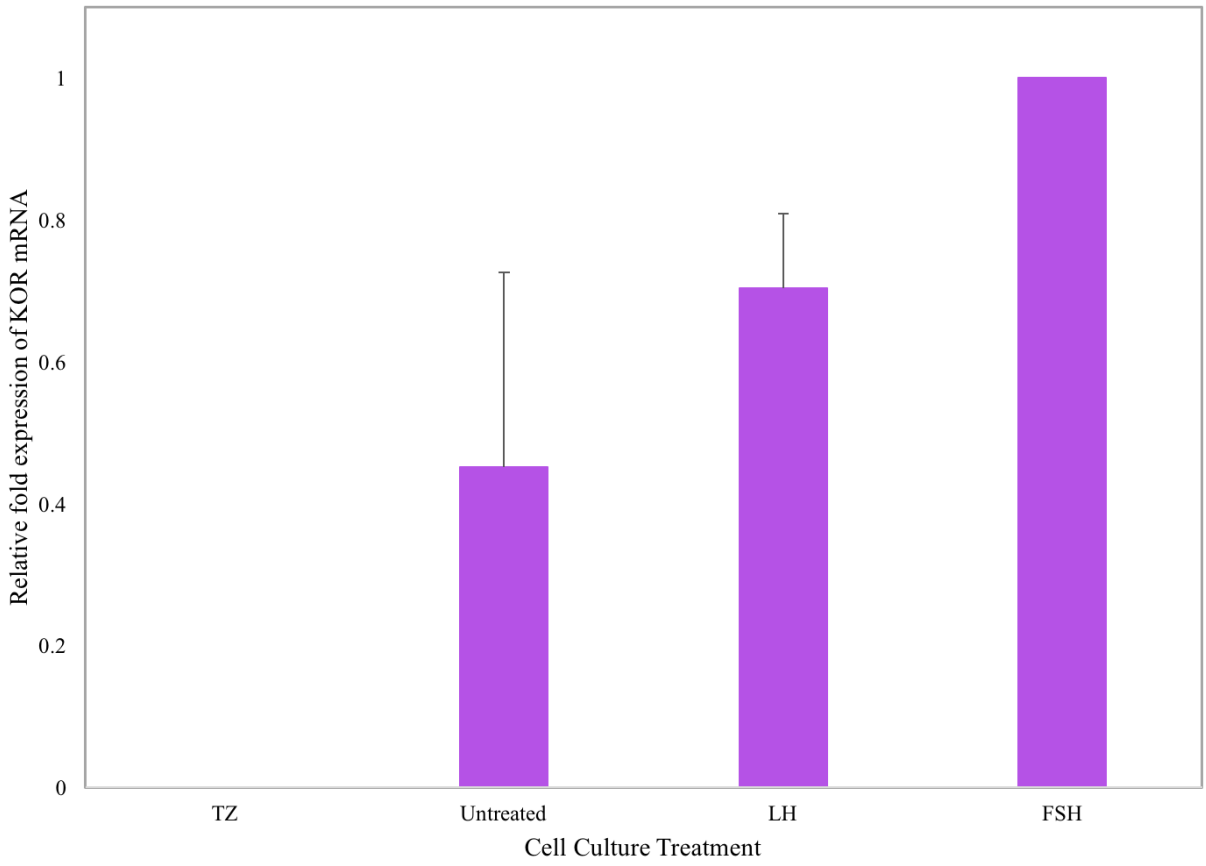

Figure 5.22. The relative fold expression of KOR mRNA in granulosa cells from the F1 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 50 ng/mL culture media of luteinizing hormone (LH) or follicle stimulating hormone (FSH; Experiment 6). KOR mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

Table 5.1. Progesterone concentrations in granulosa cell culture media from F1, F3, or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 50 ng/ml culture media of LH or FSH.

	Progesterone concentration[1] (ng/ml)
F1	
Control	356 ± 35[a]
LH	3600 ± 390[b]
FSH	1800 ± 346[c]
F3	
Control	134 ± 9[a]
LH	1900 ± 179[b]
FSH	1460 ± 154[c]
SY	
Control	0.168 ± 0.08[a]
LH	0.959 ± 0.46[ab]
FSH	1.275 ± 0.54[b]

[a-c]Values with different superscripts for a given follicle size differ ($P < 0.05$).

[1]Values are means ± SEM, n = 4 replicate experiments.

