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## Immunohistochemical Localization of Kisspeptin and Its Receptor in the Equine Ovary

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# **IMMUNOHISTOCHEMICAL LOCALIZATION OF KISSPEPTIN AND ITS RECEPTOR IN THE EQUINE OVARY**

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
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in

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Bryce Michelle Gilbert  
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## **ABSTRACT**

Kisspeptin is recognized for its role as the gatekeeper of reproduction in most mammalian species. However, its role in regulation of reproduction at the ovarian level is poorly understood in the horse. In this study, ovaries from follicular phase, luteal phase, anestrus period, and mares treated with ECP-sulpiride were subjected to immunohistochemistry to characterize kisspeptin-10 (Kp10) and its receptor (Kiss1r) protein expression throughout each reproductive stage and follicle type. Kisspeptin and receptor staining was detected in all follicle types (primordial, preantral, and antral) throughout all reproductive stages, as well as oocytes, corpora lutea, and ovulation fossa. The pattern of Kp10 and Kiss1r staining was affected by follicle type ( $P < 0.0001$ ) and reproductive stage ( $P < 0.001$ ). Kisspeptin-10 immunostaining intensity was greatest in antral follicles, with no differences ( $P > 0.05$ ) between reproductive stages. Kisspeptin receptor immunostaining intensity was greatest in antral follicles, with no differences between reproductive stages except when comparing follicular phase to anestrus phase ovaries ( $P < 0.05$ ). The results of this study indicate kisspeptin and its receptor may have potential roles in the period leading up to ovulation, as indicated by the intensity of immunostaining in antral follicles, follicular development, and steroidogenesis.

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## **CHAPTER 1. INTRODUCTION**

Whether they are used for showing, racing, or working, horses have been a part of American culture and tradition for hundreds of years. The horse industry is considered a valuable part of the United States economy in that the industry has contributed roughly \$122 billion dollars to the U.S. economy, according to the American Horse Council [1]. In order to produce valuable horses with desirable characteristics, much research has been conducted to enhance equine breeding management. As a seasonally breeding species, horses have a narrower window for conception, so manipulation of the breeding season is beneficial. Pharmacological manipulation is commonplace in equine breeding strategies to induce early cyclicity and ovulation. In order to effectively manipulate mare reproduction, a complete understanding of the physiological and endocrine mechanisms that regulate reproduction in the horse is needed.

Kisspeptin is recognized as a central regulator of reproduction by stimulating hypothalamic gonadotropin releasing hormone (GnRH), but recent discoveries have found that kisspeptin and its receptor are present in the gonads, as well as other tissues. Extra-hypothalamic sources and actions of the neuropeptide, kisspeptin, have been described. These discoveries have provided evidence that kisspeptin has the potential to affect the endocrine activity in the gonads. A recent study by Petrucci et al. [2] provided the first evidence that kisspeptin and its receptor are present in the equine testis. Kisspeptin has been localized in ovaries of many species, but has yet to be determined if kisspeptin and its receptor are present in the equine ovary. Understanding the physiological role of kisspeptin in equine reproduction, including the ability of the gonads to respond to kisspeptin, would provide additional insight into the reproductive physiology of the mare. Recently, our lab has studied the effects of incorporating kisspeptin-10 (Kp10) into an estradiol benzoate (EB)-sulpiride treatment protocol utilized to induce early ovulation in

seasonally anestrous mares [3]. The incorporation of Kp10 resulted in more mares ovulating early compared to mares receiving EB-sulpiride only. Whether the stimulatory effect of Kp10 was at the level of the ovary or the hypothalamic-pituitary (HP) axis could not be determined in that study.

Further understanding of ovarian physiology in horses has the potential to increase opportunities for pharmacological manipulation and assisted reproductive techniques. Taniguchi et al. [4] observed kisspeptin in follicular fluid and found a positive correlation between intra-follicular kisspeptin levels, serum estradiol, and number of oocytes in women undergoing assisted reproductive technologies. Pursuing to further understand the activity of kisspeptin beyond the hypothalamus could lead to potential *in vitro* applications of kisspeptin in embryo production.

The reproductive physiology of the mare is unique compared to other mammalian species and provides opportunity for further understanding reproduction in a long-day breeding species, therefore, the aim of this study is to explore the roles of kisspeptin and its receptor in the equine ovary by characterizing the immunostaining patterns of kisspeptin and its receptor throughout follicle types and reproductive stages, and evaluate the pattern of staining in ovaries from seasonally anestrous mares treated with estradiol and sulpiride to induce early ovulation.

