GENE EXPRESSION AND PROTEIN LEVELS OF GNRH ISOFORMS AND THEIR COGNATE RECEPTORS IN THE STALLION TESTIS AND SPERMATOZOA

by

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Abstract

Gonadotropin-releasing hormone (GnRH-I), as well as its receptor, GnRHR-I, once thought to be localized solely to the hypothalamus and anterior pituitary, have since been detected in the testis of numerous mammals. Another isoform of GnRH, GnRH-II, has been isolated from the testis of numerous mammals and binds a specific receptor, GnRHR-II. Our objective was to establish whether GnRH-I and GnRH-II, along with their specific receptors, are produced and present in the equine testis. Testicular tissue was collected from colts < 2 yr (n = 5) and stallions ≥ 2 yr (n = 10) of age during routine castrations. Total RNA extracted from testicular tissue was reverse transcribed and cDNA was subjected to conventional PCR using gene specific primers for GnRH-I, GnRHR-I, GnRH-II, and GnRHR-II. Protein was extracted and subjected to dot blot and Western blot using antibodies directed against GnRH-I, GnRH-II, GnRHR-I, or GnRHR-II. Transcripts for both ligands and receptors were detected in all testes. Product identity was confirmed by sequencing, which also clarified that unusual band sizes were the result of alternative splicing of GnRHR-II, and the retention of an intron in the GnRH-II mRNA was discovered. Prepro-GnRH-I and prepro-GnRH-II protein was detected in all stallion testes via dot blot technique. On Western blots, testicular samples from colts (n = 4) had 3-fold greater GnRHR-I levels compared to stallions (n = 7; $P < 0.022$). Conversely, there was a tendency for GnRHR-II protein to be greater in tissue collected from stallions compared to colts ($P < 0.0756$). Finally semen was collected from mature stallions (9 to 18 yr; n = 4) and purified using a discontinuous gradient. By utilizing immunocytochemistry, GnRHR-II was localized to the connecting piece of mature stallion spermatozoa. This is the first report identifying GnRH-I and -II and their receptors in the equine testis and GnRHR-II on mature stallion spermatozoa.

These decapeptide hormones may act via autocrine and/or paracrine signaling to affect steroidogenesis and spermatogenesis in the stallion testis.

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Dedication

The entirety of this thesis is dedicated to the loving memory of the most caring and generous man I know, Stephen Douthit, whom I had the great fortune of calling Dad. He always supported the choices I made, especially my decision to pursue a Master's degree. He may be gone, but Stephen will never be forgotten. I love you and will always miss you Dad.

Chapter 1 - Hormonal Regulation of Stallion Reproduction

An intricate network of hormonal signaling, known as the hypothalamo-pituitary-testis axis, tightly regulates stallion reproduction (Roser, 2008). The hypothalamus, pituitary, and testicles communicate through endocrine, paracrine, and autocrine signaling mechanisms (Figure 1.1). The hypothalamus is the neural control center for reproduction and is regulated via positive and negative feedback mechanisms (Roser, 1997).

Oxytocin and gonadotropin releasing hormone (GnRH) are the dominant reproductive peptides produced in the hypothalamus, with GnRH being highly conserved across species (Senger, 2012). These 2 neuropeptides differ in their hypothalamic origin as well as their secretory region. The nanopeptide oxytocin is synthesized by the supraoptic nucleus where the axon terminates in the nuerohypophysis, which is also known as the posterior pituitary. As a result, oxytocin is secreted directly into the nuerohypophysis and is released into systemic circulation (Senger, 2012).

In contrast, GnRH is synthesized in the medialbasal hypothalamus in males and in both the medialbasal hypothalamus and lateral preoptic area in females. The GnRH producing neurons contain axons that extend to the stalk region (Rissman et al., 1995). Upon axonal stimulation, GnRH is released into the median eminence where it is transported via the hypothalamo-hypophyseal portal system to the adenohypophysis (Senger, 2012). This unique portal contains small arterial capillaries in the median eminence. Neural GnRH is deposited in the primary portal plexus and then transported to a secondary capillary plexus in the adenohypophysis, or anterior pituitary (Senger, 2012). The portal system is advantageous for hormones with a short half-life and it allows for rapid response from the target tissues.

Furthermore, because the neuropeptide is not released directly into systemic circulation, GnRH is minimally diluted before reaching the anterior pituitary.

The hypothalamic structure and source of GnRH differs between males and females. The primary sources of hypothalamic GnRH in the female are from the medialbasal hypothalamus and lateral preoptic area (Rissman et al., 1995). Females require frequent pulses of GnRH released from the lateral preoptic area, which results in greater LH secretion and subsequent ovulation of the dominant follicle. In the female fetus, the hypothalamus differentiates with a medialbasal hypothalamus and lateral preoptic area as alpha-feto protein binds estradiol (E_2) and prevents E_2 from crossing the blood-brain barrier (Senger, 2012).

In contrast, the male hypothalamus is defeminized in utero and the medialbasal hypothalamus is the sole source of GnRH peptide. During male development, alpha-feto protein does not bind to testosterone (T) due to the differing steroid structure. Consequently, T crosses the blood-brain barrier and is then converted to E_2 via aromatase activity. This E_2 exposure in the hypothalamus prevents the development of the lateral preoptic area in the male fetus (Senger, 2012).

The male reproductive axis is regulated via GnRH binding its cognate receptor on gonadotrope cells in the anterior pituitary. Upon formation of the GnRH ligand:receptor complex, gonadotrope cells secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). These glycoproteins are trafficked in peripheral circulation from the anterior pituitary to the testes (Roser, 1997).

The testicle participates in the hypothalamo-pituitary-testis axis by producing T, dihydro-testosterone (DHT), and E_2, and it provides negative feedback regulation. Leydig cells in the testis express the membrane-bound receptor for LH. Upon LH binding its receptor, Leydig cells

produce T. Upon secretion, a portion of T is converted to E_2 in Sertoli cells. Testosterone, DHT, and E_2 are transported in systemic circulation to the hypothalamus where exposure to these steroids results in reduced secretion of GnRH, and, consequently, reduced LH and FSH production (Roser, 1997). Additionally, FSH binds to Sertoli cells in the testis and induces inhibin secretion. Inhibin selectively downregulates FSH secretion from the anterior pituitary (Roser, 1997). These negative feedback mechanisms play an important role in regulating reproductive hormone levels.

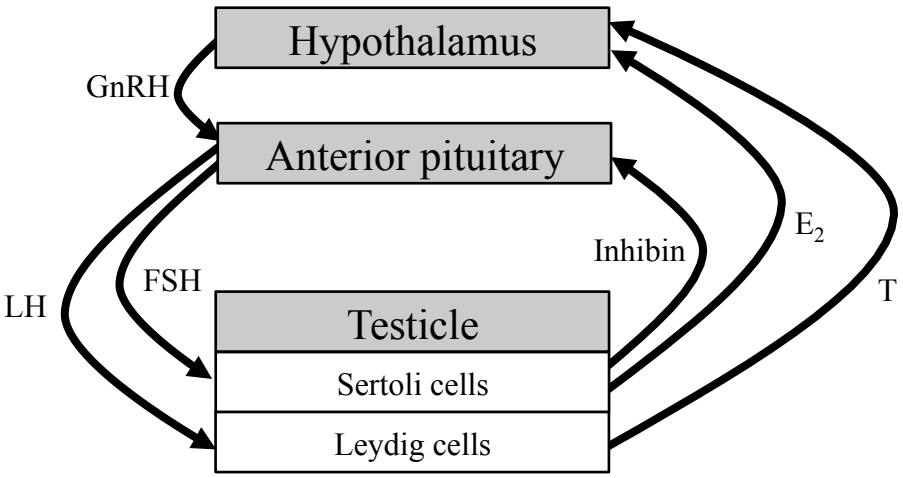

Figure 1.1. Male reproductive axis representing a negative feedback regulatory system. Steroid hormones, namely T and E_2, produced in the testis target the hypothalamus to down regulate GnRH secretion. Inhibin, secreted from Sertoli cells, targets gonadotrope cells to selectively down regulate FHS secretion.

Equine Seasonality

Horses are seasonal, long-day polyestrous breeders with the natural breeding season in the Northern hemisphere running from early spring through mid-fall. Cyclicity in the mare and sperm production in the stallion are regulated by photoperiod, although the effect is more pronounced in mares (Grosse et al., 1993). Melatonin is produced in and secreted from the pineal gland in greater concentrations during the night, or in the absence of sunlight (Gerlach and Aurich, 2000). In the equine, melatonin suppresses the release of GnRH from the hypothalamus (Minneman and Wurtman, 1975). As day length increases, melatonin production decreases which then allows GnRH to resume pulsatile secretion from the hypothalamus (Argo et al., 1991). The polyestrous activity of the mare is dependent on elevated levels of GnRH. The surge center releases greater concentrations of GnRH, resulting in an LH surge which then leads to ovulation of the dominant follicle. However, during short days, elevated melatonin suppresses GnRH release and prevents an LH surge. As a result, mares remain anestrus until the days become longer.

Unlike anestrus in the mare, spermatogenesis continues in the stallion year-round (Clay et al., 1988). Although melatonin restricts GnRH secretion from the medialbasal hypothalamus during short days, sufficient GnRH is produced to regulate and maintain spermatogenesis. The increased production of melatonin during the non-breeding season results in decreased reproductive hormonal concentrations in stallions and, consequently, reduced sperm production (Clay et al., 1988).

The testes of stallions exposed to short day length year-round still recrudesced and spermatogenesis resumed to normal levels during spring and summer (Clay et al., 1988). Basal LH concentrations eventually returned to breeding season levels, even though stallions were exposed to an inhibitory or non-stimulatory photoperiod (Clay et al., 1988). These authors

concluded a separate mechanism may regulate stallion seasonality in conjunction with melatonin secretion via the pineal gland.

The opioid antagonist naloxone can induce an acute LH release from the anterior pituitary in stallions during the non-breeding season (Aurich et al., 1994). Following LH-induced secretion stimulated by naloxone, T secretion was also found to increase (Gerlach et al., 2002). Stallions administered naloxone increased LH concentrations in March, June, and December (Gerlach et al., 2002), therefore indicating LH pulsatile secretion and endocrine testicular function also may be regulated via opioidergic neural systems (Aurich et al., 1994; Gerlach et al., 2002). In sheep, there is evidence that dopamine inactivates opioidergic regulation of GnRH release during long day length (Tortonese, 1999). In contrast, researchers have not identified an interaction between dopaminergic and opioidergic systems in regulating LH release in stallions (Gerlach et al., 2002).

Melatonin not only regulates GnRH release, but also suppresses prolactin secretion (Gerlach and Aurich, 2000). In many species, prolactin levels are closely associated with day length and fluctuate throughout the year. Because secretion of melatonin is reduced during long day length, prolactin is secreted in greater concentration during the equine breeding season (Thompson et al., 1987). In conjunction with LH and growth hormone, prolactin assists in regulating LH receptors in the testicle and subsequently affects androgen synthesis and ultimately spermatogenesis in rats, rams, and boars (Regisford and Katz, 1993; Hondo et al., 1995; Jedlinska et al., 1995).

GnRH

Extra-hypothalamic Expression

Since the 1970s the hypothalamic decapeptide GnRH has been established as the central regulator of the reproductive axis in both males and females (Senger, 2012). As part of the hypothalamic-pituitary-gonadal axis, GnRH induces LH and FSH secretion from gonadotrope cells in the anterior pituitary upon interaction with its receptor (GnRHR). Even though it is commonly accepted that GnRH is localized in the hypothalamus, GnRH has been isolated in extra-hypothalamic reproductive and non-reproductive tissues. In reproductive tissues, extra-hypothalamic mRNA coding GnRH has been identified in seminiferous tubules (Bahk et al., 1995), seminal plasma (Izumi et al., 1985), germ cells (Paull et al., 1981), the ovary, and follicular fluid (Ying et al., 1981; Aten et al., 1987).

The function of extra-hypothalamic GnRH is poorly understood. Extra-hypothalamic GnRH may have several functions aside from gonadotropin secretion, such as regulating steroidogenesis (Kang et al., 2003) and cellular proliferation (Gründker and Emons, 2003; Kang et al., 2003), or facilitating fertilization (Morales, 1998; Morales et al., 2000). Due to a short half-life of approximately 4 min (Eskay et al., 1977; Carone et al., 1984), secretion of GnRH from the hypothalamus into the periphery is unlikely. Thus, GnRH has been predicted to be synthesized locally in extra-hypothalamic reproductive tissues to exert its biological effect via autocrine or paracrine mechanisms (Hsueh and Schaeffer, 1985).

GnRH Structure

The mature GnRH peptide is comprised of 10 AA (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Residues between 5 and 8 interact to form a β-II turn (Figure 1.2; Millar, 2005). The flexible Gly at residue 6 allows the bent conformation. Agonists and antagonists substitute a

D-amino acid in this position to increase half-life, molecule stability, and binding affinity. Both the NH_2 and COOH termini are necessary for ligand binding; yet, only the NH_2 terminus is involved in receptor activation. Substitutions in this region, between amino acid (AA) residues 1 and 3, result in an antagonist molecule (Millar, 2005).

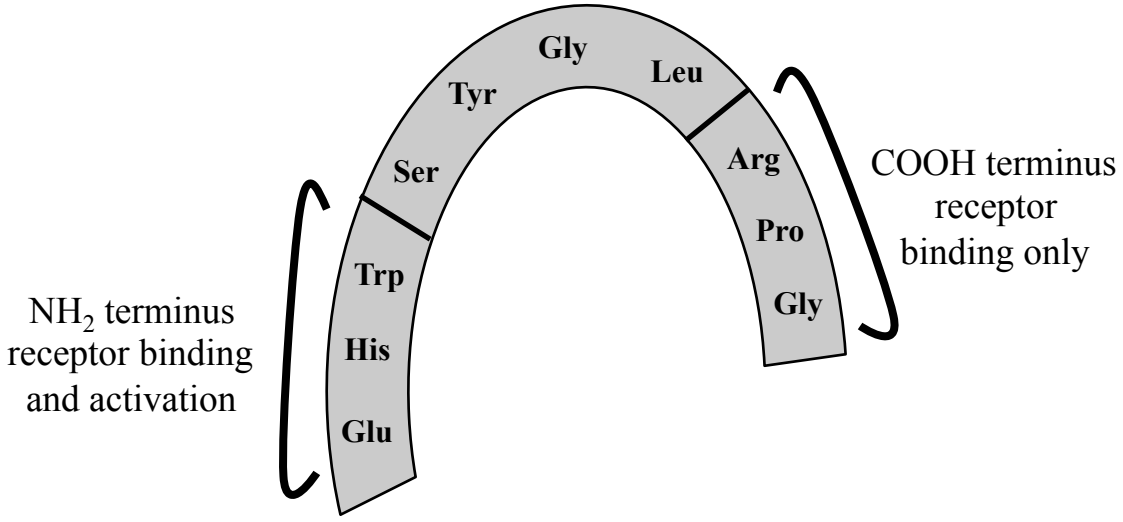

Figure 1.2. Conformation of GnRH upon binding GnRHR. The NH_2 and COOH termini of GnRH are required to bind GnRHR. However, only NH_2 terminus functions for receptor activation. Antagonists incorporate a D-amino acid substitution in the NH_2 region, while agonists substitute a D-amino acid for Gly^6.

GnRH Receptor

Structure

The receptor for GnRH is a member of the G-protein-coupled-receptor (GPCR) superfamily containing 7 transmembrane (TM) domains (Rispoli and Nett, 2005). An intracytoplasmic tail is characteristic of most GPCRs (Rispoli and Nett, 2005). However, this intracellular domain is absent in GnRHR. It has been suggested the intracytoplasmic tail assists in the desensitization and subsequent internalization of GPCRs (Willars et al., 1999), which occurs in response to constant ligand binding and continued activation of G proteins (Hislop et al., 2001). However, as GnRHR lacks a characteristic cytoplasmic tail, this may indicate an evolutionary advantage of GnRHR (Millar, 2005) as the mammalian receptor is internalized at a slower rate and is less susceptible to desensitization than other GPCRs (Willars et al., 1999).

Cellular Signaling

Prior to eliciting a cellular response GnRH must first interact with its receptor via a hydrophilic pocket within the transmembrane loops. Interaction between GnRH and GnRHR leads to the formation of a hormone:receptor complex and results in a conformational change in the TM domains of the receptor. Consequently, a cascade of intracellular events, mediated by second messengers, follows and activates signal transduction pathways. The GnRHR mediates these pathways by coupling to $G_{q/11}$ proteins (Naor et al., 1995). Activity from $G_{q/11}$ subunits leads to an upregulation of phospholipase C (Naor et al., 1995). Phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate, a membrane phospholipid, into diacylglycerol and inositol triphosphate (Naor et al., 1995). Diacylglycerol remains within the cellular membrane to stimulate protein kinase C activity, whereas inositol triphosphate solubilizes in the cytoplasm and targets its receptor on smooth endoplasmic reticulum to induce Ca^{2+} release (Naor et al.,

1995). An increase in intracellular Ca^{2+} activates protein kinase C that then phosphorylates and activates mitogen-activated protein kinase to increase gene transcription of LH and FSH subunits (Naor et al., 1995).

Gonadotropin releasing hormone regulates different cellular processes by altering the pulsatile frequency of its own secretion. With slowed pulse secretion, FSHβ subunit synthesis is upregulated (Kaiser et al., 1997). Comparatively, GnRHR and LHβ production are increased during increased frequency of GnRH pulsatile secretion (Kaiser et al., 1997). This phenomenon has been associated with the increased GnRH secretion prior to the LH surge and greater GnRHR numbers on gonadotrope cells prior to ovulation.

Extra-pituitary Tissue Expression

The receptor for GnRH also has been detected in numerous extra-pituitary tissues and systems, including the immune system, pancreas, reproductive tumors, and reproductive tissues (Walters et al., 2008). Specific reproductive tissues in which GnRHR has been identified consist of the uterus (Dong et al., 1998; Choi et al., 2006), ovary (Harwood et al., 1980; Jones et al., 1980; Reeves et al., 1980; Choi et al., 2006), placenta (Rama and Rao, 2001; Chou et al., 2004), spermatozoa (Bull et al., 2000; Lee et al., 2000; Morales et al., 2000), and testes (Ikeda et al., 1996; Bull et al., 2000) of various mammals.

Researchers have identified GnRH and GnRHR in the testis of mature rats (Botté et al., 1998), humans (Bahk et al., 1995), mice (Bull et al., 2000), alpaca (Zerani et al., 2011), sheep (Gault et al., 2004), and monkeys (Sharpe and Fraser, 1980). Gonadotropin releasing hormone receptor has been detected in interstitial cells (Bahk et al., 1995). Moreover, testicular germ cells in mice and rats express GnRHR mRNA (Bull et al., 2000). The nucleotide sequences are

identical, confirming that testicular GnRHR is homologous to pituitary GnRHR (Kottler et al., 1999; Ramakrishnappa et al., 2005).