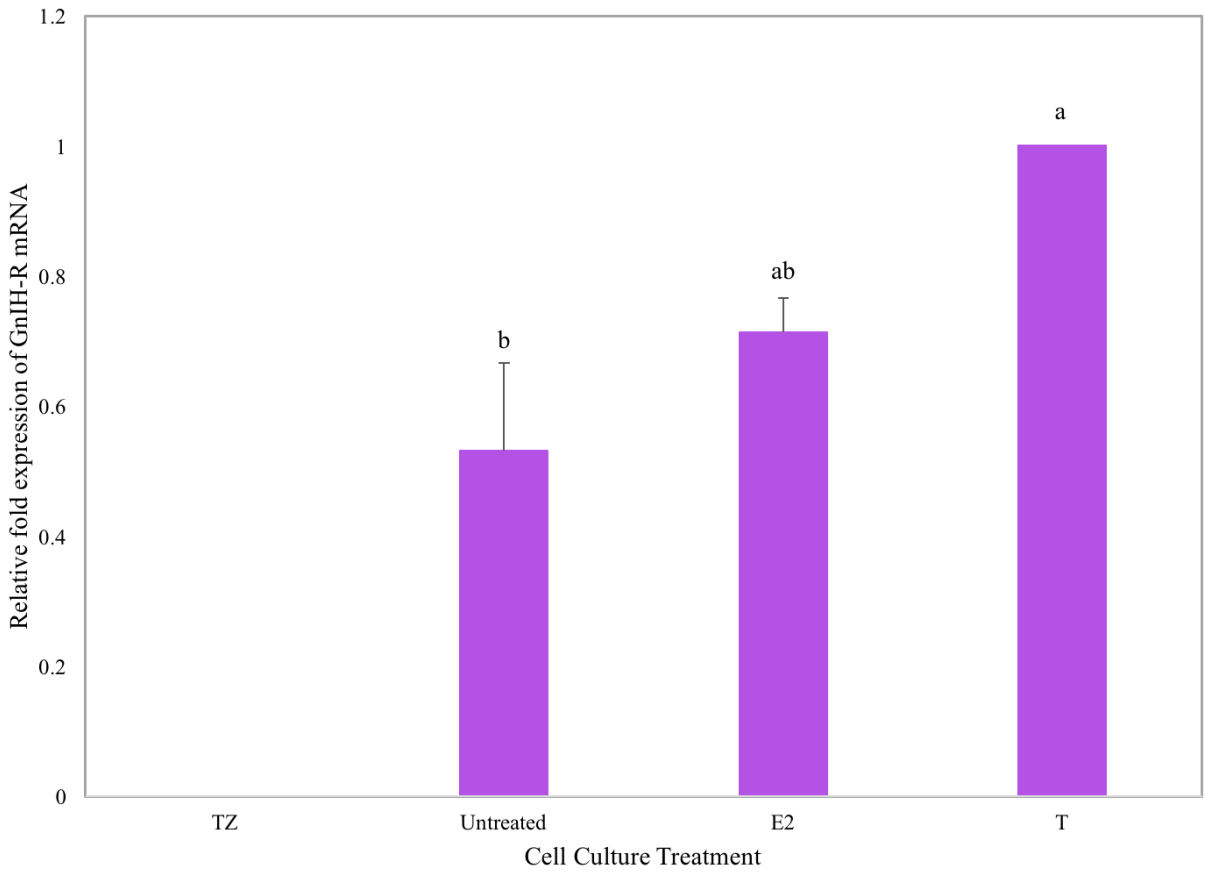

Figure 5.23. The relative fold expression of GnIH-R mRNA in granulosa cells from the F1 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). GnIH-R mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. $^{a-b}$Means with different letters differ, $P < 0.05$.

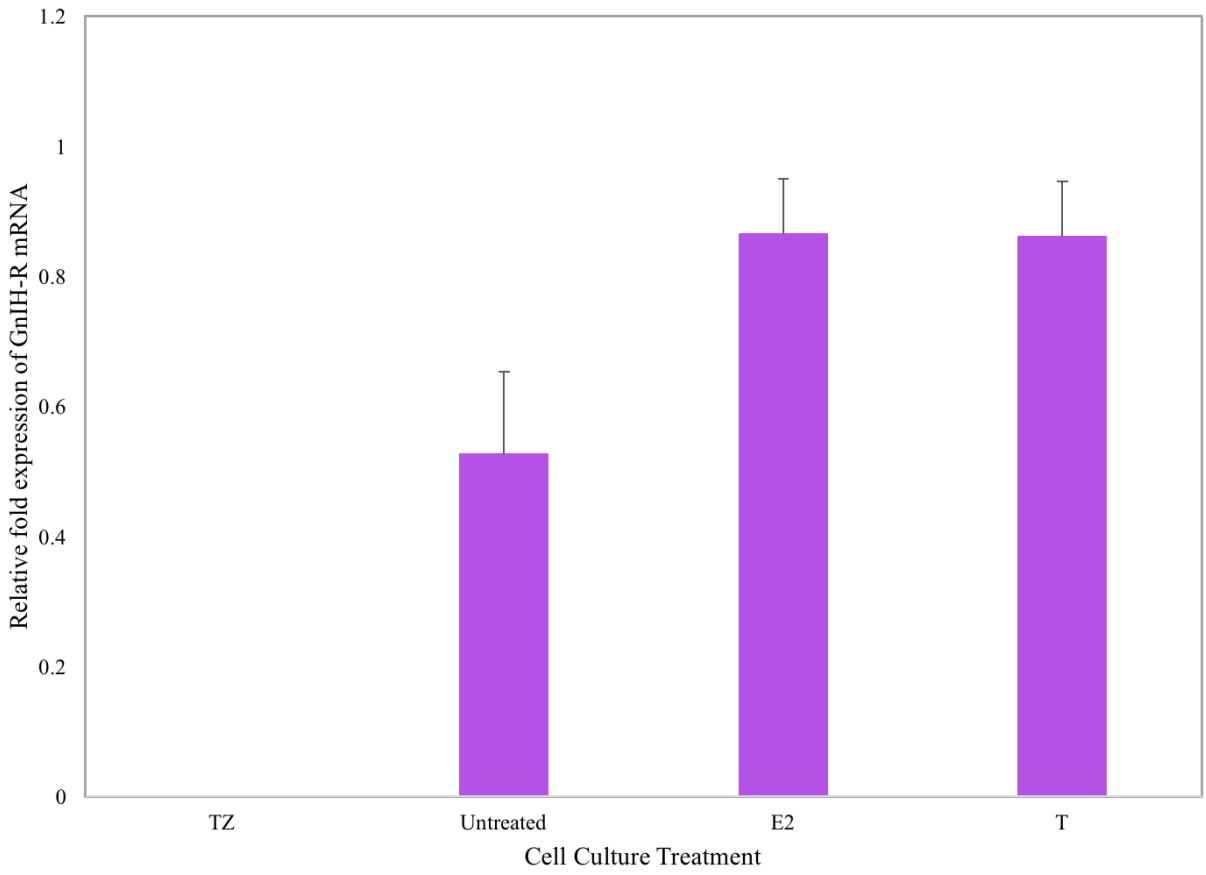

Figure 5.24. The relative fold expression of GnIH-R mRNA in granulosa cells from the F3 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). GnIH-R mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

In the SY follicle, GnIH-R mRNA expression was significantly greater in freshly dispersed granulosa cells than in cells cultured for 24 hours (Figure 5.25). There were no differences in GnIH-R mRNA expression amongst cultured SY follicle granulosa cells that were untreated, E2-, or T-treated (Figure 5.25).