## **CHAPTER 2. REVIEW OF LITERATURE**

### **2.1. Hypothalamic-Pituitary-Ovarian Axis**

The hypothalamus and the adenohypophysis, also known as the hypothalamic-pituitary (HP) axis, form a complex neuroendocrine system that is responsible for the maintenance and regulation of reproduction in most mammalian species. Neurosecretory cells in the hypothalamus release mostly stimulatory hormones into the hypophysial portal system which connects the hypothalamus and the adenohypophysis [5,6]. The hypothalamus is considered the major regulator of reproduction by controlling the synthesis and secretion of the decapeptide, gonadotropin-releasing hormone (GnRH), into the hypophysial portal system. Pulsatile GnRH release stimulates the release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the adenohypophysis [5].

Pulsatile secretion of GnRH varies with season and stage of the reproductive cycle of the mare and regulates gonadotropin secretion. Pulse amplitude and frequency determine which gonadotropins are secreted; higher GnRH pulse frequency with low amplitude favors LH secretion, while lower GnRH pulse frequency with high amplitude favors FSH secretion [6]. The HP axis regulates mare reproduction to coordinate the actions of LH and FSH on the ovary. Luteinizing hormone is critical to stimulate ovulation, formation of corpora lutea (CL), and progesterone secretion, and FSH is critical for follicular development, selection, and estradiol synthesis [7].

Ovarian steroids, estradiol and progesterone, play a role in the regulation of the hypothalamic-pituitary-ovarian (HPO) axis by providing stimulatory and inhibitory effects on gonadotropin secretion. Estradiol has a stimulatory effect on LH and an inhibitory effect on



FSH, while progesterone inhibits LH secretion but does not greatly affect FSH [6]. Estradiol can exert either positive and/or negative feedback on GnRH depending on concentrations in circulation.

Gonadal glycoproteins, inhibin and activin, act as additional feedback and feedforward mediators of FSH, respectively. Inhibin is produced by granulosa cells of growing ovarian follicles and has a suppressive effect on FSH [5–7]. Increased levels of inhibin suppress FSH which allows the follicle to become dominant in the presence of increased LH pulse frequency [7]. Activin is produced by cumulus cells of the growing follicle and acts as stimulator of FSH secretion at the level of the pituitary. Activin has also been implicated in oocyte maturation, delaying of luteinization, and ovarian steroidogenesis [6].

## **2.2. Ovarian Anatomy**

The equine ovary is unique compared to other domestic mammalian species in that the vascular zone, often referred to as the medulla, containing collagenous connective tissue surrounds the central parenchyma, often referred to as the cortex [6,8]. The cortex contains developing follicles and luteal structures, including CL and corpora albicantia. This has often led to the statement that the equine ovary is “inverted” compared to other mammalian species. Another unique feature of the equine ovary is that ovulation only occurs in one location, the ovulation fossa [7]. The ovulation fossa is the only area of the mare ovary to be covered by germinal epithelium, contrary to other species where germinal epithelium surrounds the entire surface of the ovary [6,9].

The ovary houses oocytes contained within follicles in various stages of growth. Primordial follicles, those present at birth which are arrested in meiosis until recruited, are characterized by one layer of squamous cells surrounding the oocyte. During the estrous cycle, a

cohort of primordial follicles will be recruited to grow. Early follicle growth (< 10 mm) appears to occur independent of gonadotropins; however, progression of follicular growth is mostly influenced by FSH. During a wave of follicular growth, primordial follicles will transition to primary follicles, characterized by a single layer of cuboidal cells surrounding the oocyte. Primary follicles advance to secondary follicles when two or more layers of cells are present with no antrum. Tertiary, or Graafian, follicles are characterized by a fluid-filled antrum and include three cell layers: theca externa, theca interna, and granulosa cell layer [7,8]. During this wave, typically one follicle is selected for dominance in the mare and undergoes final maturation and eventual ovulation under the influence of LH.

Ovulation is reliant on a surge of LH as a result of positive feedback from ovarian estradiol. During the periovulatory period, steroidogenesis shifts from estradiol production to progesterone production as granulosa cells differentiate into luteal cells. Granulosa cells transform into large luteal cells while theca interna transform into small luteal cells, forming the CL [5,7,8].