In the pituitary, expression of GnRHR is regulated by secretion of its ligand, GnRH. Although GnRHR in the testis is identical to the pituitary receptor, the GnRHR regulatory system in the testicle is not the same (Ramakrishnappa et al., 2005). In the testes, T upregulates GnRHR while LH downregulates the receptor's expression (Botté et al., 1999; Ramakrishnappa et al., 2005). However, LH appears to have no effect on GnRH mRNA levels in the testis of mice (Ramakrishnappa et al., 2005).

Function of GnRHR in the Testis

The function of GnRH in the testis appears to be both stimulatory and inhibitory relative to peptide concentration. A short-term, low dose of exogenous GnRH results in increased T production in adult rat Leydig cells (Sharpe and Cooper, 1982a; Sharpe and Cooper, 1982b). Comparatively, a long-term, high dose of GnRH resulted in reduced T production in hypophysectomized rats (Ramakrishnappa et al., 2005). The inhibitory function of GnRH appears to act by downregulating the GnRHR and intermediary enzymes 3-β-hydroxysteroid dehydrogenase and steroidogenic acute regulatory protein (Ramakrishnappa et al., 2005). These enzymes are necessary for the conversion of precursor steroids, such as pregnenolone and androstenediol, into T. In addition, GnRH has a stimulatory effect on germ cell proliferation, where administration of a GnRH agonist stimulated spermatogonial proliferation from damaged testes and increased the regeneration of spermatogenesis (Meistrich and Kangasniemi, 1997; Matsumiya et al., 1999; Shuttlesworth et al., 2000).

GnRH-II

Structure

Approximately 23 different isoforms of GnRH have been identified (Millar, 2005). Each isoform is comprised of 10 AA with at least 50% homology to native GnRH (Dubois et al., 2002). Because numerous forms of GnRH have been detected, the classical GnRH has now been termed GnRH-I. Two isoforms, GnRH-II and GnRH-III, are found in most vertebrates, in addition to GnRH-I (Table 1.1; White et al., 1994). However, the only other isoform identified in mammals to date is GnRH-II (Sealfon et al., 1997). This novel isoform was originally isolated from the chicken hypothalamus (Miyamoto et al., 1984).

Since its discovery, GnRH-II has been isolated in numerous mammalian species (Millar et al., 2001; Wang et al., 2001; Stewart et al., 2009). The AA sequence of GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2) differs from GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) by substitutions at positions 5, 7, and 8 (Miyamoto et al., 1984). Gonadotropin releasing hormone II is highly conserved from boney fish to humans (Temple et al., 2003); thus it is thought to be the most ancient form of GnRH (Millar et al., 2004). This conservation indicates a prominent selection pressure to retain GnRH-II, as it has been maintained over 500 million years of evolution (Millar and King, 1987).

Although GnRH-I and GnRH-II retain 70% homology (White and Fernald, 1998), the stability of the mature decapeptide isoforms differs. It has been well documented that GnRH-I has a brief half-life of about 4 min. Gonadotropin releasing hormone II has a similar β conformation to GnRH-I, but it appears to require less extensive conformational changes to activate its receptor (Pfleger et al., 2002; Millar, 2003). As a result the GnRH-II peptide exhibits approximately 6-fold greater stability than GnRH-I (Siler-Khodr and Grayson, 2001). Therefore

GnRH-II is less likely to be degraded in systemic circulation. The more stable conformation may explain the ubiquitous expression of GnRH-II (Pawson et al., 2003).

Neural Synthesis

Compared to GnRH-II, in mammals GnRH-I is synthesized predominantly in the medialbasal hypothalamus where it is trafficked to the anterior pituitary via the hypthalamo-hypophyseal portal system to interact with GnRHR-I expressed on gonadotrope cells. By contrast, the majority of neural GnRH-II originates from the midbrain. In the musk shrew, GnRH-II has been found in the hindbrain, medial habenula, midbrain central grey, hypothalamus, and medial septum (Rissman et al., 1995). However, the majority of GnRH-II synthesis takes place in the periphery in a wide range of tissues such as the kidney, bone, prostate, placenta, breast, endometrium, ovary, and testis (Millar, 2003).

Table 1.1. The AA sequences of GnRH isoforms detected in vertebrates

Isoform	Amino Acid Sequence[1]
GnRH-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH-II	pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Tyr**-Pro-Gly-NH_2
GnRH-III	pGly-His-Trp-Ser-**His**-**Asp**-**Trp**-**Lys**-Pro-Gly-NH_2

[1]Amino acid residues in bold differ from the native mammalian GnRH-I

Role in Behavior

In mammalian neural tissue, GnRH-II regulates several different physiological functions (Gründker et al., 2002; Temple et al., 2003; Kauffman and Rissman, 2004; Kauffman et al., 2005). For instance, GnRH-II in the female musk shrew plays a modulatory role between energy status and reproductive behavior (Temple et al., 2003). Restriction fed females showed reduced sexual receptivity (Temple et al., 2003). However, when dosed with GnRH-II, the inhibitory effect of food restriction was reversed and the females exhibited increased mating behavior (Temple et al., 2003). Conversely, female musk shrews that were fed ad libitum did not show a stimulatory effect when injected with GnRH-II (Schiml and Rissman, 2000). Temple et al. (2003) suggested that under ad libitum feeding conditions GnRH-II is released at basal levels, and at this concentration, females are receptive to mating. Limit-fed females released GnRH-II at a reduced rate and exhibited minimal reproductive behavior (Temple et al., 2003). This effect of GnRH-II regulating female reproductive behavior has also be confirmed in ring doves (King and Millar, 1979) and song sparrows (Maney et al., 1997). Therefore, neural GnRH-II appears to signal the energetic state in females of some species. If dietary energy is sufficient, GnRH-II is likely to act as a permissive factor that allows mating behavior (Kauffman and Rissman, 2004).

Role in Nutrition

In addition to regulating mating behavior, GnRH-II also appears to play a role in regulating food intake. When in a negative energy state, GnRH-II is released at minimal levels and stimulates increased feed intake (Kauffman and Rissman, 2004). Alternatively, excessive food intake leads to greater GnRH-II secretion, which signals the brain to reduce food consumption to basal levels (Kauffman and Rissman, 2004). An infusion of GnRH-II in ad libitum and restriction fed musk shrews resulted in reduced feed intake (Kauffman and Rissman,

2004). However, the inhibitory effect of GnRH-II on food intake persisted only a few hours (Kauffman and Rissman, 2004).

GnRHR-II

Structure

Like GnRH-I, GnRH-II binds a specific receptor (GnRHR-II). Gonadotropin releasing hormone receptor II was originally isolated from the African catfish and is a member of the GPCR superfamily containing 7 TM domains (Tensen et al., 1997). There is only 40% homology between GnRHR-II and GnRHR-I (Figure 1.3; Kauffman and Rissman, 2004). A significant difference between the receptors is the retention of an intracytoplasmic tail on GnRHR-II (379 AA) that is absent on GnRHR-I (328 AA). This intracellular domain assists in the desensitization and internalization of GPCR receptors (Willars et al., 1999) and indicates more rapid internalization and desensitization of GnRHR-II compared to GnRHR-I (Heding et al., 1998).

Tissue Expression

Similarly to GnRH-II, GnRHR-II is ubiquitously expressed across tissue types (Millar et al., 2001). In the human and marmoset, GnRHR-II mRNA has been detected in numerous cell types, such as neural tissue, cardiac muscle, digestive organs, respiratory tissue, immune system, reproductive tissues and reproductive tumors (Neill et al., 2001). Like GnRH-II, it appears GnRHR-II expression is greatest in extra-pituitary tissues (Millar et al., 2001). Expression of GnRHR-II is particularly elevated in marmoset reproductive tissues (Millar et al., 2001).

Both the ligand and receptor of GnRH-II have been characterized in the boar testis and it appears the GnRH-II system plays an important role in spermatogenesis (Bowen et al., 2006; Desaulniers et al., 2015). Immunization against GnRH-II in the boar results in reduced T production, but does not affect LH secretion (Bowen et al., 2006). Dosing the same immunized

boars with LH does not increase Leydig cell secretion of T (Bowen et al., 2006). Furthermore, GnRH-II has also been found to increase T production independent of LH secretion (Desaulniers et al., 2015). In the testis, GnRHR-II has been identified in interstitial cells, primarily Leydig cells, as well as seminiferous tubules (Desaulniers et al., 2015).

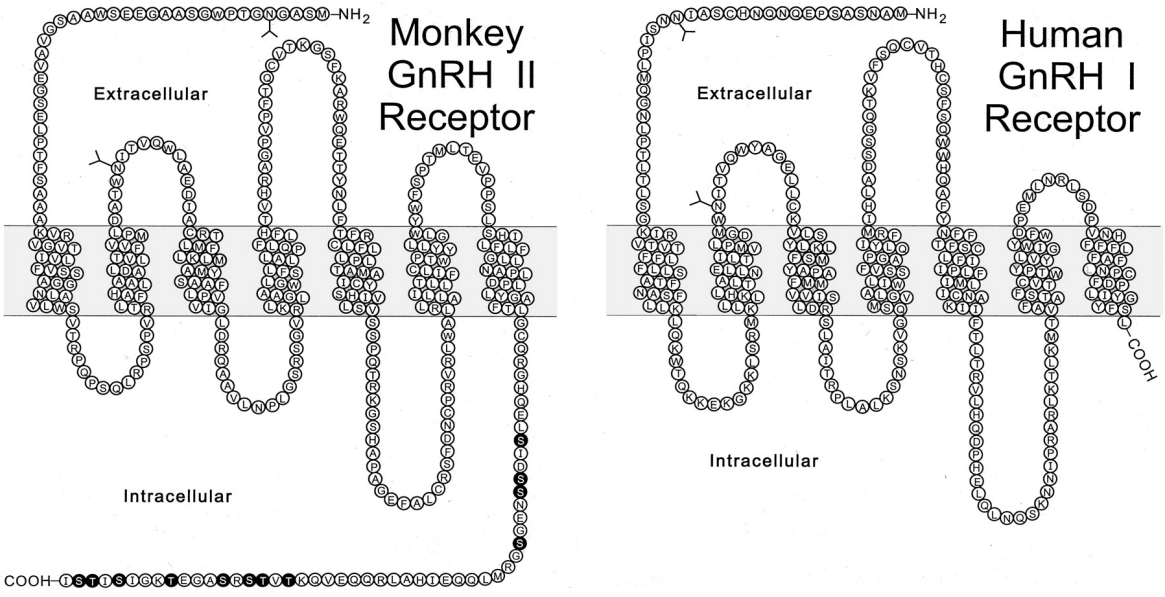

Figure 1.3. A graphical comparison of the monkey GnRHR-II and human GnRHR-I. The most notable difference is the retention of an intracytoplasmic tail in GnRHR-II. Figure from Neil (2002).

Ligand and Receptor Isoform Interaction

Ligand:Receptor Complex

The ligand:receptor complex is formed by the interaction of specific residues in the ligand and receptor. This interaction leads to receptor activation and subsequent signal transduction. The Arg^{8} residue of GnRH-I is critical for high affinity binding to GnRHR-I (Millar et al., 2004). Gonadotropin releasing hormone II also binds GnRHR-I, however, with much lower affinity as GnRH-II substitutes Tyr^{8} for Arg^{8}. Conserved residues found in both ligand isoforms, His^{2} and Trp^{3}, are necessary for activation of GnRHR-I (Sealfon et al., 1997). Comparatively, Trp^{7} and Tyr^{8} are essential for binding and activation of GnRHR-II (Wang et al., 2003).

Particular receptor residues are also important for ligand recognition. Specific regions that differ between GnRHR-I and GnRHR-II increase ligand selectivity (Li et al., 2005). For GnRHR-II, extracellular loop 3 and extracellular loop 3 proximal TM 7 are responsible for high affinity binding of GnRH-II to its receptor (Li et al., 2005; Mamputha et al., 2007). Residues Asp^{97}, Asn^{101}, and Lys^{120} are conserved in GnRHR-I and GnRHR-II, and are important for recognition of both ligand isoforms (Zhou et al., 1995; Davidson et al., 1996; Flanagan et al., 2000; Mamputha et al., 2007). Although conserved between GnRHR-I and GnRHR-II, there are subtle differences these residues play upon forming the GnRH-I:GnRHR-I or GnRH-II:GnRHR-II complex (Mamputha et al., 2007).

Cross Ligand Interaction

In addition to binding its cognate receptor, GnRH-II is also capable of interacting with the GnRHR-I binding pocket. Similarly, GnRH-I is capable of binding GnRHR-II, however with 100-fold reduced affinity compared to GnRH-II (Millar, 2003). Little is known of the effects

GnRH-I elicits upon binding GnRHR-II, but it appears GnRH-I antagonists have the ability to act as agonists when interacting with the GnRHR-II binding pocket (Millar et al., 2001).

Gonadotropin releasing hormone-II is capable of binding GnRHR-I in the anterior pituitary and mediating gonadotropin release (Gründker et al., 2002). However, the second ligand has a 10-fold lower affinity binding to GnRHR-I, making GnRH-II a weak stimulator of gonadotropin release and only 10% as effective in stimulating ovulation as GnRH-I (Gründker et al., 2002; Neill, 2002). Other potential functions of GnRH-II mediated via GnRHR-I include regulation of female reproductive behavior (Kauffman et al., 2005) and feed intake (Kauffman and Rissman, 2004). Furthermore, GnRH-II has shown anti-prolific effects on cancerous tissues that may be mediated through GnRHR-I or GnRHR-II (Gründker et al., 2002).

Disruptive Genetic Mutations

Several species code for disruptive genetic mutations in the GnRHR-II gene. In the mouse there is an absence of both GnRH-II ligand and receptor genes in the genome, while the rat genome retains a fragment of the GnRH-II gene. Of the 22 mammalian species examined by Stewart et al. (2009), 10 species retained genetic coding to produce prepro-GnRH-II, whereas only 8 species maintained the ability to produce a full-length functional GnRHR-II. Few species, including the macaque, marmoset, tree shrew, and pig, retain functional encoding genes to produce both GnRH-II and GnRHR-II transcripts (Stewart et al., 2009).

Humans maintain the ability to secrete the GnRH-II hormone; however, the human GnRHR-II gene has a premature stop codon in exon 2 (Morgan et al., 2003). This exon encodes the extracellular region of loop 2. Another start codon downstream initiates the transcription of a gene fragment, which has been termed GnRHR-II-reliquum (Pawson et al., 2003). The protein translation of this gene fragment is thought to inhibit GnRHR-I function in the anterior pituitary

(Pawson et al., 2003). Like humans, horses also retain the gene encoding prepro-GnRH-II (Stewart et al., 2009). The horse also possesses a mutated GnRHR-II gene with frameshifts in exon 2 and a premature stop codon in exon 3 (Stewart et al., 2009). However, compared to the human, the horse may transcribe a larger portion of GnRHR-II as the position of the premature stop codon in the equine is much closer to the 3' end (Stewart et al., 2009).

While it appears the GnRHR-II gene is transcriptionally active in the sheep, it encodes a premature stop codon in exon 1 (Gault et al., 2004). Furthermore, sheep also have a 51 base pair deletion in exon 2 (Gault et al., 2004). Therefore it is unlikely sheep are able to produce a functional GnRH-II receptor (Gault et al., 2004). Sheep also differ from the horse and human as they are not predicted to be able to synthesize the GnRH-II peptide (Gault et al., 2004; Stewart et al., 2009).

Investigation of numerous mammalian genomes indicates retention of a majority of the GnRH-II hormone and receptor gene (Stewart et al., 2009). Even though it appears several species have coding errors in either the hormone or receptor gene, ancestral mammalian species may have required the GnRH-II system for survival (Stewart et al., 2009). However, during evolution, this system appears to have become non-essential to the survival of mammalian species that now have coding errors (Stewart et al., 2009). Indeed, it has been proposed GnRHR-I may have replaced GnRHR-II in the evolution of certain species (Millar, 2003). Yet, the GnRH-II system must still be functionally relevant to those species, such as the pig, tree shrew, macaque, and marmoset, that retain the GnRH-II hormone and receptor gene (Stewart et al., 2009).

Role in Cancer

In recent decades, agonists and antagonists of mammalian GnRH isoforms have been used as therapeutic agents in the treatment of reproductive tumors (Cheung and Wong, 2008). The effectiveness of these therapies is predominantly attributed to the presence of GnRHR-I in reproductive cancerous tumors (Neill et al., 2001; Cheung and Wong, 2008). Gonadotropin releasing hormone receptor-I has been detected in breast, endometrial, ovarian, and prostate tumors, which are dependent on sex steroids (Limonta et al., 2001). In cancerous cells GnRHR-I couples $G_{\alpha i}$ rather than coupling to $G_{q/11}$ in the anterior pituitary (Imai et al., 1997). Coupling to $G_{\alpha i}$ results in a separate signaling cascade in tumor cells that is involved in cellular growth, metastasis, and survival (Cheung and Wong, 2008).

As with T secretion in the testis, GnRH-I is able to regulate cancerous cellular growth in a biphasic manner (Arencibia and Schally, 2000; Cheung et al., 2006; Chen et al., 2007). At a low dose, GnRH-I agonists promote cellular growth, whereas administration of a greater concentration of GnRH-I agonists inhibits cellular proliferation (Arencibia and Schally, 2000; Cheung et al., 2006). However, it appears GnRH-I antagonists more effectively inhibit cellular growth compared to GnRH-I agonists (Yano et al., 1994). It has been proposed that antagonists limit cancerous cellular proliferation by reducing gonadotropin release and subsequently decreasing sex-steroid production (Schally, 1999; Cheung and Wong, 2008).

Compared to GnRH-I, GnRH-II has even more potent anti-prolific effects on reproductive cancers. When dosed at similar levels, GnRH-II inhibits cellular growth more than GnRH-I (Gründker et al., 2002). There is contradicting evidence, however, as to whether GnRH-II acts via GnRHR-I or GnRHR-II in suppressing tumor growth. In cancer cell lines that express GnRHR-II mRNA and lack GnRHR-I mRNA, GnRH-II inhibits cellular proliferation, whereas

GnRH-I had no effect (Gründker et al., 2002). However, it is still unclear if the human is capable of producing a functional GnRHR-II (Neill, 2002; Millar, 2005).

Autocrine and Paracrine Cellular Signaling

Primary hormonal regulation of male reproduction has been attributed to the hypothalamo-pituitary-testes axis. In addition to negative feedback regulation of stallion reproduction, autocrine and paracrine systems regulate steroidogenesis and spermatogenesis locally within the testis (Figure 1.4; Roser, 2008). Autocrine mechanisms function by acting on the same cellular type; conversely, paracrine signaling molecules are produced by a cellular type and act on a neighboring cellular type (Heindel and Treinen, 1989). The autocrine and paracrine regulatory functions in the equine testis have been minimally investigated and these systems are poorly understood (Roser, 2008). Hormones that have been investigated in the stallion testis are androgens, estrogens, inhibin, activin, insulin-like growth factor-1 (IGF-1), transferrin, insulin-like peptide-3, β-endorphins, and oxytocin (Roser, 2008).