Pdyn mRNA was detected in freshly dispersed granulosa cells from the F1 follicle and in all cultured F1 granulosa cells, with no significant differences in Pdyn mRNA expression (Figure 5.26). Pdyn mRNA was not detected in the freshly dispersed granulosa cells from the F3 follicle, but it was detected in F3 follicle granulosa cells after being cultured for 24 hours in the presence or absence of the E2 and T (Figure 5.27). However, no significant differences in Pdyn mRNA expression were noted amongst cultured F3 follicle granulosa cells (Figure 5.27).

Pdyn mRNA was not detected in freshly dispersed granulosa cells from SY follicles (Figure 5.28). In the SY granulosa cells that were cultured for 24 hours, Pdyn mRNA was detected, but the expression of Pdyn did not significantly differ between untreated, E2-, and T-treated cells (Figure 5.28).

KOR mRNA was not detected in freshly dispersed granulosa cells from the F1 follicle, (Figure 5.29). Following 24 hours in culture with or without the E2 or T, KOR mRNA was detected in the granulosa cells of the F1 follicle and was significantly higher in the cells exposed to E2 and T than in the untreated cells (Figure 5.29). KOR mRNA was not detected in the freshly dispersed or cultured granulosa cells from the F3 or SY follicles.

Progesterone concentration in the media of the granulosa cells cultured with E2 did not differ significantly from the progesterone concentration in the media from untreated cultured granulosa cells from F1, F3, or SY follicles (Table 5.2). Addition of T to the granulosa cell

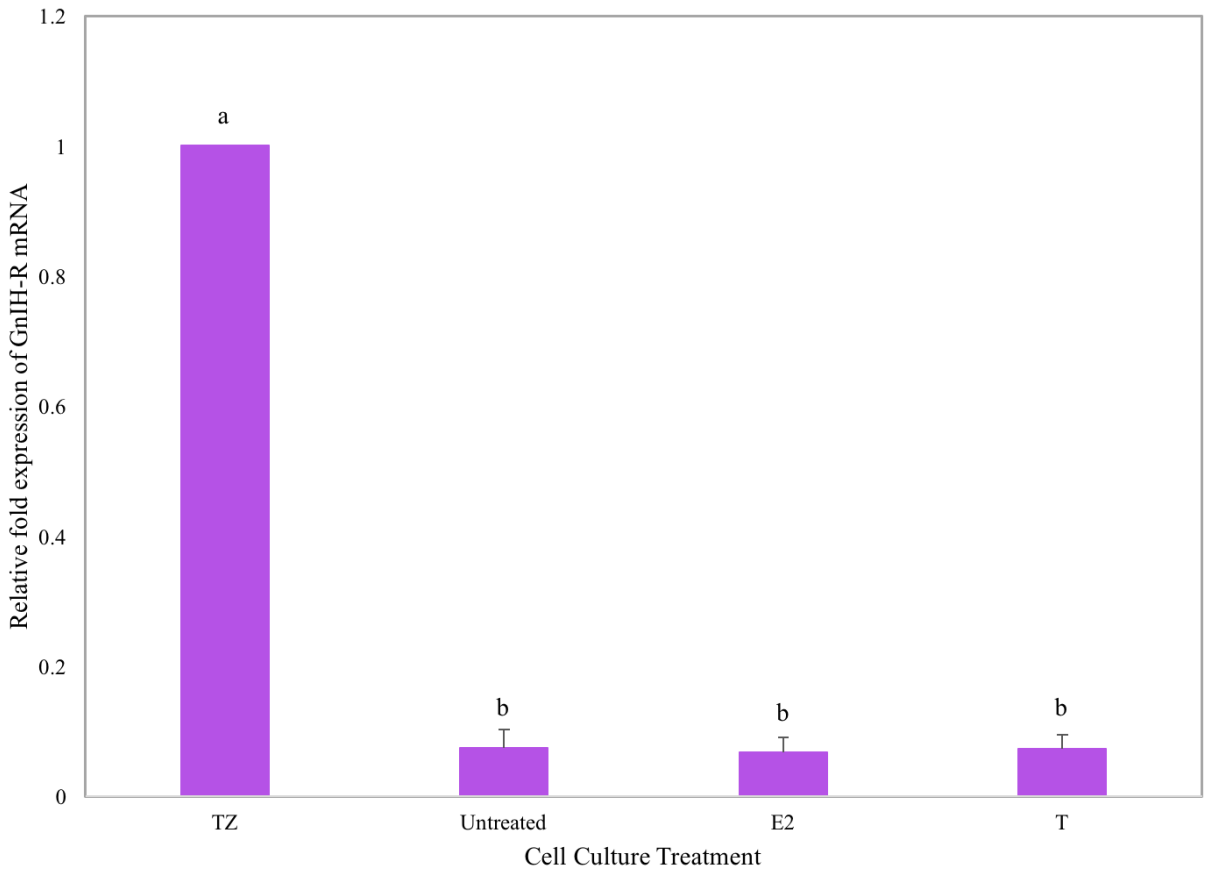

Figure 5.25. The relative fold expression of GnIH-R mRNA in granulosa cells from the SY follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). GnIH-R mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. $^{a-b}$Means with different letters differ, $P < 0.05$.