### **2.3. Estrous Cycle**

The estrous cycle of the mare averages 21 days and is defined as the period from one ovulation to the next. The estrous cycle is divided into two phases, the follicular phase and the luteal phase, based on the primary structure(s) present on the ovary during that time. The follicular phase is dominated by estradiol and is characterized by gonadotropin release, follicle preparation for ovulation, sexual receptivity, and eventually ends with ovulation [6,7]. During the follicular phase, progesterone is low and LH and FSH are secreted by the anterior pituitary. Follicle stimulating hormone is required for emergence and initial growth of small follicles [7,10]. Luteinizing hormone is responsible for final maturation of the dominant follicle and

induction of ovulation at the end of the follicular phase [6,7]. The luteal phase immediately proceeds and is characterized by formation of the CL, referred to as luteinization, synthesis and secretion of progesterone by the luteal cells of the CL, and luteolysis. Luteolysis is initiated by secretion of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) from the non-pregnant uterine endometrium 14-15 days post-ovulation [6,11]. After luteolysis, blood progesterone decreases, signaling the end of the luteal phase and the beginning of the follicular phase.

#### **2.4. Seasonality**

The mare is seasonally polyestrous, cycling only during the long-days of the year. This is unique in that other seasonally cycling species, such as sheep and goats, cycle during the short days of the year. The seasonal pattern of reproduction in the mare ensures she delivers a foal during the spring. Thus, with an 11-month gestation period, it is necessary for reproductive activity to cease during the winter months. The annual reproductive cycle of the mare can be divided into four stages: the breeding season which occurs when the days are long, such as spring and summer; the autumnal transition, when the mare transitions to a period of seasonal anovulation, also referred to as seasonal anestrus, as the days begin to shorten; the non-breeding season, in which the mare ceases to cycle during the short days of the year, occurring in the winter months; and the vernal transition period as the days begin to lengthen in early spring, which is characterized by erratic follicular activity with or without the occurrence of ovulation. During the non-breeding season, mares can be clinically defined to be in deep anestrus if follicles  $\leq 20$  mm in diameter are present on the ovaries upon transrectal ultrasonography and plasma progesterone levels are  $< 1.0$  ng/mL [12].

Many equine breeding registries in the Northern Hemisphere have a set birthdate of January 1<sup>st</sup> for foals born during that year. This sets an incentive for breeders to produce foals as

close to this universal birthdate as possible. Given the 11-month gestation period of the mare, mares would need to be bred during February in order to produce foals in January, thus promoting the manipulation of the breeding season.

## **2.5. Manipulation of the Breeding Season**

The seasonal reproductive changes in long-day breeders are governed by changes in daylength, as described before [11,13]. Therefore, manipulation of photoperiod is the most utilized method to induce early cyclicity. Artificial lighting is typically supplied 60 days prior to the start of the breeding season to induce early transition. Manipulating photoperiod by use of artificial lights has been considered an effective way to advance the breeding season by up to two months [14–16].

Exogenous hormonal therapies have also been utilized to induce early transition. The use of exogenous GnRH or its analogues have had limited success in advancing the breeding season, and repeated use can result in downregulation of GnRH receptors [17]. The use of progestogens, in conjunction with other hormones, such as estrogen or human chorionic gonadotropin (hCG), has some effectiveness with synchronization of estrous cycles and/or ovulation, but is limited in the ability to hasten the onset of the breeding season during the transition period. Many hormonal therapies are less effective if the mare is in deep anestrus. One exception is the use of dopamine receptor antagonists, such as domperidone or sulpiride. They have been utilized to induce ovulations during the anestrus season and/or vernal transition period with high success [18–20]. Sulpiride and domperidone have been utilized to stimulate prolactin secretion by blocking the inhibitory action of dopamine on prolactin secretion. An even greater prolactin response is achieved if the mare is pretreated with a conjugated estradiol [21,22]. Prolactin has been observed to have roles in seasonal reproduction and the shedding of the winter coat of mares

(reviewed by Thompson and Oberhaus [23]). There is a preovulatory prolactin surge in the mare, suggesting the role prolactin has on the stimulation of ovulation [24]. Pretreatment with estradiol, in the form of estradiol benzoate (EB) or estradiol cypionate (ECP), also stimulates LH secretion, which enhances the response of stimulated prolactin secretion with dopamine antagonists when administered to seasonally anestrus mares [21,22,25–28].

## **2.6. Kisspeptin**

### **2.6.1. Brief History**

In 1996, the *Kiss1* gene was originally discovered as a suppressor of metastasis for human malignant melanoma in Hershey, Pennsylvania, in which it was named after [29], and the *Kiss1*-derived peptide was known as metastin [30]. In 2003, a reproductive role for kisspeptin was assigned when a family of five siblings with isolated hypogonadotropic hypogonadism (iHH) underwent a genome-mapping strategy. It was determined that inactivation of kisspeptin receptor, G protein-coupled receptor 54 (GPR54), was the cause of iHH, and indicated that GPR54 and kisspeptin play an important role in the regulation of the gonadotropic axis [31].