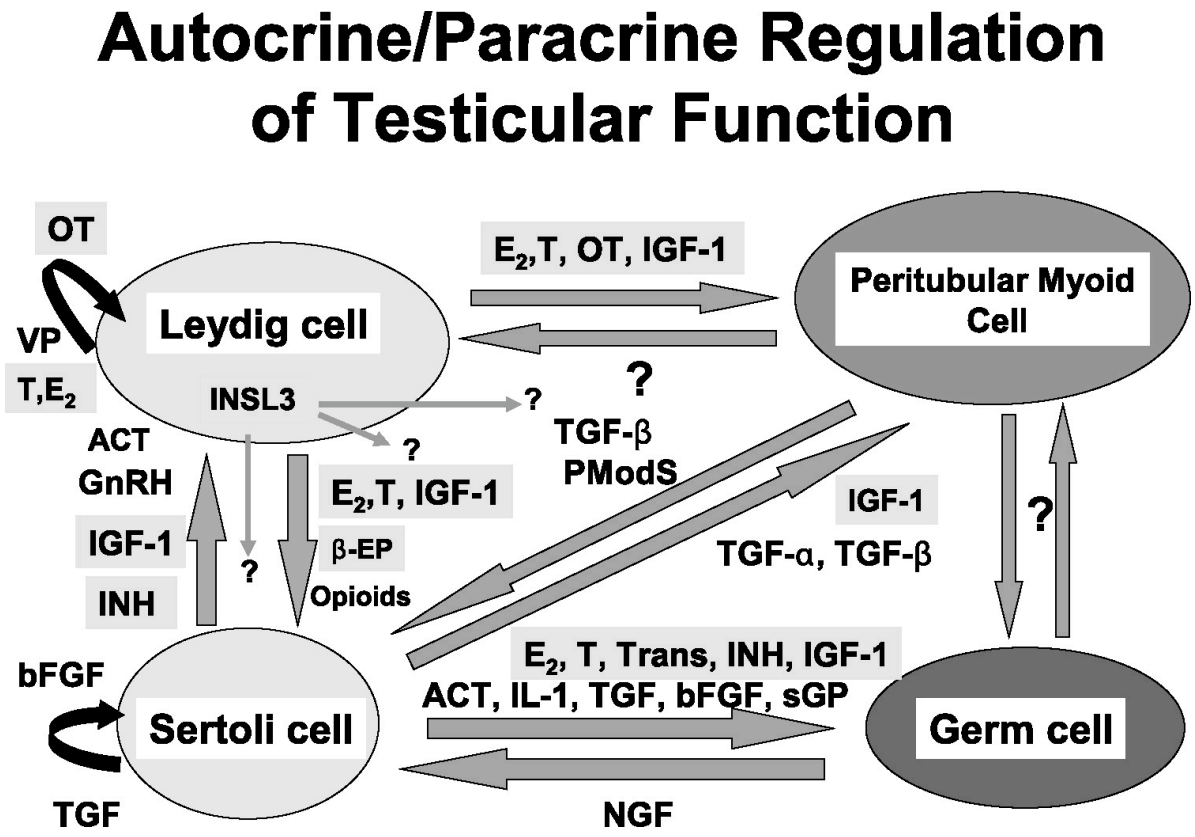

Figure 1.4. Diagram of potential local interactions in the equine testis including gonadotropin releasing hormone (GnRH), insulin-like growth factor 1 (IGF-1), insulin like peptide 3 (INSL3), inhibin (INH), activin (ACT), beta endorphin (β-EP), transferrin (Trans), basic fibroblast growth factor (bFGF), transforming growth factor (TGF), transforming growth factor beta (TGF-β), transforming growth factor alpha (TGF-α), interleukin-1 (IL-1), sulphated glycoprotein (SGP), nerve growth factor (NGF), peritubular modifying substance (PmodS), oxytocin (OT), vasopressin (VP), testosterone (T), estradiol (E_2). Highlighted factors have been confirmed in the stallion. Normal testicular function depends on intricate autocrine and paracrine hormone regulation of steroidogenesis and spermatogenesis. Figure from Roser (2008).

Androgens

The testicular environment has a 100 to 500 fold greater concentration of T than systemic blood. This is the result of androgen binding protein (ABP), produced by Sertoli cells, sequestering T, produced by Leydig cells, and retaining it in the testicle (Dohle et al., 2003). Testosterone can be converted to E_2 and it induces several paracrine and autocrine effects within testicular tissue.

Pearl et al. (2011) investigated testicular expression of the androgen receptor (AR) in pre-pubertal, peri-pubertal, and post-pubertal stallions. Immunohistochemistry revealed the AR was expressed in the nucleus of Leydig, Sertoli, and peritubular myoid cells (Pearl et al., 2011). There were no differences in expression of AR among the different age groups analyzed. There was also no evidence of AR expression in germ cells (Pearl et al., 2011; Smith and Walker, 2014). Expression of AR on Sertoli cells is greatest during the transition stage of tight junction regeneration (Bremner et al., 1994; Zhou et al., 2002). This indicates androgens may be involved in the formation of new tight junctions and the progression of spermatogongia into meiosis in the adluminal section of seminiferous tubules (Meng et al., 2005).

Further actions of T within the testis include an autocrine effect on Leydig cells (Xu et al., 2007). Removal of AR from Leydig cells via genetic knockout resulted in a disruption of spermatogenesis and a reduction in T production (Xu et al., 2007). Additionally, Sertoli cell AR knockout mice also experienced a disruption of spermatogenesis (Holdcraft and Braun, 2004). Male mice with the AR knockout gene in Sertoli cells showed a reduction in spermeiogenesis, particularly in the differentiation and elongation of spermatids (Holdcraft and Braun, 2004).

Estrogens

Estrogens are historically recognized for their biological significance in the regulation of female reproduction. However, in the last decade researchers have also examined the biological activity of estrogens in male reproduction and spermatogenesis. The final enzyme in the steriodegenic pathway, aromatase, converts T to E_2 and is the focus for scientists who quantify estrogen production. Aromatase is expressed in all testicular cells except peritubular myoid cells (Silandre et al., 2007; Bois et al., 2010; Carreau et al., 2011).

Sertoli cells are the main source of estrogen produced in the immature male (Hess and Roser, 2004; Roser, 2008). As maturity is achieved in the rat, Leydig cells increase aromatase activity 3 to 4 fold (Roser, 2008). Parlevliet et al. (2006) reported E_2 concentrations were greater in adult stallions when compared to pre-pubertal stallions. This is likely the result of greater T production in the post-pubertal animals as T is a precursor steroid of estrogens and increased aromatase activity (Senger, 2012).

Carreau et al. (2003) revealed approximately 60% of aromatase activity originates from germ cells. Furthermore, the concentration of aromatase varies at differing stages of sperm cell development. Spermatocytes express a greater amount of aromatase transcript compared to haploid spermatids and spermatozoa (Carreau et al., 2011). Aromatase and E_2 have been identified in ejaculated spermatozoa (Carreau et al., 2010). It appears human ejaculated spermatozoa have the ability to convert androgens to estrogens via aromatase (Lambard and Carreau, 2005). Compared to motile sperm after purification, immotile sperm show 28% decreased expression of aromatase (Carreau et al., 2010).

Estrogens play a role in regulating testicular function (Hess and Roser, 2004), spermatogenesis (Ebling et al., 2000), acrosome formation (Luconi et al., 1999), and fertilization (Fraser et al., 2006). Aromatase knockout mice were developed to investigate estrogen's role in

the testis. Presumably as a result of the aromatase knockout, these mice had abnormal acrosome development (Luconi et al., 1999). Fraser et al. (2006) concluded that in the presence of estradiol and phytoestrogens, sperm capacitation, acrosome reaction, and fertilizing capacity were increased.

Estrogens can bind to 2 different receptors, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ; Kuiper et al., 1997; Pearl et al., 2011). The expression of either receptor is dependent on the cellular type and animal age (Hess and Roser, 2004). In the stallion testis, ERα expression varies between pre-pubertal, peri-pubertal, and post-pubertal males (Pearl et al., 2011). In the pre-pubertal and peri-pubertal stallions, expression was identified in the nucleus of Sertoli, Leydig, and germ cells. In the mature testis ERα was restricted to most Leydig, germ, and peritubular myoid cells (Pearl et al., 2011). Pre-pubertal stallions expressed ERβ in germ cells (Pearl et al., 2011). Regardless of age, ERβ was localized to Leydig and Sertoli cell cytoplasm as well as Sertoli cell nuclei in the equine (Pearl et al., 2011).

Inhibins

The glycoprotein inhibin is a member of the transforming growth factor-β (TGF-β) super family (Chang et al., 2002). The dimeric nature of inhibin is formed by an α and β subunit. Two forms of inhibin are present in most male species. Inhibin A consists of an α and β_A subunit, whereas inhibin B is composed of an α and β_B subunit (Phillips, 2005). In most male species inhibin B is the dominant form secreted, except the ram which primarily produces inhibin A (McNeilly et al., 2002).

The testicle is the prominent source of inhibin in the male as castration results in a rapid decline of circulating inhibin levels (Ishida et al., 1990). The α subunit of inhibin is produced in Leydig cells, but it most commonly originates in Sertoli cells (Bicsak et al., 1987; Risbridger et

al., 1989). The β_A subunit is expressed in Sertoli and peritubular cells (Buzzard et al., 2004). The β_B subunit mRNA is expressed Sertoli cells and germ cells, including spermatogonia, primary spermatocytes, and round spermatids (Buzzard et al., 2004; de Kretser et al., 2004).

Contrary to other hormones that interact with a specific receptor, inhibin requires a co-receptor and has the ability to interact with the activin receptor (Lewis et al., 2000; Hedger and Winnall, 2012). The primary function of inhibin is to inhibit FSH secretion from the anterior pituitary via classical negative feedback (Bilezikjian et al., 2004). This glycoprotein acts to suppress FSH production through competitive binding of the activin receptor, effectively blocking activin from exerting its biological effect of upregulating FSH secretion (Bilezikjian et al., 2004).

Circulating concentrations of inhibin B have been associated positively with Sertoli cell numbers and concentration of sperm (O'Connor and De Kretser, 2004). Thus, inhibin has been proposed as a potential fertility marker in the male (O'Connor and De Kretser, 2004). However, immunization against inhibin has also been hypothesized to increase fertility due to greater FSH secretion (Phillips, 2005).

Aside from the endocrine role inhibin plays by regulating FSH production, little is understood pertaining to other paracrine and autrocrine factors in the testis (Suresh et al., 2011). In one in vitro study, inhibins appeared to regulate human Leydig and Sertoli cell proliferation, differentiation, and steroidogenesis (Meachem et al., 2001). Yet, these regulatory functions of inhibin have not been demostrated in vivo (Suresh et al., 2011).

Activins

Activins are structurally similar to inhibins and belong to the same super family, TGF-b (Chang et al., 2002). Activins are comprised of β dimers (Phillips and Woodruff, 2004), activin

A (β_A - β_A), activin B (β_B - β_B), and heterodimer activin AB (β_A - β_B). Activin A is the glycoprotein that has been most thoroughly studied (Phillips and Woodruff, 2004). In the testis, activin A is secreted largely from Sertoli (de Winter et al., 1993), peritubular (de Winter et al., 1994) and interstitial cells (Lee et al., 1989), which produce the β_A subunit. The testis was identified as the sole source of inhibin; however, activin levels post-castration remain unchanged (de Kretser et al., 2004). Therefore activin appears to originate from other sources, in addition to testicular tissue (de Kretser et al., 2004). Activins induce their biological effect by interacting with 1 of 2 membrane-bound receptors that are specific to the activin (Hedger and Winnall, 2012).

Activin is able to influence various biological functions in the periphery, including inflammation (de Kretser et al., 2004) and the immune response (Phillips, 2005; Hedger and Winnall, 2012). Paracrine and autocrine functions of activin in the testis have also been investigated. Steriodogenesis was decreased in cultured porcine Leydig cells in the presence of activin (Hedger et al., 2011). Additionally, T secretion from Leydig cells was inhibited by activin (Hsueh et al., 1987; Lin et al., 1989).

Insulin-like Growth Factor-1

Insulin-like growth factor-1 and the insulin-like growth factor-1 receptor (IGF-1R) have been localized in the equine testis (Yoon et al., 2011). Utilizing immunohistochemistry, IGF-1 was observed in Leydig cell cytoplasm and spermatogonia with expression increasing from pre-pubertal to post-pubertal stallions, then decreasing to moderate expression in adult and aged stallions (Yoon et al., 2011). Additionally, IGF-1R was observed in Leydig cells and spermatogonia and followed an age dependent pattern. Expression of IGF-1R was most intense

in Leydig cells from post-pubertal, adult, and aged stallions (Yoon et al., 2011). Expression of IGF-1 and IGF-1R was not observed in Sertoli cells at any age (Yoon et al., 2011).

The restriction of IGF-1 and its cognate receptor to Leydig cells and spermatogonia is indicative of a paracrine or autocrine relationship (Yoon et al., 2011). In vitro culturing of stallion Leydig cells with IGF-1 reduced the percentage of apoptotic cells (Yoon and Roser, 2010). Additional effects of IGF-1 in the testis include regulation of Leydig cell populations and spermatogonial differentiation (Tajima et al., 1995; Yoon and Roser, 2010), increased T production from Leydig cells (Lin et al., 1996), and regulation of DNA synthesis from spermatogonial cells (Söder et al., 1992).

Conclusion

While the regulatory functions of the hypothalamic-pituitary-testis axis have been characterized in the stallion, the interactions within the testis that promote steroidogenesis and spermatogenesis are poorly understood. Additionally, GnRH-I has been localized to numerous tissue types of multiple mammals. However, research investigating the localization of this peptide has been limited in the horse. And although, GnRH-II has been isolated in the equine genome, the transcription and/or translation of this gene have not been evaluated in the horse.

Chapter 2 - Identification of GnRH Isoforms and their Cognate Receptors in the Equine Testis

Introduction

The interaction between the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) and its cognate receptor (GnRHR) on gonadotropes of the anterior pituitary gland has been established as a central regulator of reproduction. The GnRH complex is typically associated with anterior pituitary function, as GnRH secreted from hypothalamic neurons targets its receptor in the anterior pituitary. Even so, researchers have identified GnRH and GnRHR in peripheral reproductive tissues such as the uterus (Dong et al., 1998; Choi et al., 2006), ovary (Harwood et al., 1980; Jones et al., 1980; Reeves et al., 1980; Choi et al., 2006), placenta (Rama and Rao, 2001; Chou et al., 2004), spermatozoa (Bull et al., 2000; Lee et al., 2000; Morales et al., 2000), and testes (Ikeda et al., 1996; Bull et al., 2000) of numerous mammals.

Approximately 23 isoforms of GnRH have been identified (Millar, 2005). As a result, the classical GnRH has now been termed GnRH-I to distinguish it from other isoforms. Only 1 other isoform has been identified in mammals: GnRH-II, which was originally isolated from the chicken hypothalamus (Miyamoto et al., 1984). The amino acid (AA) sequence of GnRH-II differs from GnRH-I by substitutions at positions 5, 7, and 8. Moreover, GnRH-II is highly conserved from boney fish to humans (Temple et al., 2003), thus it has been suggested to be the most ancient form of GnRH. There appears to be prominent selection pressure to retain GnRH-II, as it has been maintained over 500 million yr of evolution (Millar and King, 1987).

Similar to GnRH-I, GnRH-II binds a specific receptor (GnRHR-II). Originally isolated in the African catfish (Tensen et al., 1997), GnRHR-II is also a member of the G-protein-coupled-receptor (GPCR) superfamily and contains 7 transmembrane (TM) domains. Most GPCRs,

including GnRHR-II, have an intracytoplasmic tail; however, this intracellular domain is absent in GnRHR-I. The GnRHR-II gene has been detected in numerous non-mammalian vertebrates (Troskie et al., 1997) and several mammalian species, including the human, horse, and pig (Stewart et al., 2009). However, coding errors in the GnRHR-II gene of some species prevent the production of a functional receptor (Stewart et al., 2009).

In addition to binding its cognate receptor, GnRH-II also is capable of interacting with the GnRHR-I binding pocket. In the anterior pituitary, GnRH-II can bind GnRHR-I and mediate gonadotropin release (Densmore and Urbanski, 2003; Okada et al., 2003). However, GnRH-II is only 10% as effective at stimulating ovulation as GnRH-I (Gründker et al., 2002). Other potential functions of GnRH-II that are mediated by binding to GnRHR-I include regulation of female reproductive behavior (Kauffman et al., 2005) and feed intake (Kauffman and Rissman, 2004). Furthermore, GnRH-II has anti-proliferative effects on cancerous tissues that may be mediated through GnRHR-I or GnRHR-II (Gründker et al., 2002).

In the testis, GnRH-I and GnRH-II appear to affect testosterone secretion. Immunization against GnRH-II in the boar resulted in reduced testosterone production compared to control boars, without affecting LH secretion (Bowen et al., 2006). Indeed, GnRHR-II has been localized in the boar testis and it appears GnRH-II is able to stimulate testosterone secretion independent of LH (Desaulniers et al., 2015). Researchers also have suggested GnRH-I stimulates testosterone production in mouse and rat testes (Ikeda et al., 1996; Bull et al., 2000).

Locally produced GnRH-I and GnRH-II in the mammalian testis may play an important role in steroidogenesis. Therefore the objective of this study was to examine gene expression and protein production of GnRH-I, GnRH-II and their cognate receptors in the equine testis and to identify differences between pre-pubertal and mature stallions.

Materials and Methods

Tissue Collection

Testicular tissue was collected from 15 stallions ranging in age from 7 mo to 15 yr during routine castrations at the Kansas State University Veterinary Health Center (Manhattan, KS). Stallions were segregated by age (colts < 2 yr, n = 5; or stallions ≥ 2 yr, n = 10). Tissue samples were processed immediately following castration. BioProduct's RNase AWAY™ surface decontaminant (Fisher Scientific, Pittsburgh, PA) was used to maintain an RNase-free area while processing tissue samples for mRNA analysis. RNase-free forceps and razor blades were used to harvest approximately 100 mg of tissue from the testicular parenchyma and samples were submerged in 1 mL of RNA*later* (Life Technologies, Grand Island, NY), incubated at 4°C overnight, and moved to a -20°C freezer until further processing. In addition, approximately 400 mg of testicular tissue from each stallion also was transferred into a microcentrifuge tube, flash frozen in liquid nitrogen, and stored at -80°C for later protein analysis.