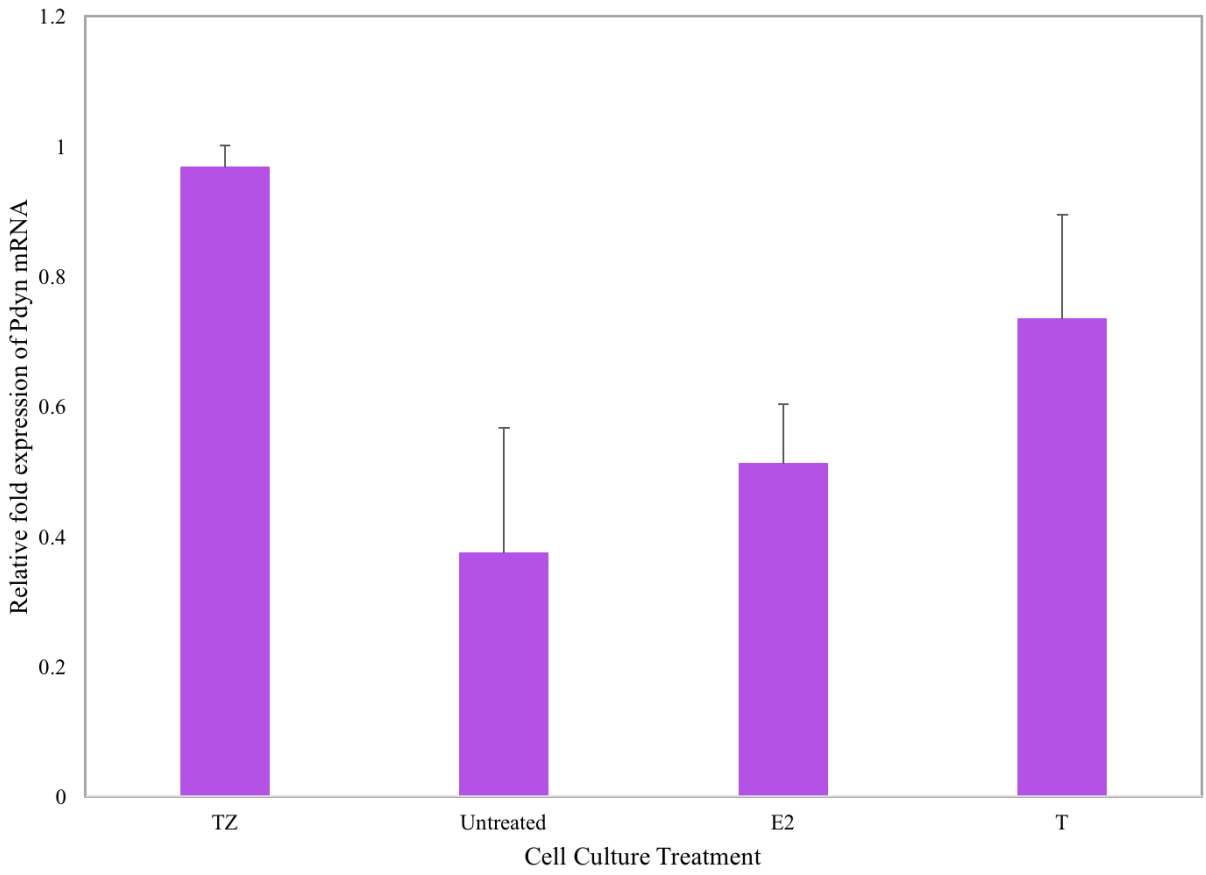

Figure 5.26. The relative fold expression of Pdyn mRNA in granulosa cells from the F1 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). Pdyn mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