### **2.6.2. Overview**

Kisspeptins are a family of peptides encoded by the *Kiss1* gene. They are cleaved from a prepropeptide 145 amino acids in length and include kisspeptin-54, 14, 13 and 10 [29,32]. All of these peptides have a common C-terminus, Arg-Phe-NH<sub>2</sub>, characteristic of the RF-amide family. Kisspeptin has natural ligands for the G protein-coupled receptor 54 (GPR54), encoded by the *Kiss1r* gene [30,33]. The kisspeptin receptor, GPR54, is a seven transmembrane domain, G<sub>q/11</sub>-coupled receptor, and the effects of the receptor are stimulated through activation of phospholipase C cell signaling pathway and result in the release of intracellular Ca<sup>2+</sup> [33]. The sequence for equine kisspeptin-10 is YRWNSFGLRY-NH<sub>2</sub> [34].

### **2.6.3. Kisspeptin Signaling in Reproduction**

The neuropeptide kisspeptin has roles in sexual differentiation and timing of puberty onset and is considered the key neuroendocrine gatekeeper for activation of the HP axis by direct interaction with the kisspeptin receptor on GnRH neurons to stimulate GnRH release, which then stimulates secretion of FSH and LH from the adenohypophysis [7,35–37]. Although the distribution of kisspeptin neurons and its receptor varies depending on species, expression has been identified in the arcuate nucleus (ARC), median eminence (ME), and preoptic area (POA) or anteroventral periventricular nucleus (AVPV) of many species [38,39].

Besides the horse, other seasonally breeding species, such as the ewe and hamster, have been investigated for the characterization of kisspeptin neuronal populations during different reproductive stages/seasons as well as the effect of puberty on these populations [40–45].

Magee et al. [46] and Decourt et al. [47] provided neuroanatomical evidence that kisspeptin neuron fibers directly contact GnRH neurons throughout the POA, ARC, and ME of the mare hypothalamus. Decourt et al. [47] examined kisspeptin neuroanatomical interactions with GnRH neurons right after (2 – 4 hours) ovulation and provided dual immunofluorescence imaging of close appositions between kisspeptin fibers and GnRH fibers in the ME at the time of the ovulatory LH surge in pony mares, as well as fluorescent imaging of a large population of kisspeptin neurons distributed throughout the ARC. Magee et al. [46] examined kisspeptin neuronal populations in the diestrous mare, and noted similar observations to Decourt et al. [47] in regards to a large population of kisspeptin neurons in the ARC, but also observed large clusters of kisspeptin neurons located in the POA. Magee et al. [46] observed that 33.7% of kisspeptin fibers had close apposition to GnRH neurons, providing neuroanatomical evidence of the kisspeptin/GnRH system in the mare hypothalamus. Magee et al. [34] also established

evidence for kisspeptin directly affecting the pituitary in the mare by stimulating a rise in intracellular  $\text{Ca}^{2+}$  in pituitary cells treated with equine kisspeptin-10 *in vitro*.

In the rat, kisspeptin neurons located in the AVPV mediate positive feedback action of estradiol [35]. Kisspeptin neurons located in the ARC are hypothesized to mediate negative steroidal feedback [42,48]. As mentioned before, estradiol from the maturing follicle provides feedback to GnRH to signal the LH surge required for ovulation, but GnRH neurons do not express estrogen receptor-alpha ( $\text{ER}\alpha$ ) [49] or progesterone receptors (PR) [50]. Kisspeptin neurons have been found to express  $\text{ER}\alpha$  [41] and PR [40]. It is hypothesized that kisspeptin neurons located in the ARC regulate the pulsatility of GnRH (i.e., the tonic center) while the kisspeptin neurons located in the POA are responsible for regulation of the LH surge (i.e., the surge center) [51–53].

Matsui et al. [37] and Messenger et al. [36] were some of the first to administer exogenous kisspeptin to rats and sheep to elicit a rise in serum GnRH concentrations, and ovulation was stimulated in rats pretreated with pregnant mare serum gonadotropin (PMSG). Although exogenous administration of kisspeptin has been shown to induce ovulation in seasonally anestrous ewes [54], it fails to induce ovulation in mares [46,55–58], likely due to the extended LH surge that lasts for several days rather than hours compared to other species [11,56,59].