RT-PCR Analysis

RNA Extraction

Testicular tissue was removed from RNA*later* and placed in 1 mL of TRIzol® Reagent (Life Technologies, Grand Island, NY) on ice. Tissue was homogenized using a Biospec Tissue Tearor (Bartlesville, OK) that had been soaked in 3% H_2O_2 to inactivate RNases. Samples were vortexed with 200 µL chloroform and centrifuged at 14,000 x *g* for 15 min at 4°C. The aqueous layer was transferred to a microcentrifuge tube with 500 µL of isopropyl alcohol. Samples were centrifuged at 12,000 x *g* for 10 min at 4°C to pellet RNA. Supernatant was removed and the pellet was rinsed with 70% ethanol and stored at -80°C. After brief centrifugation, ethanol was

removed and the pellet was allowed to air dry. The pellet was resuspended in 50 µL of nuclease free water with 0.5 µL of RNase OUT (Invitrogen, Grand Island, NY).

To remove any genomic DNA contamination, samples were incubated with 1 µL 10X RQ1 buffer, 4 µL RQ1 DNase and 0.5 µL RNase OUT for 30 min at 37°C in a MJ Research thermocycler (Hercules, CA). Finally, 1 µL of stop buffer was added to each sample, which was then incubated at 65°C for 10 min.

Reverse Transcription

Following removal of any DNA, total RNA was converted to cDNA. After quantifying total RNA using a Nanodrop spectrophotometer (ThermoFisher, Pittsburg, PA), 2 µg of RNA were diluted to a volume of 15.15 µL with nuclease free water. Then, 2 µL of 10 mM random primers (Invitrogen) or an oligo dT primer (Promega, Madison, WI) were added. Samples were incubated at 37°C for 5 min. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) was used to convert RNA to cDNA. The reverse transcription reaction was completed with 5.0 µL 5X M-MLV RT buffer, 1.25 µL 10 µM dNTP, 0.6 µL RNase out, and 1.0 µL M-MLV RT by incubation at 37°C for 1 h and 70°C for 15 min. The resulting cDNA was stored at -20°C. To verify there was no genomic DNA contamination, a second control reaction that lacked reverse transcriptase was performed on each sample and was designated the no-RT control.

Conventional PCR

Next, samples were subjected to conventional PCR analysis by following Promega's Taq Polymerase protocol. First, detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed to confirm a successful RT reaction had occurred and to verify the absence of genomic DNA. Gene specific primers for equine GnRH-I, GnRHR-I, GnRH-II, and GnRHR-II were purchased from Integrated DNA Technologies (Table 1). Each PCR began with

2 min at 95°C. Next, there were 40 cycles of 30 sec at 95°C (melting), 30 sec at the appropriate annealing temperature for each primer pair and gene, and 60 sec at 72°C (extension). The annealing temperature for GnRH-II, GnRHR-II, and GnRHR-I was 55°C and was 56°C for GnRH-I. After 40 cycles, the thermocycler remained at 72°C for 10 min for final extension.

After completion of the PCR, each product was run on a 1% agarose gel via gel electrophoresis in sodium borate running buffer and imaged under an ultraviolet light. A representative PCR product for each gene was column purified using a Gel/PCR DNA Fragments Extraction Kit (IBI Scientific, Kapp Court Peosta, IA) and sequenced at the University of Nebraska Medical Center (Omaha, NE) to confirm product identity. The online translation tool ExPASy (Bioinformatics Research Portal) was used to predict the AA sequence of sequenced transcripts.

Primers initially used for GnRHR-II targeted a small region of the gene in exons 1 and 2. Sequencing confirmed the product identity as equine GnRHR-II. For further characterization, multiple gene specific primers were purchased from Integrated DNA Technologies. Forward primers targeted the 5' end of exon 1 and exon 2 and reverse primers targeted the 3' end of exon 3. Additionally, due to low abundance of transcript, a second round of PCR was completed using a nested primer pair to amplify a greater volume of product.

Table 2.1. Gene specific primers utilized to target equine GnRH-I and GnRH-II ligand and receptor cDNA

Gene	Sequence Name	Primer Sequence
GnRH-I	hGnRH1F79	5'-AGCCAACACTGGTCCTATGG-3'
	hGnRH1R269	5'-TCCTCTTCAATCAGGCTTTC-3'
	hGnRH1R278	5'-TGCCAAGTTTCCTCTTCAAT-3'
GnRHR-I	hGnRHR1F436	5'-CCCCAGCCTTCATGATGGTA-3'
	hGnRHR1F453	5'-GTAGTGATCAGCCTGGACCG-3'
	hGnRHR1R633	5'-AACCTTCTGTCTGTCCGGAG-3'
GnRH-II	hGnRH2F2021	5'-CACTGGTCACATGGCTGGTA-3'
	hGnRH2R2230	5'-CTGTACCAGTGTCTGCTTCC-3'
	hGnRH2F2055	5'-GAGCCTCTAGTTCACCCCAG-3'
	hGnRH2R2257	5'-GCCTGTCACCCTACTTACCA-3'
GnRHR-II	hGnRHR2F116	5'-AGGTCCGAGTGGGAGTGAC-3'
	hGnRHR2F151	5'-TCTTCTGCTGGTGGGAACTT-3'
	hGnRHR2F232	5'-CGACTCTTTGCCCATTTAGC-3'
	hGnRHR2F251	5'-CAGCTGCCGACTTACTGGT-3'
	hGnRHR2F515	5'-TGTTCCTGTTCCATACTGTCCG-3'
	hGnRHR2F545	5'-GCCCAGTCTCCTTCACTCAA-3'
	hGnRHR2F564	5'-ATGTATCACCAAAGGCAGCT-3'
	hGnRHR2R617	5'-AGGTTATAGGTGGTCTCTTG-3'
	hGnRHR2R638	5'-AAGGCAGCAGAAGGTGAGAA-3'
	hGnRHR2R1086	5'-TTTGTTTGTACTTGCAGCTGTCT-3'
	hGnRHR2R1105	5'-TGCCCTTCTTCCTGCCACAT-3'
	hGnRHR2R1123	5'-CGTCTCTTTTGTTTCTCCTGCC-3'
	hGnRHR2R1159	5'-GTATACTCTGTTGTGAGCAAATGG-3'

Antibody Validation

To detect GnRHR-I protein, a goat polyclonal antibody directed against GnRHR-I (sc-8681; Santa Cruz Biotechnology; Santa Cruz, CA) was utilized. The epitope for this peptide was mapped against the c-terminus of the human GnRHR-I between AA 270 and 320. Protein Basic Local Alignment Search Tool (BLAST) alignment identified 96% homology for equine GnRHR-I (National Center for Biotechnology Information; NCBI accession number NP_001075305.1) to the antibody epitope. Mouse testis protein, previously reported to express GnRHR-I (Bull et al., 2000), was included in immunoblot analysis for GnRHR-I as a positive control. This antibody has been reported to successfully detect GnRHR-I in male rat thymus, rat ovary, human neurons, and human cancerous tissues (Keller et al., 2005; Szabó et al., 2005; Wilson et al., 2006; Sengupta et al., 2008; Su et al., 2013).

A goat polyclonal antibody directed against GnRHR-II (sc-162889; Santa Cruz Biotechnology) was used to detect GnRHR-II protein in the stallion testis. This antibody was prepared against a synthetic human peptide derived between AA 60 and 75 of GnRHR-II. Using protein BLAST, the peptide corresponded to AA 147 to 162 of the predicted equine GnRHR-II protein (NCBI accession number XP_005610261.1). Protein BLAST software also identified 89% homology between porcine GnRHR-II and the antibody epitope, while the equine GnRHR-II maintained 86% homology to the antibody peptide. The validity of this antibody was confirmed via ELISA (Desaulniers et al., 2015).

For detection of prepro-GnRH-I, a rabbit polyclonal antibody (sc-20941; Santa Cruz Biotechnology) was utilized. The epitope, AA 1 to 92, corresponded to the full-length prepro-GnRH-I of human origin and was reactive against GnRH-I precursor, mature GnRH-I peptide, and GnRH-associated peptide-I (Santa Cruz Biotechnology). According to BLAST alignment, 88% homology was retained from the antibody epitope to the predicted equine prepro-GnRH-I

hormone and 100% homology was retained for the mature GnRH-I peptide of equine origin (NCBI accession number XP_00364546.2). This antibody had previously been reported to detect GnRH-I in the female rat brain (Smarr et al., 2012) and mouse preoptic neurons (Szymanski and Bakker, 2012).

To detect prepro-GnRH-II in the stallion testis, a rabbit polyclonal antibody (sc-20942; Santa Cruz Biotechnology) was utilized, which previously has been confirmed to detect GnRH-II in human ovarian cancer cells (Wilkinson et al., 2008). The immunogen used corresponded to AA 1 to 120 of the full-length prepro-GnRH-II hormone of human origin and was reactive against the GnRH-II precursor, the mature GnRH-II, and the GnRH-associated peptide-II (Santa Cruz Biotechnology). Alignment in Protein BLAST revealed 33% homology between the epitope and the predicted equine prepro-GnRH-II and 100% homology with the mature GnRH-II peptide of equine origin (NCBI accession number XP_005604572.1).

Further validation for all primary antibodies included positive and negative control samples to verify antibody accuracy via Western blot technique (Table 2.2). To verify primary antibodies reacted to their specified peptide sequences in Western blot analysis, a testis tissue sample was collected from a species known to express the specific protein. Additionally, to confirm the antibody's ability to recognize different concentrations of the specific protein, 3 increasing protein levels were used. Finally, the secondary antibody was incubated with protein samples that did not contain the primary antibody to verify the secondary antibody did not interact with protein samples and provide false signal.

Table 2.2. Protein sources used as positive and negative controls to validate antibody specificity for the detection of GnRH-I and –II in the stallion testis using Western blot technique

	Positive Control	Negative Control
GnRH-I	Mouse Testis	Mouse Bladder
GnRHR-I	Mouse Testis	Boar Testis
GnRH-II	Boar Testis	Mouse Testis
GnRHR-II	Boar Testis	Mouse Testis

Immunoblot Analysis

Protein was extracted from 400 mg of each testis sample via homogenization in 1 mL radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid, 2 mM EDTA, 1 mM PMSF, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail) using a Biospec Tissue Tearor. A bicinchoninic acid assay (BCA; Pierce Biotechnology, Rockford, IL) was used to quantify extracted protein. A 4X loading dye [2% Tris (pH 6.8), 28% glycerol, 20% SDS and Orange G] containing dithiothreitol (DTT; 100 mM) was mixed with each sample. Next, a 10% SDS-PAGE gel was used to separate 20 µg or 40 µg of each sample. This was followed by semi-dry electroblotting to an Immobilion-FL polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Membranes were incubated in Odyssey blocking buffer to block against non-specific binding for 2 h at room temperature (RT). Membranes were incubated with a primary antibody directed against GnRHR-I (1:300; goat polyclonal; sc-8681; Santa Cruz Biotechnology) or GnRHR-II

(1:1000; goat polyclonal; sc-162889; Santa Cruz Biotechnology) by shaking at 4°C overnight (OVN). The primary antibody was diluted in Odyssey blocking buffer with 0.1% Tween-20.

The following day, membranes were washed and incubated with a secondary donkey anti-goat antibody (1:20000; Alexa Fluor 680; Invitrogen, Carlsbad, CA). Secondary antibodies were diluted in Odyssey blocking buffer with 0.1% Tween-20 and 0.01% SDS and incubated with blots for 1 h at RT. Washes were performed with Tris-buffered saline with 0.1% Tween-20 (TBS-T). Immunoblots were visualized with an Odyssey Scanner and Image Software (LI-COR Biosciences). Membranes were stained with GelCode® Blue Stain Reagent (Pierce Perbio Science Company, Rockford, IL) and destained with a 50% methanol and 1% acetic acid solution (Welinder and Ekblad, 2011). Blots were reimaged using an Odyessey Scanner to determine total protein in each lane. Total protein in each lane was used to normalize immunoblots. Normalized values were used to determine relative protein concentrations of GnRHR-I and GnRHR-II in the testicular tissue of all animals.

Immunodot Blot

Protein was boiled for 5 min to denature proteins and expose epitope-binding sites. Then, 1.6 µg of protein was applied directly onto a pre-wetted Immobilion-FL PVDF membrane and allowed to dry for 30 min. Saturated filter paper was used to keep the PVDF membrane hydrated while allowing protein to bind the membrane. Next, immunoblots were blocked against non-specific binding by incubation in Odyssey Blocking Buffer for 2 h. Primary antibodies directed against GnRH-I preprohormone (1:150; rabbit polyclonal; sc-20941; Santa Cruz Biotechnology) and GnRH-II preprohormone (1:200; rabbit polyclonal; sc-20942; Santa Cruz Biotechnology) were used. Blots were incubated with primary antibody by shaking at 4°C OVN and then with secondary mouse anti-rabbit antibody (1:5000; Alexa Fluor 800; Invitrogen) for 1 h while

shaking at RT. Immunoblots were visualized with an Odyssey Scanner. Finally, immunoblots were stained for total protein as described previously.

Statistical Analysis

Statistical analyses were performed on Western blot data using SAS (Version 9.2; Cary, NC). The General Linear Model (GLM) procedure was used to analyze immunoblot data with animal as the experimental unit and age as the fixed effect. Immunoblot data were confirmed to be normally distributed using the Shapiro-Wilk test. A P-value ≤ 0.05 was considered significant. Results are presented as means ± the standard errors of the mean (SEM).

Results

Sequencing Transcripts of GnRH Ligand and Receptor Isoforms in the Stallion Testis

Utilizing gel electrophoresis, a band for each reverse transcription PCR (RT-PCR) product was identified in all animals (n = 15) at the expected product size for GnRH-I, GnRHR-I, and GnRHR-II (Fig 2.1). The product generated with GnRH-I primers showed the most homologous match to predicted *Equus caballus* GnRH-I mRNA (NCBI accession number XM_003364498.2) with query coverage of 100%, an *e*-value of 6e-56, and maximum alignment identity of 100%. Alignment for the product generated with GnRHR-I primers revealed *E. caballus* GnRHR-I mRNA (NCBI accession number NM_001081836.1) as the optimal match with 100% query coverage, an *e*-value of 1e-42, and 94% alignment identity. The BLAST alignment for the GnRHR-II product identified 100% query coverage to the NCBI predicted *E. caballus* GnRH-II receptor-like mRNA (NCBI accession number LOC100065736) with an *e*-value of 3e-158.

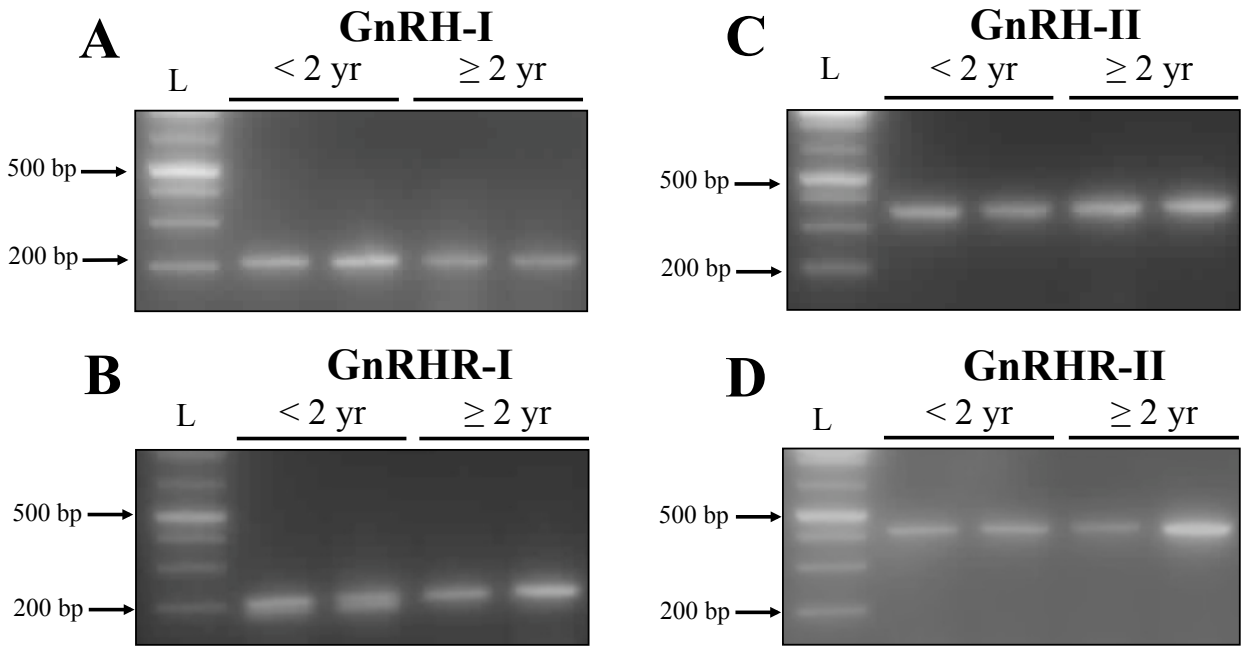

Figure 2.1. Representative agarose gels containing RT-PCR products generated from mRNA isolated from equine testicular tissue. The ladder is represented by L on each agarose gel. Primers were specific for GnRH-I, GnRH-II and their cognate receptor isoforms. Expected product size was observed for **(A)** GnRH-I [200 base pair (bp)], **(B)** GnRHR-I (200 bp), and **(D)** GnRHR-II (400 bp). The expected product size for **(C)** GnRH-II was 200 bp, however, the observed product size was 350 bp.

Equine GnRH-II Retain Intron through Transcription

Examination of the equine genome indicates the horse retains the GnRH-II precursor gene located on chromosome 22 (NCBI database; Stewart et al., 2009). However, researchers have not investigated the equine GnRH-II transcript. The predicted RT-PCR product size for equine GnRH-II was 200 base pair (bp), yet the RT-PCR product was approximately 350 bp (Fig. 2.1). Alignment for this product showed the greatest query coverage (100%) to predicted *Equus przewaslkii* GnRH-II mRNA (NCBI accession number XM_008527598.1). Comparatively, predicted *E. caballus* GnRH-II mRNA (NCBI accession number

XM_005604515.1) had query coverage of only 40%. However, alignment of the sequenced product directly against the predicted *E. caballus* genomic GnRH-II gene (NCBI accession number NC_009165.2) showed 100% query coverage, an *e*-value of 6e-127, and 100% alignment identity (Fig 2.2). Thus it appears the horse retains the intron between exon 1 and exon 2 through transcription of the GnRH-II gene and is transcribed differently than predicted by bioinformatics.