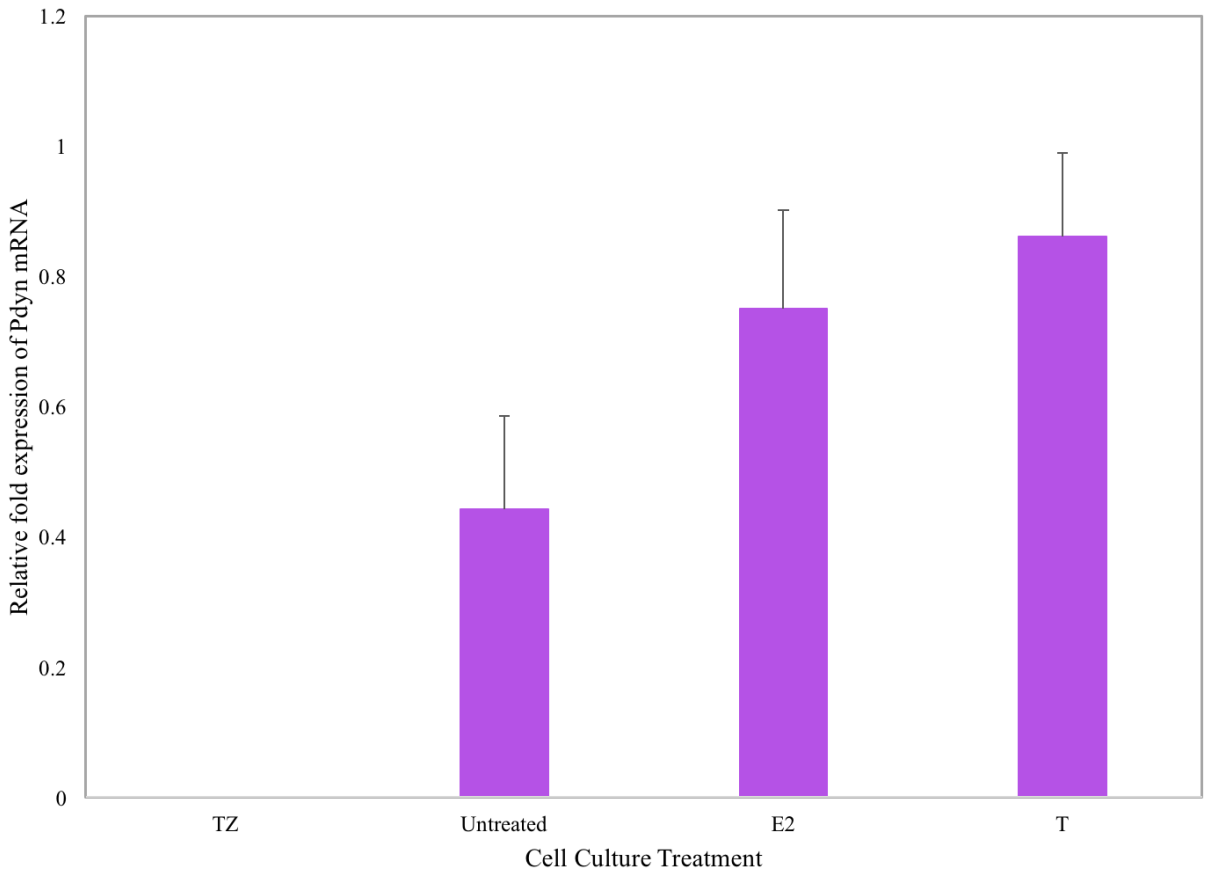

Figure 5.27. The relative fold expression of Pdyn mRNA in granulosa cells from the F3 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). Pdyn mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

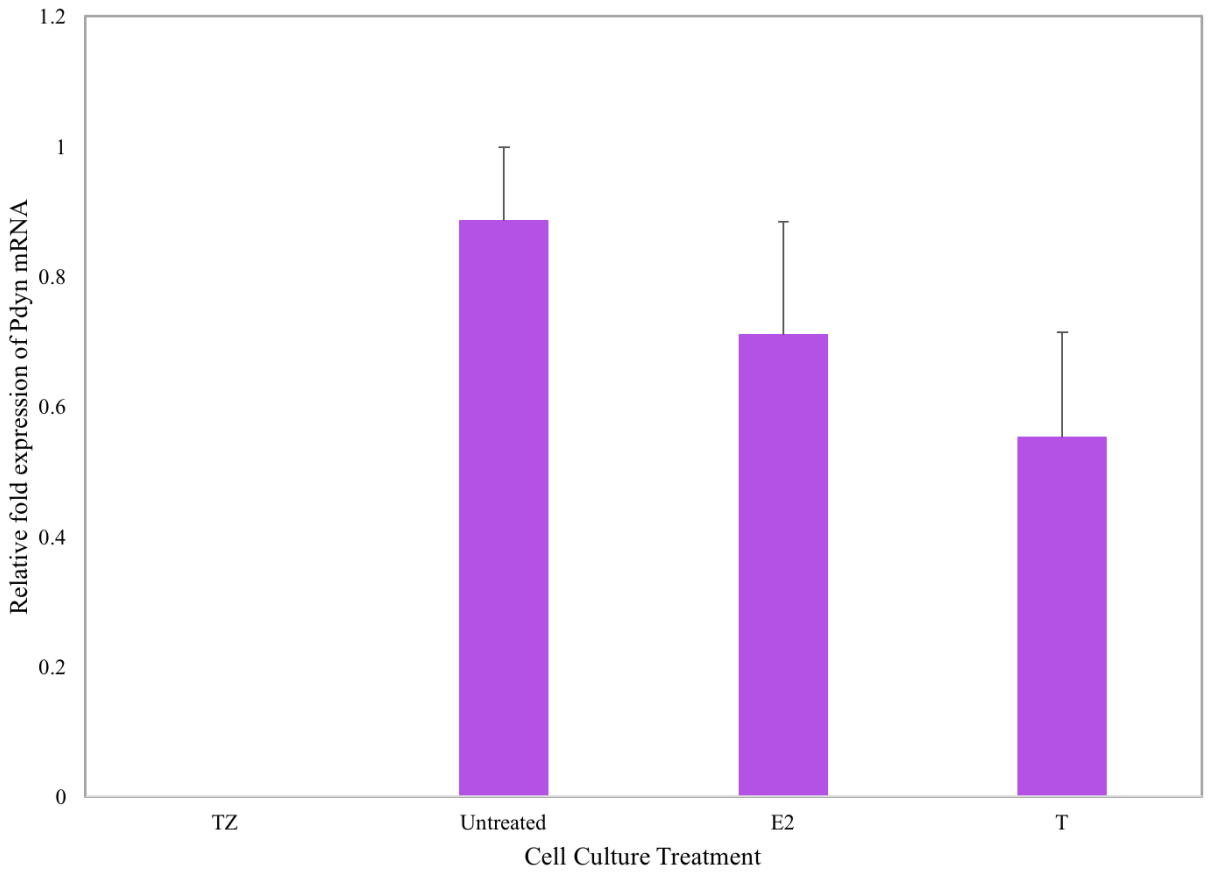

Figure 5.28. The relative fold expression of Pdyn mRNA in granulosa cells from the SY follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). Pdyn mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4.