Decourt et al. [57] established the minimal effective dose to stimulate an increase in LH, but failed to induce ovulation in the mare regardless of reproductive status or season [58]. Decourt et al. [58] also found that treatment with equine kisspeptin-10 was most effective at stimulating an increase in LH and FSH during the early follicular phase, but still failed to hasten the date to ovulation. Magee et al. [56] found that kisspeptin treatment post-ovulation had no effect on progesterone secretion by the CL, but treatment with kisspeptin did have a negative

effect on serum estradiol and sexual receptivity in estrous mares. McGrath et al. [55] was unable to induce ovulation in mares during the vernal transition when given a constant rate of infusion of kisspeptin. Interestingly, Briant et al. [60] was able to hasten the date to ovulation in synchronized cycling Welsh pony mares when given an intravenous bolus injection of kisspeptin when the dominant follicle was 33 mm in diameter, but Magee et al. [46] was unable to replicate similar results. The results of Briant et al. [60] and Magee et al. [46] provide a comparison for the use of kisspeptin to stimulate ovulation when a preovulatory-sized follicle is present, as comparable to the use of GnRH analogues or hCG in similar circumstances [61].

Administration of native kisspeptin to mares is ineffective at stimulating ovulation in the absence of preovulatory-sized follicle, regardless of season likely due to the unique endocrinologic profiles of the horse and the inability of native kisspeptin to resist proteolytic degradation. Kisspeptin analogs have been developed in an attempt to combat the rapid proteolytic degradation and maintain stability and potency of the molecule. Analogs developed include FTM080 [62,63], TAK-683[64,65], Compound 17 [66], and C6 [67]. Kisspeptin analog C6 has been the most successful at inducing ovulation and advancing puberty in Ile de France ewes [67,68], and can be used as an alternative to PMSG in the Alpine goat [69]. Fanelli et al. [70] compared the ability of C6 to trigger ovulation in comparison to buserelin acetate, a GnRH agonist, in jennies. While C6 was successful at triggering ovulation in the presence of a preovulatory-sized follicle, similar to the effects of GnRH agonists, there is no current research published assessing the effects of C6 during the nonbreeding season in jennies or mares. Currently, administration of kisspeptin or kisspeptin analogs to mares to advance the breeding season or trigger ovulation is considered unsuccessful.



#### **2.6.4. KNDy Neurons**

A subpopulation of kisspeptin neurons located in the ARC, named KNDy neurons, express the tachykinin neurokinin B (NKB) and the endogenous opioid peptide dynorphin (Dyn) which cooperate in the regulation of the HP axis by combining steroidal and metabolic feedback to GnRH neurons in the hypothalamus [71]. It has been suggested that the colocalization of Dyn and kisspeptin mediates progesterone negative feedback, and that the colocalization of NKB with kisspeptin mediates positive feedback actions of estradiol [42]. Goubillon et al. [72] investigated that the majority of NKB neurons in the ovine ARC expressed ER $\alpha$ . Foradori et al. [73] investigated a similar distribution of Dyn neurons in the ovine ARC with most of them expressing progesterone receptors. These key findings led to the possibility of both NKB and Dyn being contained within the same neurons [74,75].

#### **2.6.5. Kisspeptin in the Gonads**

Kisspeptin and/or its receptor localization has been characterized in the gonads of many species, including horse testes [2]; cat ovary [76]; canine ovary [77]; Siberian Hamster ovary [45]; human and marmoset ovaries [78]; rat ovary [79]; swine ovary [80]; buffalo ovary [81–83]; and chicken ovarian cells [84]. Recently, Petrucci et al. [2] evaluated the kisspeptin/GnRH1 system in the Leydig cells of horse testes via immunohistochemistry (IHC) and found immunopresence of kisspeptin and its receptor in Leydig cells, providing evidence for the kisspeptin/receptor system at the level of the gonads in the horse. Immunocytochemistry of *in vitro* cultured granulosa cells from chicken ovary revealed immunosignal for kisspeptin-10 [84].

In the rat ovary [79], the pattern of kisspeptin staining was affected by reproductive stage and cell type. Kisspeptin immunosignal was present in the oocyte and thecal cells of growing and preovulatory follicles throughout the estrous cycle, but there was no evidence of signal in

granulosa cells of the same follicles. Ovarian surface epithelium (OSE) and developing CL presented positive immunolabeling for kisspeptin. Kisspeptin receptor immunolabeling had a similar pattern of staining to kisspeptin in growing and preovulatory follicles and CL [79].

In the human and marmoset ovaries [78], kisspeptin immunosignal was detected in theca cells of developing follicles, luteal cells of the CL, and the OSE. This is similar to the observation made by Castellano et al. [79] in the rat ovary. Kisspeptin receptor immunostaining patterns in the human ovary are similar to that of kisspeptin, except there was no reported staining in the OSE for receptor. Kisspeptin receptor immunolocalization was not assessed in marmoset ovary in this study [78].