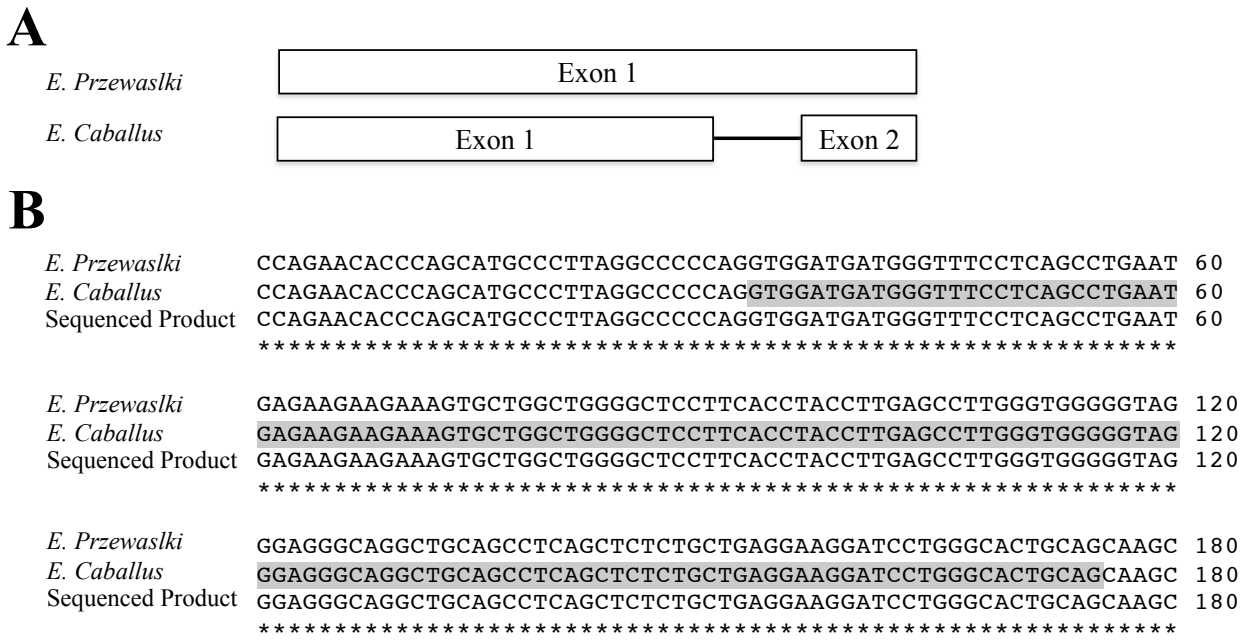

Figure 2.2. (A) Model of intron and exon location of GnRH-II gene for *E. przewaslki* and *E. caballus* predicted by NCBI database. **(B)** Sequence comparison of the GnRH-II gene for *E. przewaslki* RNA, *E. caballus* DNA, and the sequenced product indicates predicted intron area (highlighted) is identical to the sequenced RT-PCR sample. (*indicates an identical sequence)

Alternative Splicing of Equine GnRHR-II mRNA

Several products were generated in the second PCR using nested primers to amplify GnRHR-II mRNA. Sequencing confirmed the product identity as *E. Caballus* GnRHR-II mRNA and provided clarification for unusual band sizes, as alternative splicing was discovered at the junction between exon 2 and 3 (Fig. 2.3). Splice variant 1 appeared to retain the entire 536 bp of the intron between exon 2 and 3. However, splice variant 2 retained only 196 bp of the intron and lacked 167 bp at the beginning of exon 3. Similar to splice variant 2, variant 3 also partially retained the intron; however, only 186 bp of the intron were retained and it lacked 174 bp of exon 3. Splice variant 4 deleted 75 bp from the terminal portion of exon 2 as well as 100 bp from the start of exon 3. If translated, equine GnRHR-II splice variants 1, 2, and 3 are each predicted to generate a 241 AA protein with a premature stop codon located 4 bp into the retained intron. Comparatively, a 235 AA protein was predicted for splice variant 4. Although variant 4 does not retain the intron between exon 2 and 3, this transcript still encodes a premature stop codon 61 bp into the retained portion of exon 3.

In addition to alternative splice transcripts, sequencing of RT-PCR products also identified 2 transcripts spliced as predicted between exon 2 and 3. The first transcript was amplified from the 5' end of exon 2 to the 3' end of exon 3. Translation of this mRNA sequence predicts a 244 AA protein. This predicted protein was compared to known sequences in mammals reported to express the full-length GnRHR-II (Fig 2.4). A separate transcript amplified from the 5' end of exon 1 to the 3' end of exon 3 included 'CACT' at bp 649 in exon 2 (Fig 2.5). These 4 bases are encoded in *E. Przewaslkii* GnRHR-II mRNA (NCBI accession number XM_008528236.1), but they are not predicted to be included in the equine GnRHR-II mRNA (NCBI accession number XM_005610204.1). Translation of this product is predicted to generate a 220 AA protein.

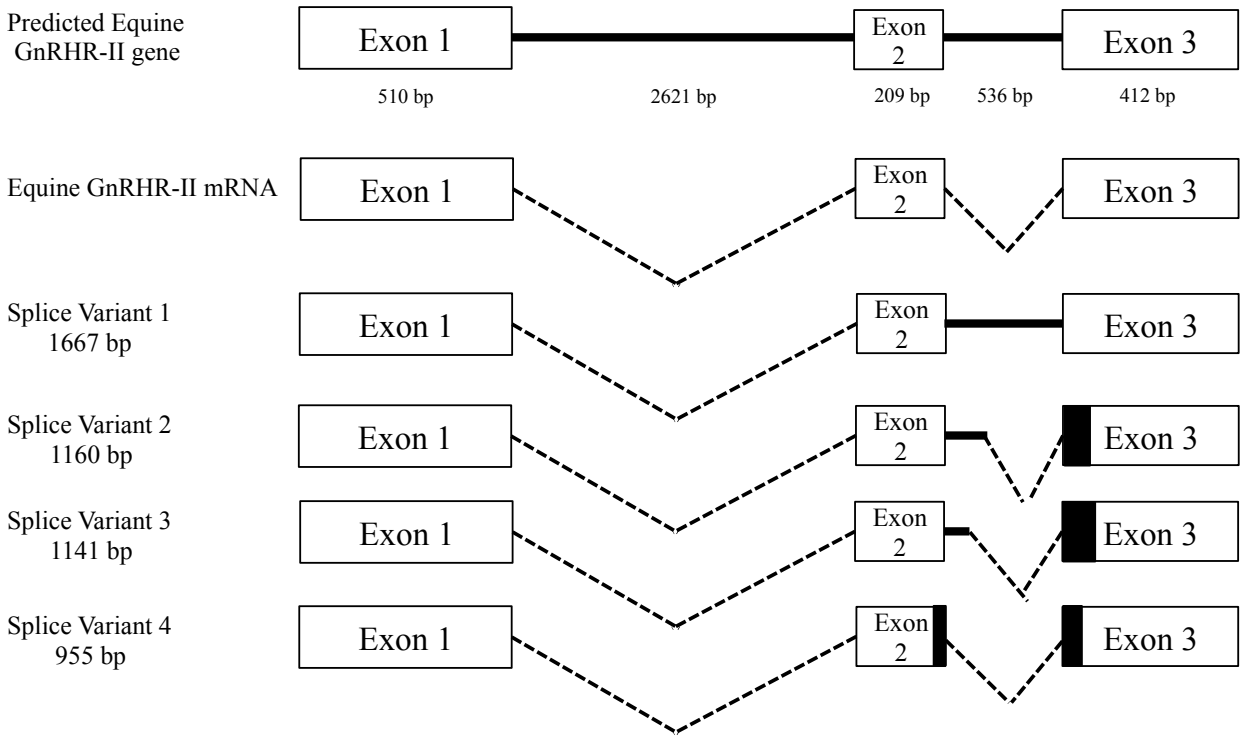

Figure 2.3. A model indicating the relative position of the 3 exons and 2 introns for the predicted *E. Caballus* GnRHR-II gene sequence. Sequencing of RT-PCR products revealed several alternatively spliced transcripts for the equine GnRHR-II. A dashed line represents the intronic region that was removed as predicted. A solid line is representative of a transcribed intron, while a black box indicates a portion of an exon that was spliced out. Splice variant 1 retained intron 2 through transcription, while splice variants 2 and 3 partially retained 196 or 186 bp of intron 2 as well as deleted 167 or 174 bp of exon 3 respectively. Splice variant 4, however, deleted 75 bp from exon 2 and 100 bp from exon 3.