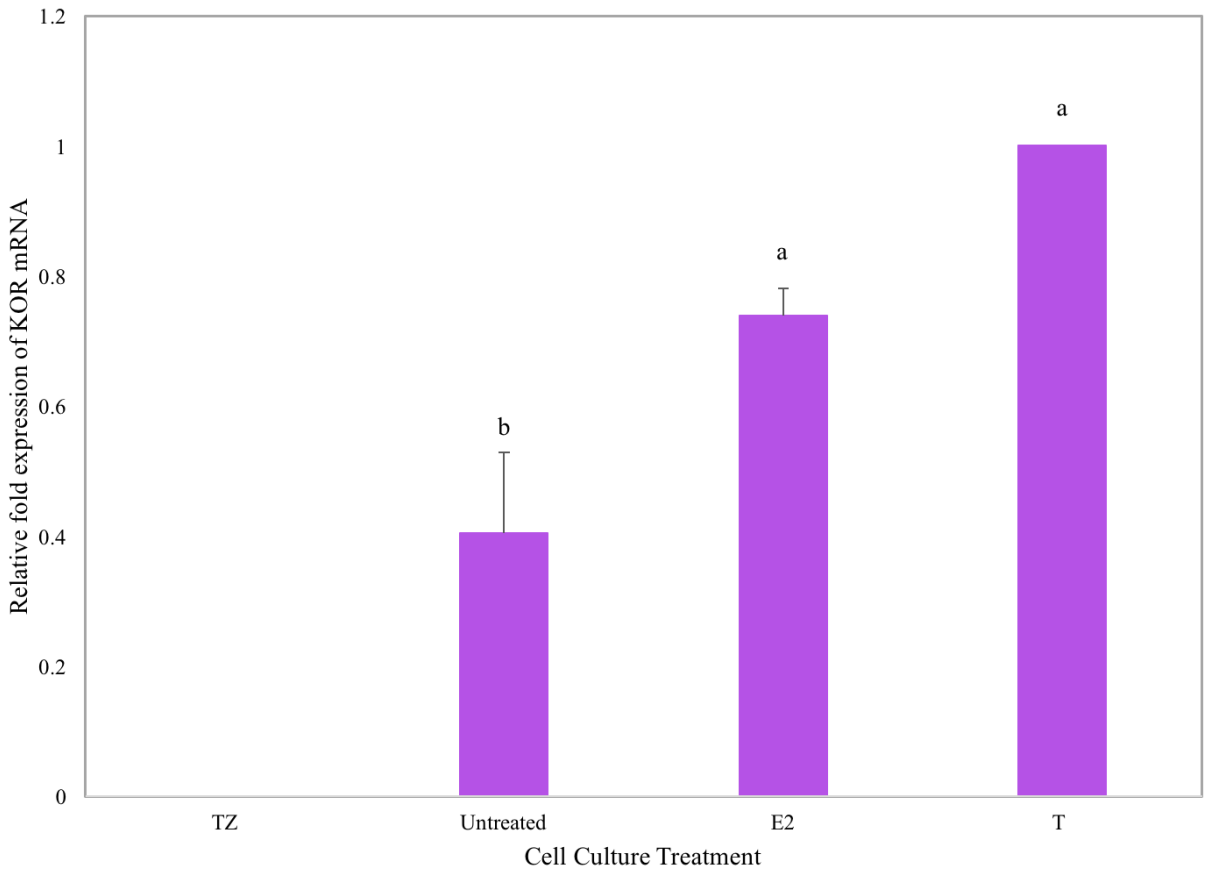

Figure 5.29. The relative fold expression of KOR mRNA in granulosa cells from the F1 follicle cultured for 0 (time zero, TZ) or 24 hours in the presence of 0 (control) and 1 x 10^{-6} M culture media of 17-ß-estradiol (E2) or testosterone (T; Experiment 7). KOR mRNA expression values were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$) ± SEM, n = 4. $^{a-b}$Means with different letters differ, $P < 0.05$.

Table 5.2. Progesterone concentrations in granulosa cell culture media from F1, F3, or small yellow (SY) follicles cultured for 24 hours in the presence of 0 (control) and 1 x 10^{-6} M of estrogen or testosterone.

	Progesterone concentration[1] (ng/ml)
F1	
Control	335 ± 39[a]
Estrogen	297 ± 40[a]
Testosterone	619 ± 68[b]
F3	
Control	150 ± 16[a]
Estrogen	137 ± 12[a]
Testosterone	679 ± 36[b]
SY	
Control	0.40 ± 0.1[a]
Estrogen	0.29 ± 0.1[a]
Testosterone	3.24 ± 0.49[b]

[a-b]Values with different superscripts for a given follicle size differ ($P < 0.05$).

[1]Values are means ± SEM, n = 4 replicate experiments.

culture media significantly increased the production of progesterone by the granulosa cells from all three follicle sizes (Table 5.2).

CHAPTER 6

DISCUSSION

Gonadotropin Inhibitory Hormone and Gonadotrophin Inhibitory Hormone Receptor

In the current research, GnIH mRNA expression was detected in the pituitary tissue of the broiler breeder hen. Furthermore, the expression of GnIH mRNA in the pituitary tissue significantly increased after 72 hours of fasting. Given that Chowdhury et al. (2012) previously reported an increase in hypothalamic GnIH precursor mRNA expression following a heat-induced fasting period in broiler chicks, the increased expression observed in the broiler breeder hen following a 72 hour fast is reasonable. However, pituitary expression of GnIH precursor mRNA had not been previously investigated in chickens; in fact, the only documented reports of GnIH mRNA expression specific to the pituitary tissue were made by Bentley et al. (2008), who detected the neuropeptide in the pituitary gland of the quail but not the European starling.

Additionally, the mRNA for the GnIH-R was detected in broiler breeder hen pituitary tissue, which mirrored previous work completed in Leghorn hens (Maddineni et al., 2008a). However, contrary to its neuropeptide ligand, the mRNA expression of the GnIH-R in the pituitary tissue did not differ based on feeding state.