The hamster provides a model for long-day seasonal breeders, like the horse. Shahed and Young [45] characterized kisspeptin and receptor immunolocalization in cycling Siberian hamster ovaries throughout each phase of the reproductive cycle and during a photoperiod induced recrudescence. Immunostaining for kisspeptin and its receptor were localized to steroidogenic cells of preantral and antral follicles throughout the estrous cycle and steroidogenic cells of the CL. In contrast to the rat [79] and human [78] studies, in which kisspeptin and/or receptor immunoreactivity was not present in granulosa cells follicles. Staining was most intense during proestrus and estrus of the cycle, and staining decreased during diestrus. During the photoperiod induced recrudescence, intensity of immunostaining for kisspeptin and its receptor increased which suggests a role for kisspeptin and its receptor in restoring ovarian function [45].

In the canine ovary [77], kisspeptin and its receptor were localized in all oocytes in pre-pubescent, cycling, and anestrus females and the CL of cycling ovaries. Immunostaining for kisspeptin was present in granulosa cells of follicles in cycling ovaries. Kisspeptin receptor immunoreactivity was present in granulosa cells of follicles in cycling, anestrus, and pre-

pubescent ovaries. Similar to the Siberian hamster [45], staining was present for both kisspeptin and receptor in granulosa cells of the follicle, in contrast to the rat [79] and human [78] studies.

In domestic cat ovaries [76], kisspeptin and receptor immunostaining was present throughout all stages of the cycle with no differences in cell types throughout each stage of the cycle. Kisspeptin and receptor immunoreactivity were present in the oocyte, granulosa cells, and theca cells of follicles and the CL. Kisspeptin staining was also present in follicular fluid of antral follicles. Tanyapanyachon et al. [76] suggest that the kisspeptin/kisspeptin receptor system may have a role in folliculogenesis and oocyte survival.

Kisspeptin and its receptor localization has been characterized in the buffalo (*Bubalus bubalis*) ovary [81–83]. Mishra et al. [82,83] assessed immunofluorescence signal for kisspeptin and receptor in the cyclic CL and observed intense signal during the early and middle stage CL, but weak signal was observed in the late CL. Rajin et al. [81] assessed the pattern of kisspeptin and receptor staining throughout follicles of different functional status (i.e., active, intermediate, atretic). Immunostaining for kisspeptin and receptor was present in granulosa cells of active and intermediate follicles, but kisspeptin immunostaining was present in theca interna cells of active follicles only.

Basini et al. [80] characterized the localization of kisspeptin and its receptor in swine ovaries. Intense kisspeptin and receptor immunostaining was present in the granulosa cells of preantral and antral follicles, but staining was less obvious in theca cells of the same follicles observed. In the same preantral and antral follicles, kisspeptin and receptor immunostaining was observed in the zona pellucida surrounding the oocyte and the oocyte cytoplasm.

Although the pattern of kisspeptin and kisspeptin receptor immunostaining varies depending on species, reproductive cycle stage, and cell type, the presence of kisspeptin and

receptor staining in the ovaries of many species suggests that kisspeptin may have a role in regulating the HPO axis at the level of the ovary or ovarian follicle development.

## **2.7. Rationale for Present Experiment**

Kisspeptin and its receptor have been implicated in the seasonality of reproduction in the mare, but much is still unknown regarding the physiological roles at the level of the ovary. The aim of this study was use standard immunohistochemical procedures to evaluate and localize kisspeptin and kisspeptin receptor staining in mare ovaries and compare the pattern of immunostaining between follicle sizes and reproductive states.

## **CHAPTER 3. MATERIALS AND METHODS**

### **3.1. Ovary collection**

A single ovary from 11 mares was used for immunohistochemistry, including three follicular phase ovaries, two luteal phase ovaries, three anestrus ovaries, and three ovaries from ECP-sulpiride treated mares in winter. Follicular and luteal phase ovaries were collected post-mortem in June and July. Follicular phase ovaries were defined as cycling ovaries lacking active luteal structures with follicles greater than or equal to 25 mm. Luteal phase ovaries were defined as cycling ovaries with active luteal structure(s) present. Corpora albicantia were not considered active luteal structures.