Figure 2.4. The amino acid sequence of GnRHR-II compared between *Macaca mulatta, Sus scrofa,* and *E. caballus*. The online transmembrane (TM) prediction tool, Tmpred, was used to predict TM domains which are indicated by boxes. GnRHR-II has 7 TM domains as indicated by *M. mulatta* and *S. scrofa*. However, a 244 AA protein with 5 TM domains is predicted to be translated from the equine transcript that spliced correctly between exon 2 and 3 and did not include 4 bases in exon 2. This transcript encodes a premature stop codon in exon 3. [(*) indicates an identical sequence, (:) represents an AA alignment with very similar properties, (.) represents an AA alignment with slightly similar properties]

```
E. caballus         GGCCCAGTCTCCTTCACTCAATGTATCACCAAAGGCAGCTTCAAGGCTCGATGGCAAGAG 600
E. przewalskii      GGCCCAGTCTCCTTCACTCAATGTATCACCAAAGGCAGCTTCAAGGCTCGATGGCAAGAG 600
Sequence product    GGCCCAGTCTCCTTCACTCAATGTATCACCAAAGGCAGCTTCAAGGCTCGATGGCAAGAG 600
                    ************************************************************

E. caballus         ACCACCTATAACCTCTTCTCACCTTCTGCTGCCTTTTTCTGCTGCCACT----ACTATGA 656
E. przewalskii      ACCACCTATAACCTCTTCTCACCTTCTGCTGCCTTTTTCTGCTGCCACTCACTACTATGA 660
Sequence product    ACCACCTATAACCTCTTCTCACCTTCTGCTGCCTTTTTCTGCTGCCACTCACTACTATGA 660
                    ********************************************    *******

E. caballus         CCATCTGCTACAGCCGCATTGTCCTCAGTGTGTCCAGTCCCCAGACAAGGAAAGGGAACT 716
E. przewalskii      CCATCTGCTACAGCCGCATTGTCCTCAGTGTGTCCAGTCCCCAGACAAGGAAAGGGAACT 720
Sequence product    CCATCTGCTACAGCCGCATTGTCCTCAGTGTGTCCAGTCCCCAGACAAGGAAAGGGAACT 720
                    ************************************************************
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Figure 2.5. Sequenced equine GnRHR-II product spliced as predicted between exon 2 and 3, yet encoded 4 additional bases beginning at bp 649. Alignment via BLAST shows 99% homology of the predicted *E. caballus* GnRH-II Receptor-like mRNA to the predicted *E. przewalskii* GnRH-II Receptor-like mRNA. This transcript is predicted to only produce a protein with 220 AA and a stop codon (TGA) at 658 bp. (*indicates an identical sequence)

Protein Expression of GnRH Ligand and Receptor Isoforms in the Stallion Testis

Immunodot blot analysis (Fig 2.6) was used to identify the presence of prepro-GnRH-I and prepro-GnRH-II protein in colts < 2 yr (n = 4) and stallions ≥ 2 yrs (n = 10), thus confirming that the GnRH-I and GnRH-II transcripts detected via RT-PCR are translated to protein within the stallion testis. The presence of both GnRHR-I and -II protein in the testis of all horses sampled was verified using immunoblot technique. However, colts < 2 yr (n = 4) had 3-fold greater GnRHR-I than stallions ≥ 2 yrs (n = 7; $P < 0.022$; Fig 2.7). Conversely, quantification of the relative band density of GnRHR-II protein indicated a trend for stallions ≥ 2 yr to have greater levels compared to colts < 2 yr ($P = 0.0756$; Fig 2.8). The approximate molecular weight of the GnRHR-II protein was approximately 55 kDa and the band for GnRHR-I was 50 kDa.

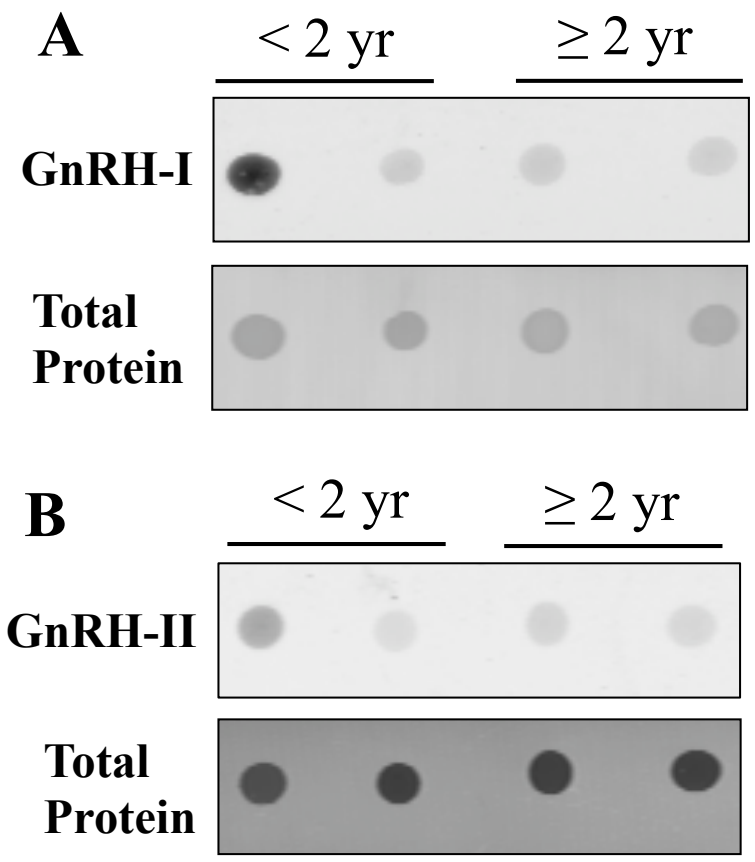

Figure 2.6. (A) Representative dot blots showing the presence of prepro-GnRH-I and **(B)** prepro-GnRH-II in the testis of colts < 2 yr and stallions ≥ 2 yr.

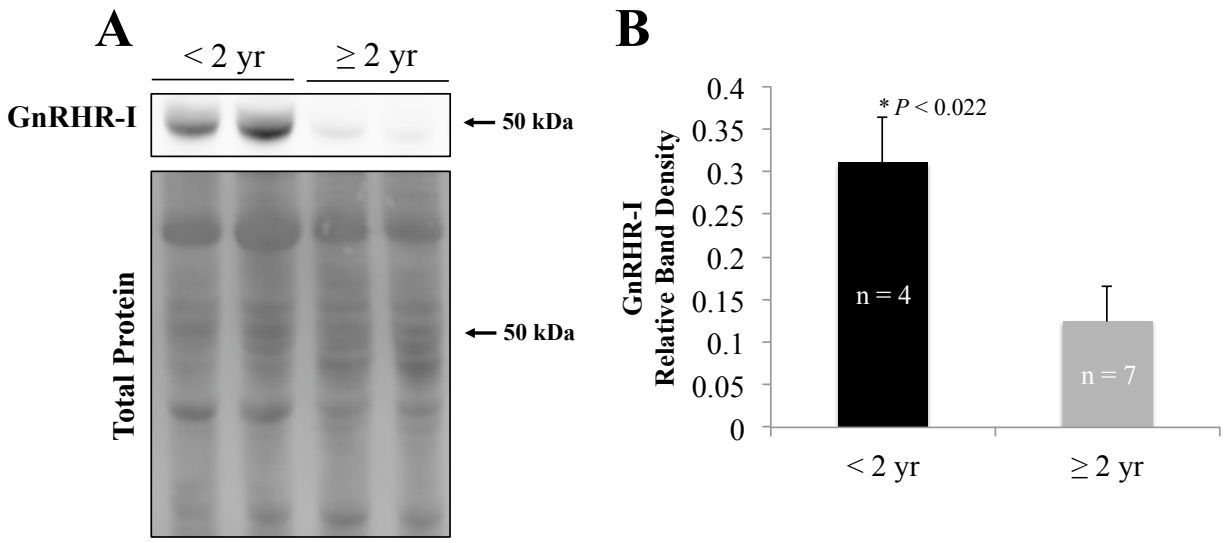

Figure 2.8. (A) Representative Western blot of testicular tissue from colts < 2 yr and stallions ≥ 2 yr using an antibody directed against GnRHR-I. **(B)** Quantification of Western blots showed 3-fold greater levels of GnRHR-I in colts < 2 yr compared to stallions ≥ 2 yr ($P < 0.022$).

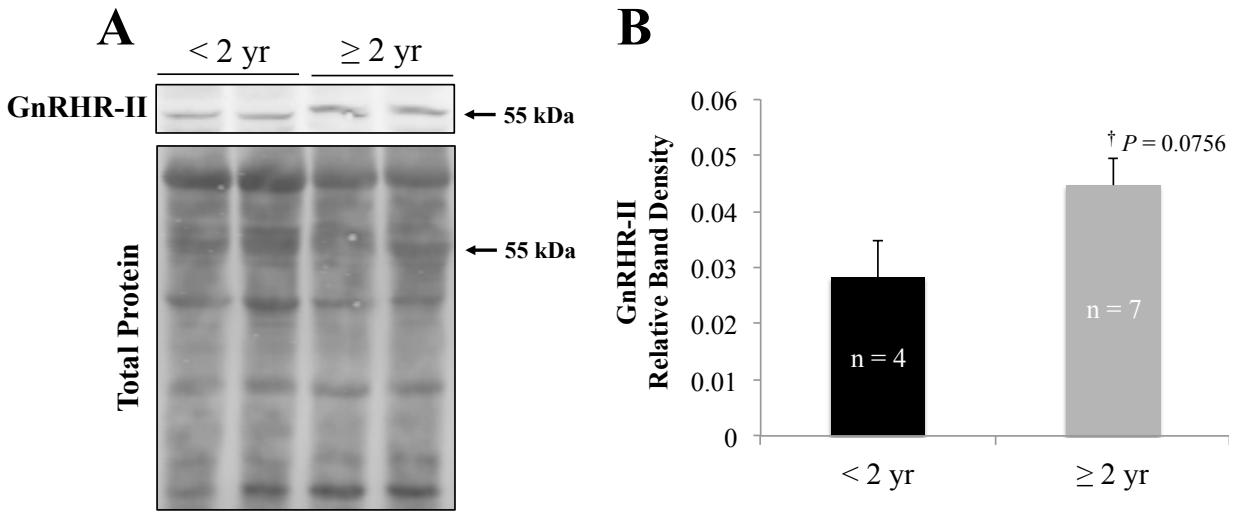

Figure 2.7. (A) Representative Western blot of testicular tissue from colts < 2 yr and stallions ≥ 2 yr using an antibody directed against GnRHR-II. **(B)** There was a tendency for GnRHR-II to be produced in greater levels in stallions ≥ 2 than in colts < 2 yrs ($P = 0.0756$).

Discussion

Although previous researchers have identified GnRH-I and GnRHR-I in the testis of mature rats (Sharpe and Fraser, 1980; Botté et al., 1998), humans (Bahk et al., 1995), mice (Bull et al., 2000), alpacas (Zerani et al., 2011), and frogs (Pierantoni et al., 1984; Minucci et al., 1986), very little is known regarding the role of the testicular GnRH-I:GnRHR-I complex. While production of GnRHR-I in mice and rats is greater after sexual maturity (Botté et al., 1998, Anjum et al., 2012), the current study provides the first evidence that GnRHR-I expression patterns differ in the stallion compared to other mammals. In equine testicular tissue, GnRHR-I protein levels were greater in colts < 2 yrs compared to stallions ≥ 2 yrs. Therefore, it appears GnRHR-I protein levels in the testis decrease as stallions age. The testicular GnRH-I complex may play a more important role in testicular development of pre-pubertal stallions compared to other mammals.

The *E. przewalskii* species is the most ancient living relative to the modern horse, thus it is appropriate to compare its mRNA to that of *E. caballus*. The GnRH-II gene structures for *E. przewalskii* and *E. caballus* were predicted using bioinformatics to indicate the relative position of exons and introns. The *E. przewalskii* species is predicted to encode a single exon for GnRH-II transcript (NCBI accession number XM_008527598.1). Comparatively, *E. caballus* is predicted to encode 2 exons separated by an intron with 140 bp (NCBI accession number XM_005604515.1). Sequencing of our RT-PCR product and BLAST alignment indicated that the equine transcribes the intron between bases 2102 and 2242 of the GnRH-II gene. A reverse transcription reaction with no reverse transcriptase confirmed there was no genomic DNA contamination in our samples. The sequenced product also has an identical alignment to the *E. przewalskii* GnRH-II mRNA. As *E. przewalskii* is not predicted to encode an intron in its GnRH-

II mRNA, an identical alignment may indicate that the horse encodes a single exon for GnRH-II, similar to *E. przewalskii*, and is not as predicted via bioinformatics.

It appears numerous species have coding errors in the GnRHR-II gene (Stewart et al., 2009). Indeed, of the genomes examined thus far, only the pig, tree shrew, marmoset, and macaque retain a functional GnRH-II:GnRHR-II complex (Stewart et al., 2009). Previous researchers believed that the horse was unable to produce a functional GnRHR-II because of disruptive mutations in the gene (Stewart et al., 2009). The GnRHR-II gene in the horse is located on chromosome 5 and encodes the expected 3 exon/2 intron structure for the GnRHR-II gene similar to numerous other mammals (NCBI database). Researchers suggested that the equine encodes frameshifts in exon 2 and 3 with a premature stop codon located at codon 94 in exon 3 in the GnRHR-II (Stewart et al., 2009). We have shown that the GnRHR-II gene is transcriptionally active in the equine testis, as the presence of GnRH-II mRNA was confirmed via RT-PCR.

Transcriptional regulation of GnRHR-II is poorly understood. Humans and sheep are reported to have coding errors in their GnRHR-II coding sequences (Morgan et al., 2003; Gault et al., 2004). However, it appears that the GnRHR-II gene is transcriptionally active in these mammals (Morgan et al., 2003; Gault et al., 2004). Based on our findings, the same appears to be true for the horse.

Multiple splice variant transcripts were sequenced for GnRHR-II mRNA isolated from stallion testes. Alternatively spliced products of GnRHR-II also have been detected in the chicken and human (Morgan et al., 2003; Shimizu and Bédécarrats, 2006). Furthermore, it appears several GnRH receptor isoforms, including GnRHR-I (Kakar, 1997; Kottler et al., 1999) and GnRHR-III (Wang et al., 2001), generate multiple mRNA transcripts via alternative splicing.

Alternative splicing of the human GnRHR-II transcript circumvents a frameshift in exon 1 (Morgan et al., 2003). As a result of this splicing, a 5 TM receptor may be produced in the human (Neill, 2002). In the human and chicken, there was no intron retention in the GnRHR-II variant transcripts. However, the bullfrog GnRHR-III gene is reported to exhibit spliced transcripts that retain a portion of intron 2 (Wang et al., 2001). Similar to what has been observed in the bullfrog, we also sequenced several transcripts that showed intron retention of the equine GnRHR-II gene. Yet, splice variants in the equine that retained the intron portion introduced a premature stop codon that would produce a truncated protein with 241 AA.

While multiple equine GnRHR-II transcripts were sequenced, these transcripts were predicted via an online translation tool to generate truncated proteins. However, we detected a large protein using Western blot. The molecular weight of GnRHR-II in the boar testis, which was incorporated as a positive control in this study, is 60 kDa (Desaulniers et al., 2015), whereas our data revealed a molecular weight of 55 kDa using an antibody specific to GnRHR-II. The differently sized products of GnRHR-II may be the result of differing glycosylation or a truncated protein.

Reseachers have suggested a full-length GnRHR-II may be produced in the human (Neill et al., 2004). Although a frameshift and premature stop codon are documented in the human genome, there is precedence for a functional human GnRHR-II (Neill et al., 2004). In human cancer cells where GnRHR-I was suppressed, the administration of GnRH-II still showed anti-prolific effects; thus GnRH-II may be acting via GnRHR-II (Gründker and Emons, 2003).

Even though the horse appears to encode frameshifts and a premature stop codon, post-transcriptional mechanisms may be employed in the equine testis to produce the full-length GnRHR-II protein that was detected via Western blot. Suppression of premature stop codons has

been reported in numerous mammalian genes (Robinson and Cooley, 1997). Rather than translation of the stop codon, UGA is translated as a selenocysteine (Low and Berry, 1996). Moreover, RNA editing may also recode or repair frameshifts in GnRHR-II transcript, thus allowing a full-length receptor to be translated (Namy et al., 2004). Post-transcriptional modification of the GnRHR-II transcript is a potential mechanism to overcome coding errors and allow the horse to generate a full-length, potentially functional receptor.

The numerous other effects reported for GnRH-II, such as regulation of female reproductive behavior, feed intake, gonadotropin release, and anti-prolific effect on cancerous tissues, are likely caused by GnRH-II binding GnRHR-I. Evidence from the boar is the first to indicate an effect of GnRH-II binding its cognate receptor and eliciting a response (Desaulniers et al., 2015). The boar does not express GnRHR-I in the testis (Zanella et al., 2000), thus it is thought GnRH-II interacts solely with GnRHR-II. However, as the stallion expresses both receptor and ligand isoforms in the testis, the potential functions of GnRH-I and -II increase with their ability to bind the other's receptor and illicit separate responses. To date, the stallion is the only other mammal, in addition to the human, that has been identified to potentially express both ligand and receptor isoforms in the testis (Neill et al., 2001; Neill, 2002; Neill et al., 2004).

Indeed, the intricacies of equine autocrine and paracrine regulation of steroidogenesis and spermatogenesis in the stallion testis are very complex. As reviewed by Roser (2008), numerous hormones are produced in the testis to modulate the microenvironment and aid germ cell development. Hormones known to be produced in the stallion testis include androgens, estrogens, inhibin, activin, insulin-like growth factor-1, transferrin, insulin like peptide 3, β-endorphins, and oxytocin (Roser, 2008). To this list, GnRH isoforms -I and -II can now be included, however their physiological effects are still unknown in the stallion.