While GnIH expression was detected in the pituitary tissue of the broiler breeder hen in the current research, GnIH mRNA was not detected in the theca or granulosa tissues of the hierarchical or prehierarchical follicles from fed or fasted hens. Previously, GnIH mRNA was

detected in the ovary of the Japanese quail and the European starling (Bentley et al., 2008). However, Maddineni et al., (2008b) was unable to detect GnIH in Leghorn hen ovarian tissue.

To further verify that the real time PCR GnIH primer-probe set used in the current research was robust and that the lack of detection of GnIH mRNA in broiler breeder hens was real, testes expression of GnIH in broilers and broiler breeder roosters was examined. Previously, GnIH mRNA had been detected in the testes of the Japanese quail (Bentley et al., 2008), European starling (Bentley et al., 2008), and house sparrow (McGuire and Bentley, 2010). Therefore, the detection of GnIH mRNA in the testicular tissues of sexually immature broilers and sexually mature broiler breeder roosters provided further confirmation for the absence of GnIH expression observed in the follicular tissues of the broiler breeder hen. Additionally, the greater expression of GnIH mRNA observed in immature testicular tissues when compared to mature testicle tissues is sensible, given that increased GnIH production in the testes may be involved in inhibition of testicular development until sexual maturation is reached.

The mRNA encoding for the GnIH-R was detected in the ovarian follicular tissues of fed and fasted broiler breeder hens, and while there were no significant differences in GnIH-R expression detected amongst follicles based on feeding state, a similar pattern of expression was displayed in follicular tissues collected from fed and fasted hens. Generally, GnIH-R mRNA expression was higher in prehierarchical follicles when compared to hierarchical follicles. The heightened responsiveness of these smaller follicles to GnIH could be associated with the atretic fate of the overwhelming majority of these prehierarchical follicles (Gilbert et al., 1983a).

This heightened sensitivity of prehierarchical follicles to GnIH relative to hierarchical follicles was also supported by the results of the cell culture experiments. GnIH-R mRNA was not detected in freshly isolated granulosa cells of the F1 or F3 follicles in either the gonadotropin

or steroid cell culture experiments. However, after 24 hours in cell culture, GnIH-R mRNA was expressed in the granulosa cells isolated from these follicles, which indicates that the expression of GnIH-R is normally repressed in vivo in the granulosa cells of these hierarchical follicles. Interestingly, FSH and LH as well as estrogen and testosterone, the very hormones that are necessary for follicular maturation and follicular hierarchy maintenance, are not the hormones directly involved in repressing GnIH-R mRNA expression. In addition, because, as expected, culturing granulosa cells in the presence of LH, FSH, and testosterone stimulated progesterone production, GnIH-R mRNA expression is also not negatively regulated by this sex hormone in hierarchical follicles.

Actually, in cultured F1 follicle granulosa cells, the addition of testosterone to the culture media resulted in a significantly increase in GnIH-R mRNA expression. At the time of their collection, the granulosa cells of the F1 follicle have halted production of androgens for exclusive production of progesterone to stimulate ovulation. The abnormal presence of high levels of testosterone in the cell culture media surrounding F1 follicle granulosa cells could be a signal to these cells that conditions are not normal, and the cells may respond by upregulating expression of GnIH-R mRNA as a means of heightening their response to GnIH for inhibition of normal ovulatory function.

In contrast to the F1 and F3 hierarchical follicle granulosa cells, there is abundant GnIH-R mRNA expression in the freshly isolated granulosa cells of the small yellow (SY) follicles (experiments 2, 6, and 7). After 24 hours in cell culture, the expression of GnIH-R significantly decreases, and the presence of LH, FSH, estrogen, testosterone, and progesterone in the cell culture media does not alter this decrease. It is important to remember that the granulosa cells used for the cell culture experiments come from a pool of all the SYF on the ovary of the broiler

breeder hens. Only one of these small yellow follicles will be selected to join the hierarchy, while the other unselected SYF will undergo atresia (Gilbert et al., 1983a; Johnson et al., 1996a). If sensitivity to GnIH induces follicular regression, it would be expected that high levels of GnIH-R would be found in all the SYF except the one that is going to be selected, and thus, GnIH-R expression would be high in a pooled sample of granulosa cells collected from the SYF, as is seen in the current research. However, once these granulosa cells from the pool of predominately unselected SYF is put in culture, the in vivo signals leading them towards atresia are no longer present, and the sensitivity to GnIH decreases. This hypothesis is further supported by the stimulation of progesterone in these cultured SYF granulosa cells. Based on research findings (Johnson et al., 1996a, 1999) in vivo, it is assumed that it is only the SYF that is selected, which has the capability to produce progesterone. However, the cultured SYF granulosa cells, which would have been predominately derived from the unselected follicles, produced progesterone and augmented this production when stimulated by gonadotropins or testosterone. Thus, this supports the contention that, in culture, the granulosa cells from these unselected SYF were no longer functioning as cells heading for apoptosis and atresia. Future research needs to examine individual SYF to determine if GnIH-R expression is limited to those SYF destined not to be selected.

Given the absence of GnIH mRNA expression in the ovarian follicular tissues, locally produced GnIH is not able to regulate reproductive function within the ovary of the broiler breeder hen. However, the presence of GnIH-R mRNA within the follicular tissues indicates that there is a functional role for this receptor. Our finding that GnIH mRNA is expressed in the pituitary gland and is up-regulated by fasting in the broiler breeder hen could provide an explanation for the purpose of the GnIH-R within the theca and granulosa cells of the developing

follicles, as pituitary-produced GnIH could travel systemically to the ovary to interact with the GnIH-R in order to negatively regulate follicular development. It is important to note that fasting Leghorn hens for about 24 to 30 hours and fasting broiler breeder hens for 80 to 96 hours results in atresia of the hierarchical follicles (Freeman, 2008). The signal that initiates this fasting follicular atresia is unknown, but it could be GnIH. While the current results indicate that GnIH-R expression is normally repressed in the granulosa cells of hierarchical follicles, fasting for 72 hours increased GnIH-R expression to detectable levels in F2 follicle granulosa cells and tended to stimulate its expression in the F3 and F4 follicles.