#### **3.1.1. Treatment with estradiol cypionate and sulpiride**

As part of a larger, separate experiment, deep anestrus mares were treated with 50 mg ECP one day prior to treatment with 3 g sulpiride, both intramuscularly. One ovary was collected from each of three treated mares once a follicle  $\geq 30$  mm was detected by ultrasonography. Standing colpotomy was achieved using a chain ecrasser after sedation. Each day an ovary from a treated mare was collected, an ovary from an untreated, deep anestrus mare was also collected in the same manner. Mares were determined to be in deep anestrus if follicles  $\leq 20$  mm in diameter are present on the ovary upon transrectal ultrasonography and plasma progesterone levels were  $< 1.0$  ng/mL [12].

### **3.2. Tissue preparation**

Ovaries were bisected longitudinally at the midline through the ovulation fossa. One half of each ovary was immediately immersed in 10% neutral buffered formalin for transport. Ovarian sections were further bisected creating three or four sections per hemi-ovary before further fixation in 4% paraformaldehyde. Sections were processed by dehydration in ethanol

(EtOH) and embedded in paraffin. Paraffinized tissue blocks were cut at 5  $\mu$ m using a microtome and mounted onto positively charged slides.

### **3.3. Immunohistochemistry**

Mounted sections were deparaffinized in two changes of xylene for 3 minutes each, transferred to a solution of xylene with 100% EtOH (1:1) for 3 minutes, then two changes of 100% EtOH for 3 minutes each, then 95% EtOH for 3 minutes, then 70% EtOH for 3 minutes, then 50% EtOH for 3 minutes, and then rinsed in deionized water. For antigen retrieval, tissue sections were steamed in Na Citrate buffer (10 mM, pH 6) solution using a rice cooker at 95°C for 20 minutes and allowed to cool on ice for 20 minutes. Tissues were then rinsed in deionized water for 5 minutes. To quench endogenous peroxidase activity, slides were immersed in 0.3% H<sub>2</sub>O<sub>2</sub> and methanol for 30 minutes at 23°C and then rinsed in deionized water for 5 minutes. Tissue was rinsed again in tris-buffered saline (TBS) containing 0.3% Triton X-100 (TBSt) for 5 minutes. Background labelling was prevented by incubating tissue sections with 1.5% (v/v) normal goat serum (NGS) for 30 minutes at 23°C and then lightly shaken to remove serum. Tissues were incubated with either anti-Kiss1r polyclonal rabbit antibody diluted 1:100 (AKR-001; Alomone Labs, Jerusalem, Israel) or anti-Kp10 polyclonal rabbit antibody diluted 1:200 (AB9754-I; Millipore, Massachusetts, USA) at 23°C for one hour and then at 4°C overnight. Hypothalamus tissue from a mare served as positive control for validation of both antibodies. Primary antibodies were diluted in TBSt containing 1.5% NGS. Primary antibodies were omitted from negative control tissues and were instead incubated with TBSt containing 1.5% NGS. Tissues were rinsed three times in TBSt for 5 minutes each and incubated in biotinylated goat anti-rabbit IgG secondary antibody (PK-6101; Vector Laboratories, Burlingame, CA, USA) for one hour at 23°C. Tissues were rinsed again in TBSt three times for 5 minutes each and then

incubated in avidin-biotin complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) for one hour at 23°C. Tissues were then rinsed once with TBS for 5 minutes. Immunolabelling was visualized by incubating tissues with diaminobenzidine solution (ImmPACT DAB Peroxidase Substrate; Vector Laboratories, Burlingame, CA, USA) for 15 minutes. Slides were rinsed once with deionized water and then counterstained with Mayer's hematoxylin for 1 minute. Slides were rinsed with deionized water for 5 minutes and then dehydrated and cleared by transferring to 70% EtOH for 3 minutes, then 80% EtOH for 3 minutes, then 95% EtOH for 3 minutes, then two changes of 100% EtOH for 3 minutes each, and then two changes of xylene for 3 minutes each. Slides were mounted with coverslips using a xylene-based mounting medium (Eukitt; Sigma, St. Louis, MO, USA).

### **3.3. Evaluation and Statistical Analysis**

A single observer assessed immunostained slides using a light microscope at 200x. A minimum of 30 follicles from each developmental category, or every structure in that category found on the ovary where fewer than 30 were present, were assessed from each ovary. A total of 1,244 follicles were observed for Kp10 staining and 1,258 follicles observed for Kiss1r staining. All follicles were counted and graded for staining intensity using a scale of 0-3. A scoring system of 0–3 was used in which 0 described no staining or staining equivalent to background staining and 3 described intense staining. Corpora lutea were also visualized and graded based on staining density.