The current study is the first to establish GnRH-I and GnRHR-I mRNA and protein are produced in the stallion testis. The equine genome has been examined for GnRH-II and GnRHR-II genes and bioinformatics has predicted the potential mRNA transcripts of these genes. However, we are the first to report that the GnRH-II and GnRHR-II genes are transcriptionally active and translated into protein within the stallion testis. Therefore, the presence of GnRH-I and GnRH-II and their cognate receptors in the stallion testis infers a possible autocrine and/or paracrine function of these decapeptide hormones to assist in steroidogenesis and/or spermatogenesis.

These discoveries may have implications in developing novel pharmacologic agents for the treatment of sub-fertility or infertility in stallions, as researchers suggest a pivotal role for GnRH-I and GnRH-II in male reproduction. Additionally, detection of the GnRH-II complex within the stallion testis may provide insight into why current GnRH vaccines do not consistently suppress sperm production in stallions. The inclusion of GnRH-II in GnRH vaccines may improve male contraceptive methods and assist in the control of wild horse populations. Further research is required to fully elucidate the localization and function of GnRH-I and -II and their cognate receptors in the equine testis.

Chapter 3 - GnRHR-II Detected on Ejaculated Equine Spermatozoa

Introduction

Spermatogenesis is the process whereby spermatogonial germ cells develop into spermatozoa in seminiferous tubules. The seminiferous tubules, in turn, contain both germ cells and Sertoli cells. Within seminiferous tubules, basal and adluminal compartments are formed via tight junctions between Sertoli cells. These tight junctions form the blood-testis barrier, isolating germ cells from the interstitial tissue. The purpose of the blood-testis barrier is to provide a microenvironment conducive to germ cell development and to prevent an autoimmune response of the body to its own germ cells (Senger, 2012).

There are 3 major stages of spermatogenesis: proliferation, meiosis, and differentiation. Proliferation occurs in the basal compartment of seminiferous tubules and results from mitotic divisions of spermatogonial germ cells. Meiosis and differentiation take place in the adluminal compartment. Primary spermatocytes are haploid cells that are produced from meiosis 1. To increase genetic diversity, crossing over of DNA occurs in secondary spermatocytes and results in spermatozoa that encode unique nuclear DNA (Johnson et al., 1997).

Next, spermatids differentiate via spermiogenesis into highly specialized spermatozoa. Spermatozoa are comprised of a head that stores nuclear material and a flagellum that includes a midpiece and principal piece. Upon differentiation, spermatozoa are released into the lumen of the seminiferous tubule where they migrate to the epididumal caput (Johnson et al., 1997). Spermatozoa mature as they migrate through the epididymis. During maturation the cytoplasmic droplet is removed from the midpeice and the spermatozoa become progressively motile (Johnson et al., 1997; Senger, 2012).

The GnRH isoforms, GnRH-I and GnRH-II, along with their receptors, have been detected in seminiferous tubules of several mammals (van Biljon et al., 2002; Ramakrishnappa et al., 2005; Ciaramella et al., 2015; Desaulniers et al., 2015). Like GnRH-I, GnRH-II, has been isolated in the testis of humans (van Biljon et al., 2002), boars (Desaulniers et al., 2015), and our previous work in stallions. Receptors for both GnRH-I and GnRH-II (GnRHR-I and GnRHR-II) have been localized to spermatogenic cells throughout several stages of spermatogenesis in seminiferous tubules (Ciaramella et al., 2015; Desaulniers et al., 2015).

In terms of function, several roles for GnRH-I have been postulated that affect spermatogenesis. In the rat and mouse, GnRHR-I has been identified on spermatogonial germ cells and elongated spermatids (Ciaramella et al., 2015), while, in the human, GnRHR-I has been detected on ejaculated spermatozoa (Morales et al., 2002). Because both GnRH-I and GnRHR-I are expressed in the same cell type during acrosome biogenesis, it has been postulated that GnRH-I may function through an autocrine mechanism during this stage of spermatogenesis (Ciaramella et al., 2015). As well, it has been suggested that GnRH-I secreted from Sertoli cells functions via paracrine signaling to assist in sperm release from seminiferous tubules and transport to the epididymal caput (Ciaramella et al., 2015). The percentage of ejaculated human spermatozoa that bind to the zona pellucida is increased when incubated with a GnRH-I agonist, thus GnRH-I may interact with its receptor to facilitate spermatozoa binding (Morales et al., 2002).

Similarly, GnRHR-II has been detected in human and porcine spermatogonial germ cells. Transcripts for GnRHR-II have been isolated from ejaculated human spermatozoa (van Biljon et al., 2002). In the boar, GnRHR-II has been localized to the membrane of germ cells and the

connecting piece of mature ejaculated spermatozoa (Desaulniers, 2013; Desaulniers et al., 2015). Although GnRHR-II is clearly present in germ cells, its function is poorly understood.

Previously, we detected GnRHR-I and GnRHR-II mRNA transcripts and protein expressed locally in the stallion testis (Chapter 2). Because localized GnRHR-I and GnRHR-II in mammalian spermatogenic cells may play an important role in the maturation of spermatozoa and in their ability to fertilize an ovum, our next objective was to determine whether these receptor isoforms are present on ejaculated equine spermatozoa.

Materials and Methods

Collection and Purification of Equine Spermatozoa

Using standard industry practices, semen was collected from 6 Quarter Horse stallions (9 to 18 yr) via a Missouri model artificial vagina equipped with a collection bottle and gel filter. After collection, semen was transferred to a 50 mL conical tube where 1 mL of semen was layered on 6 mL of EquiPure^{TM} (Nidacon, Healdsburg, CA) and centrifuged at 300 x *g* for 30 min at room temperature (RT). The EquiPure^{TM} solution was removed and the purified sperm pellet was rinsed by centrifugation with phosphate buffered solution (PBS; Life Technologies, Grand Island, NY) at 300 x *g* for 10 min. Next, PBS was removed and each sample was diluted in 1 mL of fresh PBS. A drop of diluted sperm was air-dried on an UltraStick slide (Gold Seal Products, Portsmouth, NH) for immunocytochemistry. Purified sperm were also flash frozen in liquid nitrogen for protein extraction and immunoblot analysis. Remaining semen was centrifuged for 10 min at 300 x *g* to separate seminal plasma, which was then removed and stored at -20°C. Four semen samples were used for immunoblotting and 4 semen samples were used for immunocytochemistry.

Immunoblot

To extract protein from spermatozoa, 400 µL of RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid, 2 mM EDTA, 1 mM PMSF, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail) was added to purified spermatozoa, which were then passed through a 21 gauge needle followed by a 27 gauge needle. Samples were incubated in RIPA buffer for 90 min on ice. The protein in each sample was quantified using a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford, IL). A 4X loading dye [2% Tris (pH 6.8), 28% glycerol, 20% SDS and Orange G] containing dithiothreitol (DTT; 100 mM) was mixed with each sample. Twenty µg of protein was then separated on a 12% SDS-PAGE gel. This was followed by semi-dry electroblotting to an Immobilion-FL polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Membranes were incubated with Odyssey blocking buffer for 2 h at RT to block against non-specific binding. Immunoblots were probed with a goat polyclonal antibody directed against GnRHR-II (1:750; sc-162889; Santa Cruz Biotechnology, Santa Cruz, CA) and GnRHR-I (1:300; sc8681; Santa Cruz Biotechnology) diluted in Odyssey blocking buffer with 0.1% Tween-20. Blots were incubated with primary antibody by shaking at 4°C overnight (OVN). The following day, membranes were incubated with a secondary donkey anti-goat antibody (1:20000; Alexa Fluor 680; Invitrogen, Carlsbad, CA) in Odyssey blocking buffer with 0.1% Tween-20 and 0.05% SDS for 1 h at RT. Following primary and secondary antibody incubation, membranes were washed in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Immunostaining was visualized with an Odyssey Scanner and Image Software (LI-COR Biosciences). To determine total protein in each lane, membranes were stained with GelCode® Blue Stain Reagent (Pierce Perbio Science Company, Rockford, IL) and destained with a 50% methanol and 1%

acetic acid solution (Welinder and Ekblad, 2011). Blots were then reimaged using an Odyssey Scanner.

Immunocytochemistry

Sperm were fixed to an UltraStick slide with 4% paraformaldehyde at RT in a humidified chamber. Once sperm were fixed, the slides were rinsed in TBS. Autofluorescence was abolished with a 30 min exposure to ultraviolet light. Slides were incubated in TBS-T (0.5% Tween-20) for 10 min at RT to permeablize spermatozoa. To remove excess TBS-T, slides were then rinsed with TBS. A 10% normal serum diluted in TBS-T (0.05% Tween-20) was incubated with the slides for 30 min at RT to reduce non-specific binding. After blocking, slides were treated with either a monoclonal mouse primary antibody directed against GnRHR-II (1:25, sc-100301, Santa Cruz Biotechnology) or a polyclonal goat primary antibody directed against GnRHR-I (1:25; sc-8681; Santa Cruz Biotechnology) diluted with 10% normal serum in TBS-T (0.05% Tween-20) and incubated OVN at 4°C.

The next morning, excess primary antibody was removed with 3 washes in TBS-T (0.05% Tween-20). Slides were treated with an Alexa Fluor 488 secondary antibody (rabbit anti-goat or goat anti-mouse; Invitrogen) for 1 h at RT in a dark, moist chamber. The secondary antibody was diluted 1:400 in 10% normal serum TBS-T (0.05% Tween-20). Finally, slides were rinsed with TBS-T (0.05% Tween-20) to remove residual antibody. An 80% glycerol and 20% Tris mounting solution was utilized to seal the slides. An inverted confocal microscope (Olympus IX 81) with FITC filter sets was utilized at the University of Nebraska-Lincoln microscopy core to view the spermatozoa.

Results and Discussion

While GnRHR-I was not detected in purified stallion sperm protein via Western blot analysis nor immunocytochemistry using the sc-8681 antibody directed against GnRHR-I, we identified a 55 kDa band for GnRHR-II using immunoblot (Fig 3.1). A similar molecular weight was reported for GnRHR-II isolated in purified boar spermatozoa (Desaulniers et al., 2015) and equine testicular tissue (Chapter 2). Furthermore, GnRHR-II was localized to the neck region of mature purified equine spermatozoa through the use of immunocytochemistry (Fig 3.2). The GnRHR-II has also been detected at the same location on the connecting piece of mature boar spermatozoa while GnRH-II was also identified in boar seminal plasma (Desaulniers, 2013). It is thought that in boars the GnRH-II complex may play a role in motility or tail development (Desaulniers, 2013).

In contrast, GnRHR-I has been identified on spermatogenic cells of rats, mice and humans (Morales et al., 2002; Ciaramella et al., 2015). Depending on the stage of development, multiple functions have been postulated for the GnRH-I complex. Even though we previously detected GnRHR-I in the testis of stallions, this receptor appears to be absent on mature stallion spermatozoa. Identifying the exact location of GnRHR-I within the testis may elucidate the presence of GnRHR-I on immature spermatogenic cells or other testicular cells.

Although we are the first to identify GnRHR-II protein on stallion spermatozoa via immunoblot and immunocytochemistry, the significance of this finding is unclear. Because of the localization of GnRHR-II to the connecting piece of equine spermatozoa, GnRH-II may have numerous functions including assisting fertilization, increasing motility, and assisting in capacitation. In the human, GnRHR-I has been reported to assist sperm binding to the zona pellucida (Morales et al., 2002). As GnRHR-I was not present on equine spermatozoa, GnRHR-II may have a similar function in the stallion. Because mitochondria located in the midpiece