To help further elucidate the potential role of GnIH in fasting induced atresia of preovulatory follicles, future research should determine if plasma GnIH levels mirror that of the mRNA for GnIH in the pituitary tissue of fed and fasted broiler breeder hens. Further, it will be important to determine what signal associated with fasting in the broiler breeder hen is responsible for the upregulation in GnIH mRNA expression in the pituitary gland. Future research should also identify the factor(s) responsible for normally repressing transcription of the GnIH-R gene in the granulosa cells of the hierarchical follicles. Finally, it will be important to determine with future research if GnIH has a negative impact on hierarchical follicle granulosa cell function by exposing these cells to GnIH in cell culture.

Dynorphin and Kappa Opioid Receptor

To our knowledge, the present research is the first to characterize the existence and expression of Pdyn and KOR in the pituitary and follicular tissues of any avian species. The detection of Pdyn and KOR mRNA in the pituitary tissues of the broiler breeder hen aligns with

previous findings in mammals (Goldstein et al., 1979; Khachaturian et al., 1985, 1986; Mansour et al., 1988, 1995).

In addition to expression in the pituitary tissue, Pdyn mRNA was also detected in the ovarian follicular tissues of the broiler breeder hen. In follicles collected from hens fed daily, the expression of Pdyn mRNA was exclusive to the granulosa tissue, and Pdyn expression was highest in the F1 follicle, when compared to the other hierarchical follicles. Expression of Pdyn mRNA in follicular granulosa tissue has also been observed in female rats (Kaynard et al., 1992b). Fasting did not alter the mRNA expression of Pdyn in the granulosa tissue, but it did consistently induce Pdyn mRNA expression in the theca tissue of the F4 follicle. The significance of this induction needs to be investigated in future research.

The mRNA for KOR was detected primarily in the theca tissue of hierarchical follicles, which suggests that the granulosa-produced Pdyn is having a paracrine effect on neighboring theca cells. The expression of KOR mRNA was greater in the theca tissues of follicles collected from fed hens in comparison to theca tissues of follicles collected from fasted hens. Because extended fasting results in follicular atresia, the results may suggest that granulosa produced Pdyn is positive on the maintenance of the follicular hierarchy, and the loss of theca sensitivity to granulosa produced Pdyn in fasted hens contributes to the loss in the maintenance of the follicular hierarchy.

Overall, the cell culture experiments with Pdyn and KOR are difficult to interpret. For the fed and fasted experiment, granulosa tissue was collected and RNA isolation commenced in less than an hour. However, for the cell culture experiments, once the granulosa tissue is collected, the tissue has to be digested and dispersed to get individual cells that are counted and then plated. As a result, it is about 5 hours after being removed from the hen that the freshly

dispersed cells (TZ) are ready for RNA isolation. In the case of GnIH-R mRNA expression, there was very close agreement in the expression between the freshly isolated granulosa cells of the fed birds and the TZ granulosa cells. For the fed hens, the mRNA for Pdyn was expressed in granulosa tissue from all the follicles, but in the cell culture experiments, Pdyn was not detected in the TZ granulosa cells from the F3 and SY follicles. In fed hens, KOR mRNA was not expressed in the F1 follicle and was expressed in the F3 and SY follicles, but in the cell culture experiments, KOR was not detected in the F3 and SY follicle TZ cells.

While the timing differences between the collection of the TZ samples and the fed hen samples likely account for the differences in the mRNA expression of Pdyn and KOR in these samples, further research is needed to confirm this hypothesis. Future research should also investigate whether protein expression for dynorphin and KOR in the pituitary, theca, and granulosa cells mirrors the mRNA expression of dynorphin and KOR. In addition, it will be important to determine if pituitary produced dynorphin is released into the general circulation and has a half-life that is long enough to interact with gonad expressed KOR.

Summary

The overall goal of this research was to determine if GnIH and dynorphin could be translating metabolic hormonal inputs to the reproductive axis at both the central and gonad level. Although the mRNA for Pdyn and it receptor, KOR, is present in the pituitary and preovulatory follicles of broiler breeder hens, the mRNA expression of Pdyn and KOR was not altered by fasting at the central level. In preovulatory follicles from fed broiler breeder hens, the expression of Pdyn is restricted to the granulosa layer, while KOR is primarily expressed in the theca layer. Fasting significantly reduces the theca expression of KOR, and this loss of

dynorphin sensitivity could lead to detrimental effects on the maintenance of the follicular hierarchy, which are seen with prolonged fasting. However, unlike dynorphin, the current research suggests GnIH could be translating metabolic status at both the central and ovarian level. Fasting increased GnIH mRNA levels in the pituitary, and if this translates to an increased blood level of GnIH, the presence of the GnIH receptor mRNA in preovulatory follicles suggests that these follicles could be influenced by the increase in blood GnIH. Furthermore, given that it appears based on mRNA expression, that the GnIH receptor is normally repressed in the fed state in hierarchical follicles and upregulated with fasting, it is possible that the effect of increased centrally produced GnIH is negative on hierarchical follicular maturation. Further research is needed to determine the exact roles GnIH and dynorphin may have in regulating the negative effects that caloric insufficiency associated with restricted feeding causes in broiler breeders.

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