enable the whipping motion of the tail and subsequent motility of spermatozoa, GnRH-II may also play a role in progressive motility. Sperm capacitation requires calcium release (Ho and Suarez, 2003). Intracellular stores of calcium are located near the redundant nuclear envelope in the neck region of sperm (Ho and Suarez, 2003). These stores of calcium may be released as a result of GnRH-II binding GnRHR-II on the connecting piece of stallion spermatozoa and assist capacitation.

While GnRHR-II was detected on stallion spermatozoa, we did not analyze stallion seminal plasma for the presence of GnRH-II. Furthermore, GnRHR-II mRNA has been identified in the female reproductive tract of the human and marmoset (Neill et. al., 2001; Millar et al., 2001). Additional research investigating the presence of GnRH-II in stallion seminal plasma and the mare reproductive tract may elucidate potential functions for the GnRH-II complex in stallion reproduction. Identifying the role of GnRHR-II on spermatozoa may have implications for increasing post-thaw motility of frozen semen, and improving conception rates of sub-fertile stallions by increasing the percentage of motile sperm after storage.

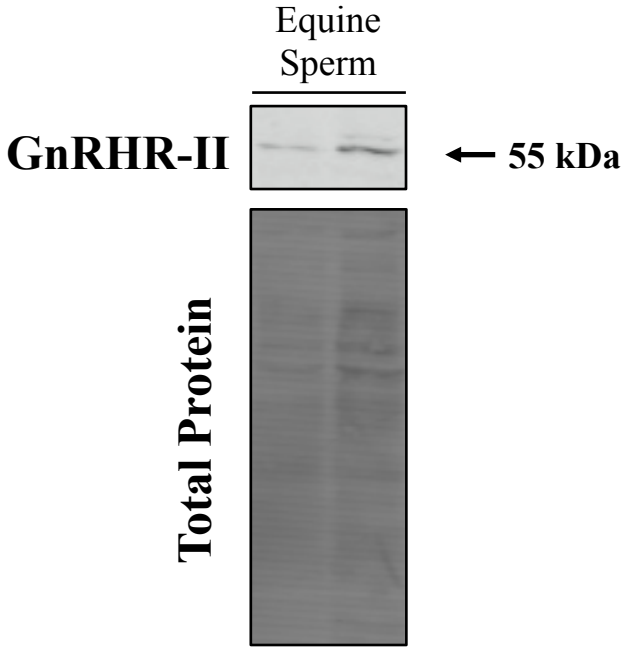

Figure 3.1. Representative Western blot of purified stallion spermatozoa protein using the GnRHR-II antibody sc-162889 (Santa Cruz Biotechnology).

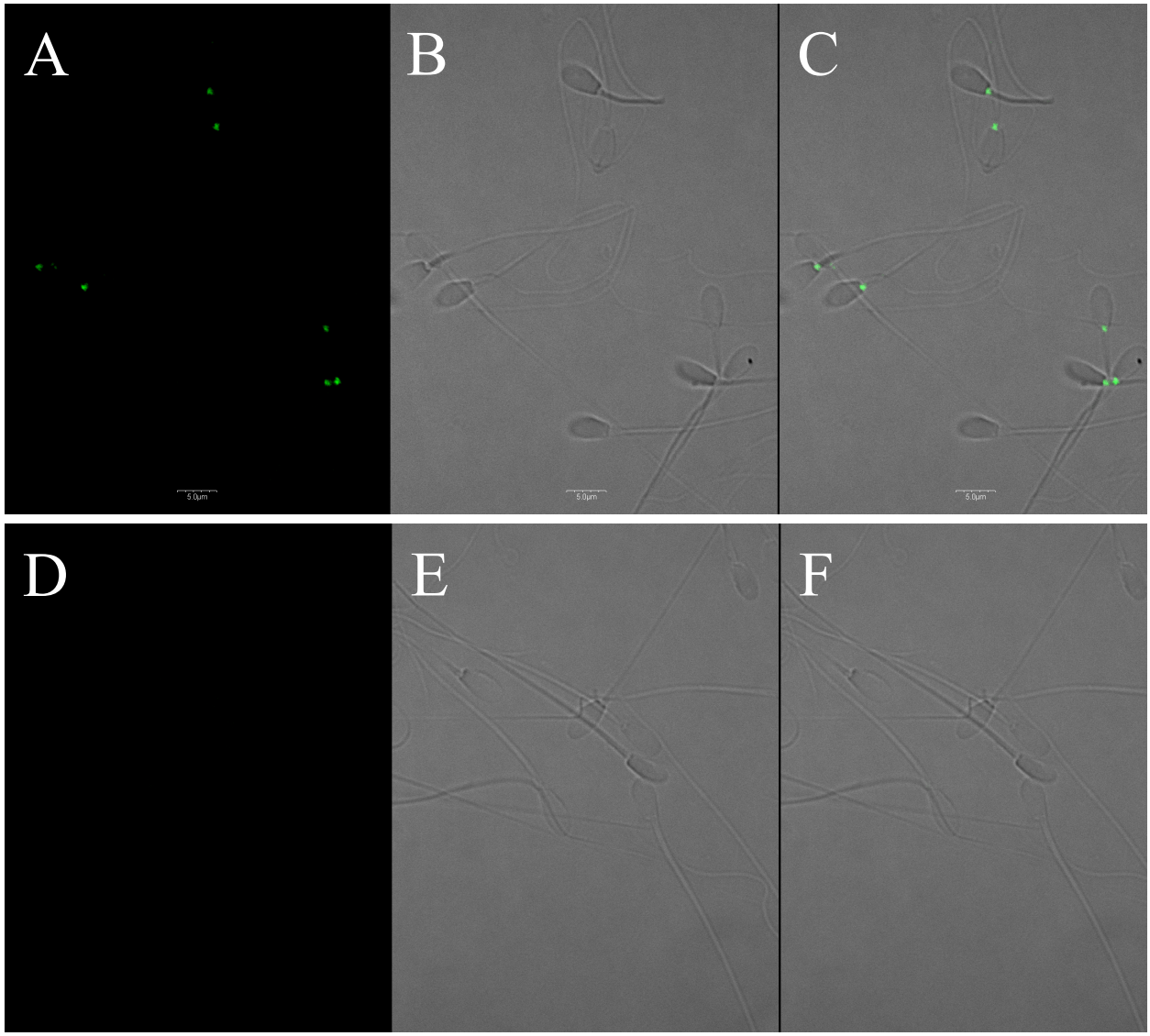

Figure 3.2. Representative confocal microscopy images of purified stallion spermatozoa using an antibody directed against GnRHR-II (sc-100301) labeled with an Alexa Fluor 488 secondary antibody. Panel A shows green fluorescence, B shows brightfield, and C represents an image merged with both fluorescence and brightfield. A secondary only control is shown by panels D-F.

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Appendix A - Utilizing Fecal pH to Predict Cecal pH in the Equine

Introduction

Equine digestive disorders including, colic, hindgut acidosis, and laminitis, are detrimental to the horse and the entire equine industry. Treatment of these diseases can be challenging and expensive. Thus, preventative measures often implemented through dietary management, are often the best strategy for dealing with these ailments. Cecal cannulation is can be used for direct assessment of cecal pH in equines. However, collecting cecal material requires cannulation or post-mortem sample collection. While cecal cannulation allows access to the cecum, this surgery is expensive and invasive.

Analysis of fecal material is considered 1 of few viable methods for monitoring digestive health in intact horses (Berg et al., 2005; Vörös et al., 2009; Berg et al., 2013, as collecting digesta directly from the gastrointestinal tract can be challenging. Therefore, analysis of volatile fatty acids, pH, and microbial content of fecal material this is often used to represent the hindgut environment (Berg et al., 2005). Indeed, analysis of fecal material has been used to assess colonic health in rats (Campbell et al., 1997), pigs (Howard et al., 1995), and humans (Benno and Mitsuoka, 1992). Even though analysis of fecal material is commonly utilized to represent the hindgut environment of the equine, few researchers have investigated the true relationship between cecal and fecal material. In this retrospective study, we assessed the association between fecal pH and cecal pH and attempted to develop a predictive equation for cecal pH.

Materials and Methods

Management

Nine cecally cannulated Quarter horses were utilized. The group was comprised of 5 geldings and 4 mares, aged 8 to 10 yr, and body weight ranging from 455 to 590 kg. Horses were

housed in heated individual stalls with ad libitum access to water and white salt blocks. They were fed 1.5% BW prairie grass hay split into twice daily feedings (0700 and 1930) and 0.5% BW concentrate (Omolene 200, Purina Animal Nutrition, LLC, Gray Summit, MO) which was fed in the mornings only (0700). Horses were maintained on this diet for 3 separate 21-d periods.

Cecal and Fecal Collection

Cecal and fecal pH were measured on d 19 to 21 of each period at -1, +1, +4, +8, +12, +16, +20, and +24 h relative to feeding the concentrate meal (Figure A.1). Fecal and cecal samples were collected concurrently from each horse at each timepoint. While biosamples were collected, horses were restrained in stocks. Cecal fluid was obtained from the cannula via gravity directly into sterile collection cups and filtered through a layer of cheesecloth to remove large particles. Cecal pH was immediately measured by immersing a pH probe (Thermo Scientific orion 3 Star Portable pH Meter, Waltham, MA; Accument probe) directly into the cecal fluid. Once a stable reading was established, the measurement was recorded.

Fecal samples were retrieved via the rectum and placed into sterile collection cups. Equal parts deionized water and fecal material were included in a sterile collection cup and homogenized. A pH reading was then recorded for the homogenous mixture. Between each horse, cecal, and fecal measurements, the pH probe was rinsed in deionized water.

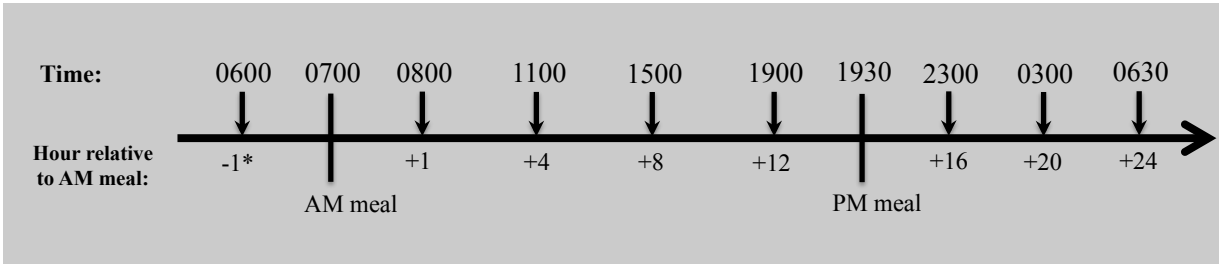


Figure A.1. Timeline depicting cecal and fecal sampling protocol. Arrows depict times of sampling on d 19 to 21; *d 19 only

Statistical Analysis

Given inherent animal-to-animal variability in the pH dynamics of the cecum and rectum over time, the minimum cecal pH observed after feeding was utilized for each animal-day combination and modeled as a function of the corresponding minimum fecal pH using a general linear mixed model. Kenward-Roger's procedure was used to estimate degrees of freedom and to make the corresponding adjustments in estimated standard errors. The model was fitted using the GLIMMEX procedure of Statistical Analysis Software (SAS; Version 9.2; SAS Institute, Cary, NC).

Results and Discussion

There was a moderate association between minimum cecal pH and minimum fecal pH ($P = 0.03$; Figure A.2). The estimated rate of change for minimum cecal pH per unit increase in minimum fecal pH was 0.131 with a 95% confidence interval of 0.011 to 0.251. The prediction equation for estimated cecal pH was $Y = 0.131*X + 5.8969$, where X was the observed fecal pH.

However, the amount of predictive variability was considerable. Multiple factors alter the transit time of digesta from cecum to rectum, including feed composition, animal weight, and physiological state. Indeed, there was a -0.059 correlation coefficient for actual cecal pH vs predicted cecal pH (Figure A.3). This indicates there is no relationship between actual cecal pH and the estimated cecal pH generated via prediction equation.

Although analysis of fecal material has been used to assess the colonic environment of other monogastrics, analysis of equine fecal material may not accurately reflect what is occurring in the cecal environment. Horses differ greatly from monogastric mammals, as they are hindgut fermenters. Based on the data available and these analyses, it appears fecal pH has limited usefulness in predicting cecal pH.

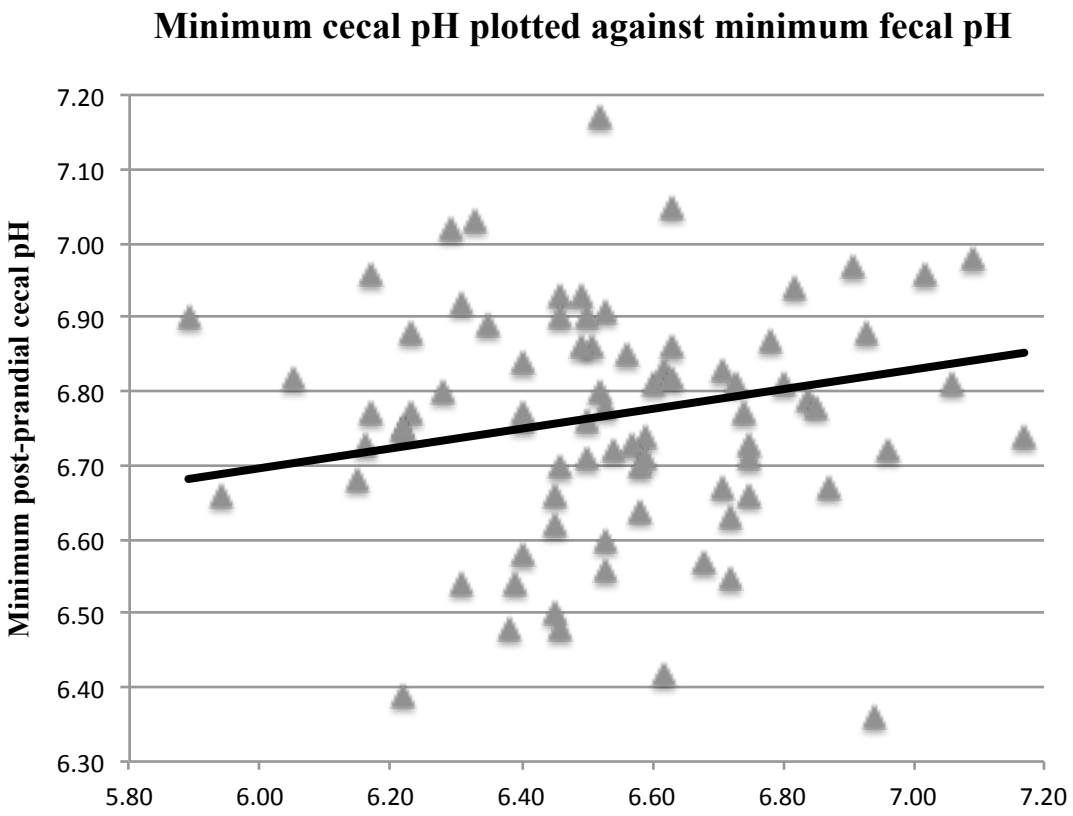


Figure A.2. Minimum post-prandial cecal pH plotted against minimum post-prandial fecal pH for each horse on each day (n = 81). Analysis revealed evidence for an association between minimal cecal pH and minimal fecal pH ($P = 0.03$).

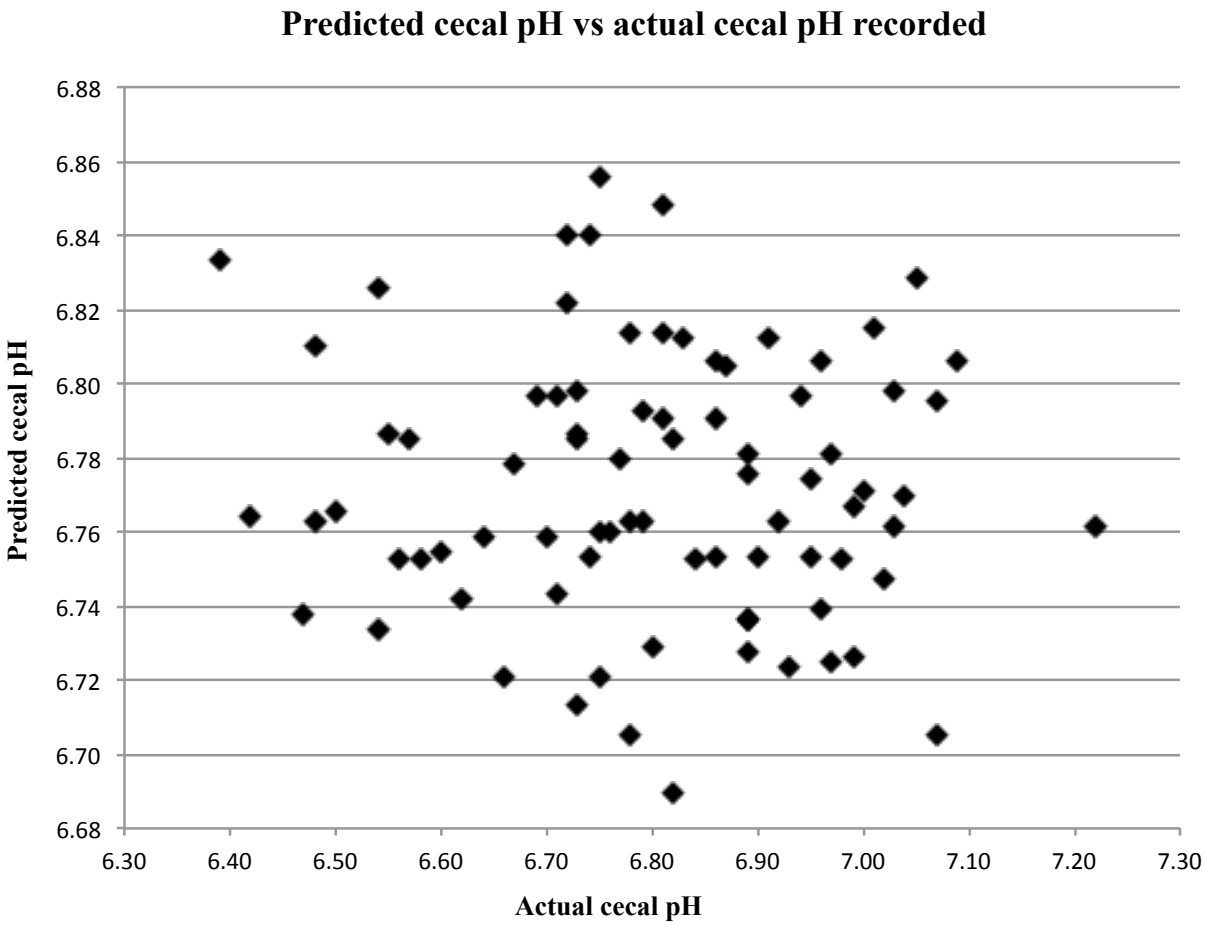
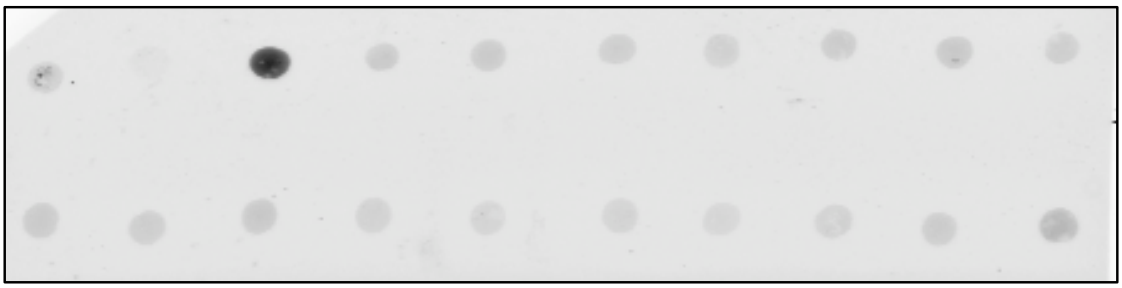


Figure A.3. Using the prediction equation generated, this graph represents predicted cecal pH vs. actual cecal pH recorded. A 4 h time lag between minimal cecal and minimal fecal pH values was detected (Delano et al., 2014). Therefore, fecal pH at 12 h post-prandially was utilized in the prediction equation to estimate cecal pH at 8 h post-prandially for each horse on each day. The correlation coefficient for actual cecal pH vs predicted cecal pH was -0.059.

Appendix B - Supplemental Data

A



B

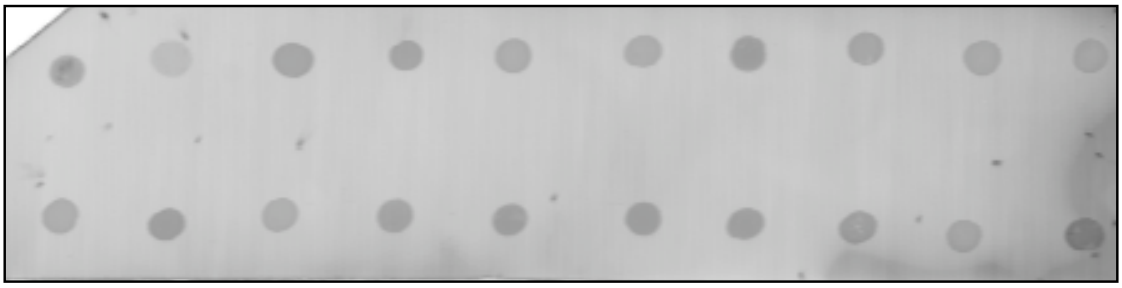


Figure B.1. (A) Full GnRH-I dot blot with extracted equine testicular protein (n = 18) from Chapter 2. The first sample is a positive control (mouse testis) followed by a negative control sample (mouse bladder). **(B)** Total protein stain from the above blot was used to confirm protein was present on the membrane.

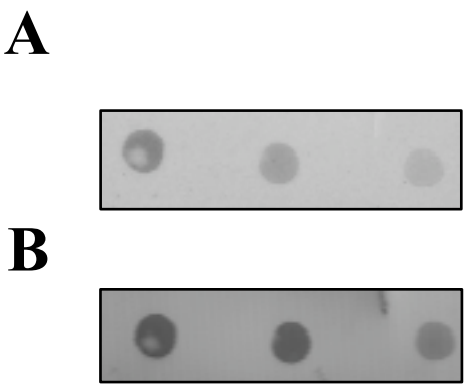
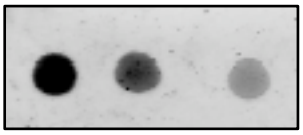


Figure B.2. (A) Antibody validation of GnRH-I antibody (sc-20941; Santa Cruz Biotechnology) with decreasing protein concentrations (1600, 800, 400 ng/μL protein). **(B)** Total protein stain from the above blot.

Figure B.3. Membrane incubated in only the secondary antibody (mouse anti-rabbit; 1:5000; Alexa Fluor 800; Invitrogen) to verify the secondary antibody did not interact with protein samples and provide fase signal. Samples include positive control (mouse testis), negative control (mouse bladder), and protein collected from the horse testis. No signal was detected.

Figure B.4. (A) Full GnRH-II dot blot with extracted equine testicular protein (n = 18) from Chapter 2. The first sample is a positive control (boar testis) followed by the negative control sample (mouse bladder). **(B)** Total protein stain from the above blot was used to confirm protein was present on the membrane.

A



B

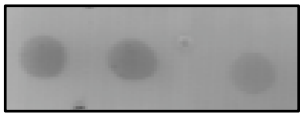
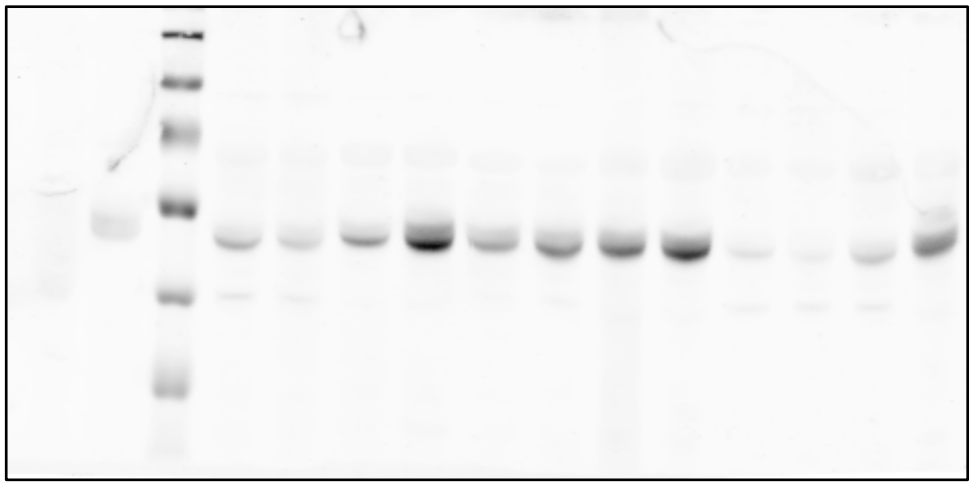


Figure B.5. (A) Antibody validation of the GnRH-II antibody (sc-20942; Santa Cruz Biotechnology) with decreasing protein concentrations (400, 200, 100 ng/μL protein). **(B)** Total protein stain from the above blot.

Figure B.6. Membrane incubated in only the secondary antibody (mouse anti-rabbit; 1:5000; Alexa Fluor 800; Invitrogen) to verify the secondary antibody did not interact with protein samples and provide fase signal. Samples include positive control (boar testis), negative control (mouse testis), and protein collected from the horse testis. No signal was detected.

A



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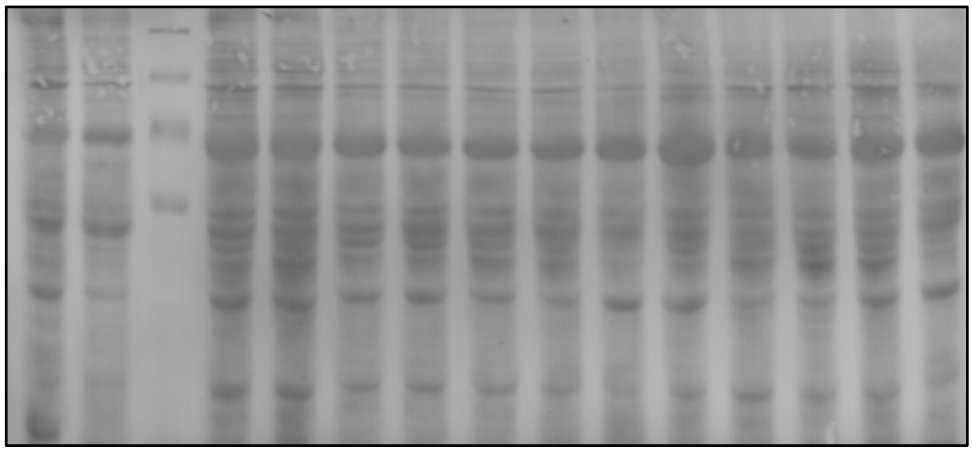
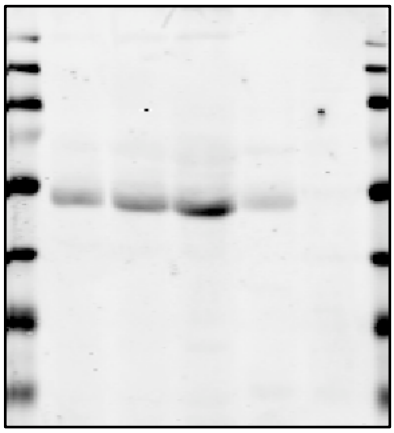


Figure B.7. (A) Western blot of testicular tissue from colts < 2 yr (n = 4) and stallions ≥ 2 yr (n = 7) using an antibody directed against GnRHR-I. These blots were replicated 3 times. The first lane includes a negative control sample (boar testis) and the second lane includes a positive control sample (mouse testis). The third lane contained the pre-stained ladder. **(B)** Total protein stain from the above blot was used to confirm protein was present on the membrane.

A



B

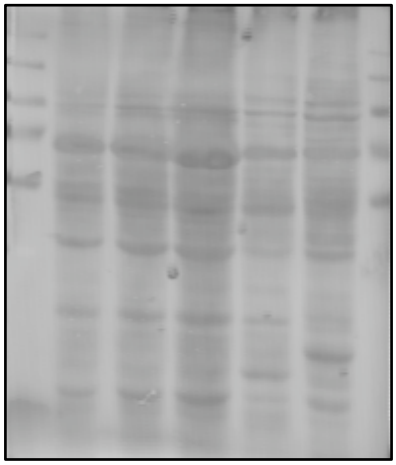


Figure B.8. (A) Antibody validation of GnRHR-I antibody (sc-8681; Santa Cruz Biotechnology) with increasing protein concentrations (10, 20, 40 µg/µL protein). The forth lane includes positive control (mouse testis) and fifth lane includes negative control (boar testis). **(B)** Total protein stain from the above blot was used to confirm protein was present on the membrane.

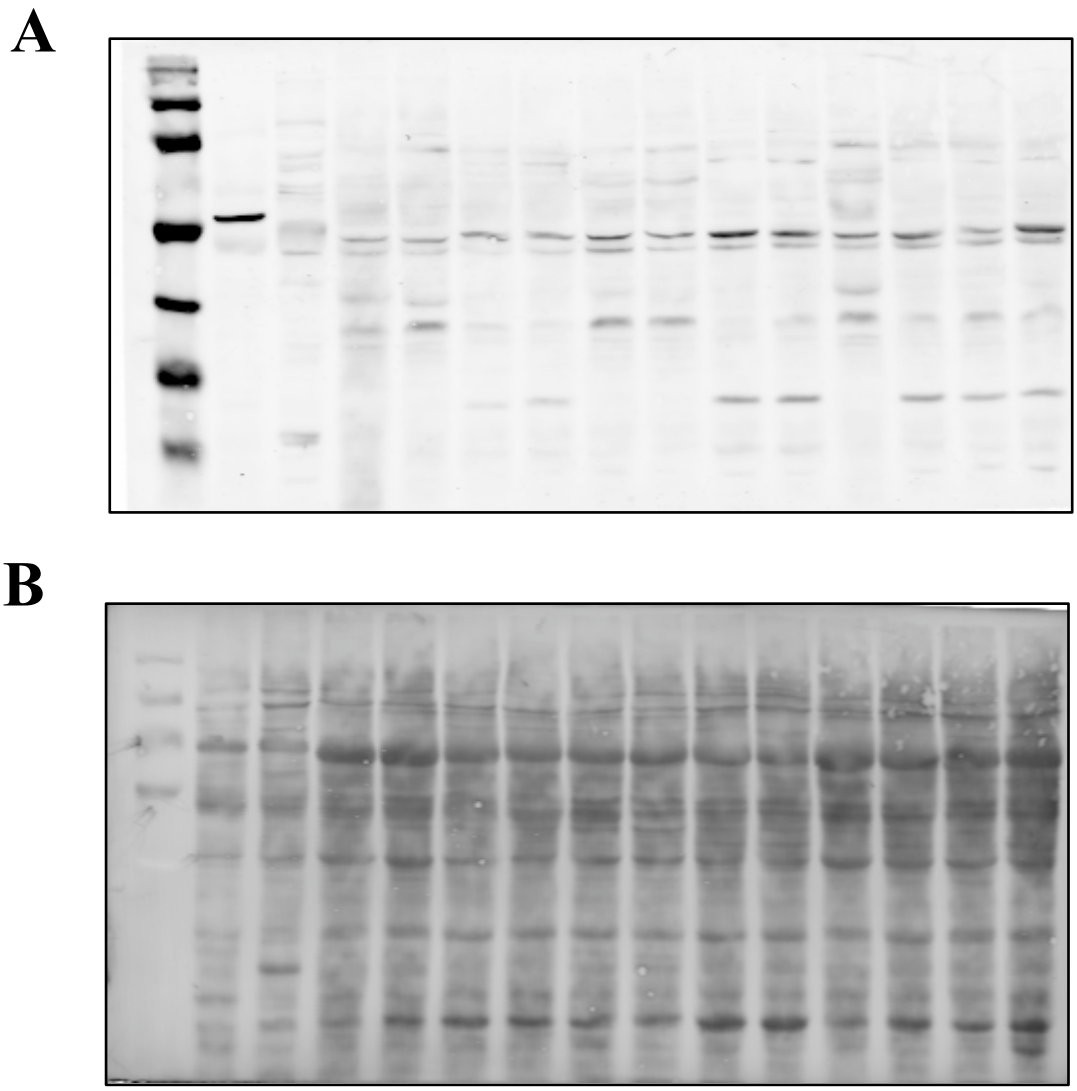


Figure B.9. (A) Western blot of testicular tissue from colts < 2 yr (n = 4) and stallion ≥ 2 yr (n = 7) using an antibody directed against GnRHR-II. These blots were replicated 3 times. The first lane contains the pre-stained ladder. The second lane includes a positive control sample (boar testis) and the third lane includes a negative control sample (mouse testis). Only the largest band detect was quantitated to total protein. **(B)** Total protein stain from the above blot was used to confirm protein was present on the membrane.

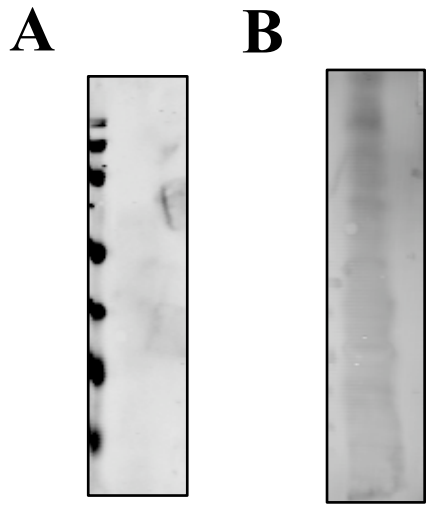


Figure B.10. Membrane was incubated in only the secondary antibody (donkey anti-goat; 1:20000; Alexa Fluor 680; Invitrogen) with horse testis protein to verify the secondary antibody did not interact with protein samples and provide fase signal. The same secondary antibody was utilized for both GnRHR-I and -II antibodies.

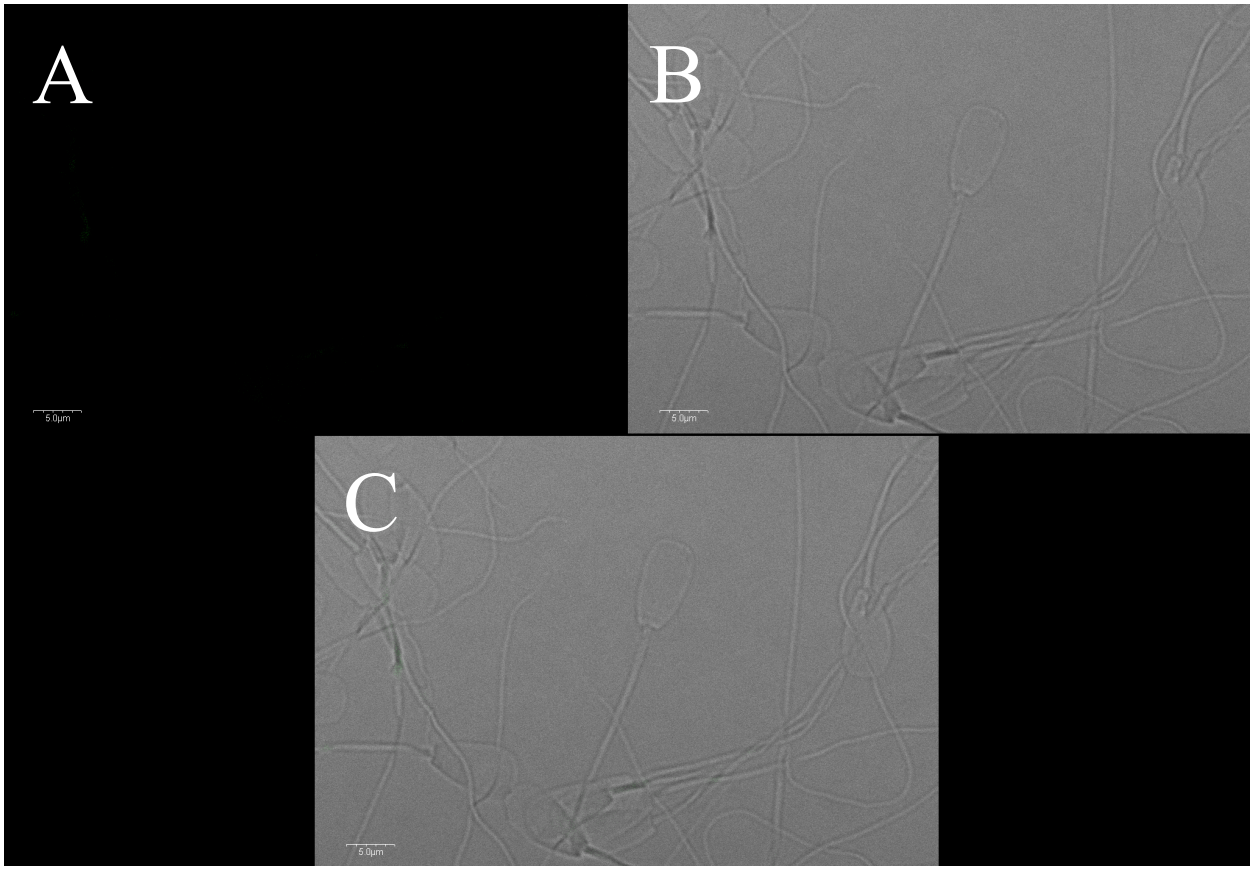


Figure B.11. Representative confocal microscopy images of purified bull spermatozoa (n = 1) using an antibody directed against GnRHR-II (sc-100301) labeled with an Alexa Fluor 488 secondary antibody. Panel A shows green fluorescence, B shows brightfield, and C represents an image merged with both fluorescence and brightfield. This bull sample was included as a negative control to verify antibody specificity.