Staining intensity was compared between different follicle sizes within a reproductive state as well as between reproductive states using two-way ANOVA (GraphPad Prism version 8.0.0 for Windows; GraphPad Software, San Diego, CA). Follicle size served as one factor and reproductive state as a second factor. Parametric tests were chosen based on determination of a

normal distribution for all data sets. Significance was determined if  $P \leq 0.05$  and, if significant, means were separated using Tukey`s test.



## CHAPTER 4. RESULTS

### 4.1. Staining intensity of kisspeptin-10 and kisspeptin receptor

Equine hypothalamus was used as positive control tissue for validation of anti-Kp10 (panel A) and anti-Kiss1r (panel B) antibodies, as shown in Figure 4.1 with immunosignals present in neurons near the III ventricle of the hypothalamus.

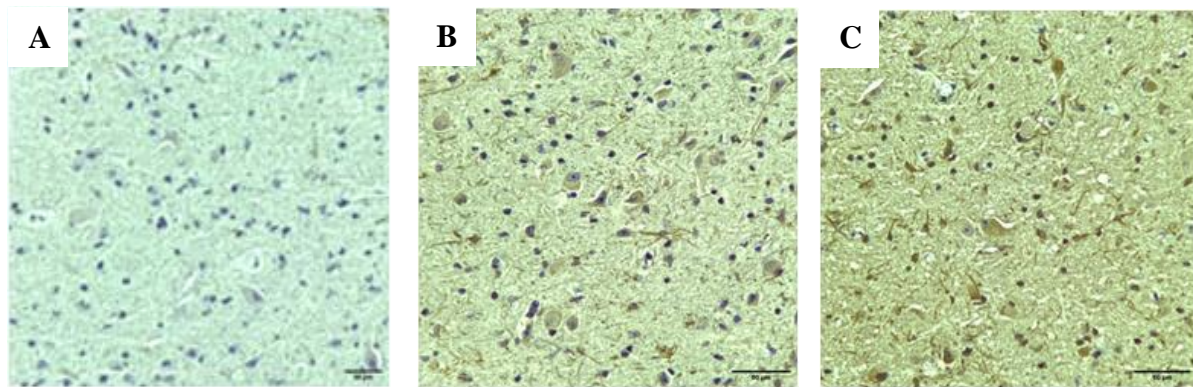


Figure 4.1. Hypothalamus tissue from a mare was used as a positive control for both kisspeptin-10 and kisspeptin receptor antibodies. Omission of antibody (panel A) from hypothalamus tissue served as negative control. Panels B and C represent positive staining for kisspeptin-10 and kisspeptin receptor, respectively. Scale bars represent 50  $\mu\text{m}$ .

A total of 1,244 follicles were assessed for Kp10 immunoreactivity and 1,258 follicles were assessed for Kiss1r immunoreactivity. Positive immunostaining of Kp10 and Kiss1r was present throughout reproductive structures in the mare ovary, including all follicle types and corpora lutea. Regardless of reproductive state, intensity of Kp10 and Kiss1r staining increased as follicle size increased ( $P < 0.0001$  and  $P < 0.0001$ , respectively). An interaction ( $P < 0.01$ )

between follicle size and reproductive state was also observed for Kiss1r staining. The intensity of staining was affected by follicle size and reproductive state.

#### **4.2. Differences in kisspeptin-10 staining intensity in follicles within different reproductive states**

Mean kisspeptin-10 staining intensity between follicle sizes within different reproductive states is presented in Figure 4.2. In primordial follicles, staining was most intense and similar in ovaries from follicular phase mares and mares stimulated with ECP-sulpiride. Staining in primordial follicles was least intense in ovaries from luteal phase mares and differed ( $P < 0.01$ ) from all other reproductive states. Staining in primordial follicles from anestrus mares was less ( $P < 0.001$ ; Figure 4.3.) than ECP-sulpiride treated mares but did not differ from follicular phase mares.

Staining intensity in preantral follicles was least in luteal phase ovaries and differed ( $P \leq 0.05$ ; Figure 4.2.) from anestrus ovaries and ovaries from mares stimulated with ECP-sulpiride. Staining intensity in preantral follicles tended to be less ( $P \leq 0.1$ ) in luteal phase ovaries compared to follicular phase ovaries. Figure 4.4 illustrates staining observed in anestrus ovaries and ovaries from mares stimulated with ECP-sulpiride.

Staining for Kp10 was most intense in antral follicles and did not differ between follicular, luteal, or ECP-sulpiride stimulated ovaries; however, staining intensity tended to be less ( $P = 0.06$ ) in anestrus ovaries compared to follicular and ECP-sulpiride stimulated ovaries. Figure 4.5 illustrates staining observed in luteal and follicular phase ovaries.

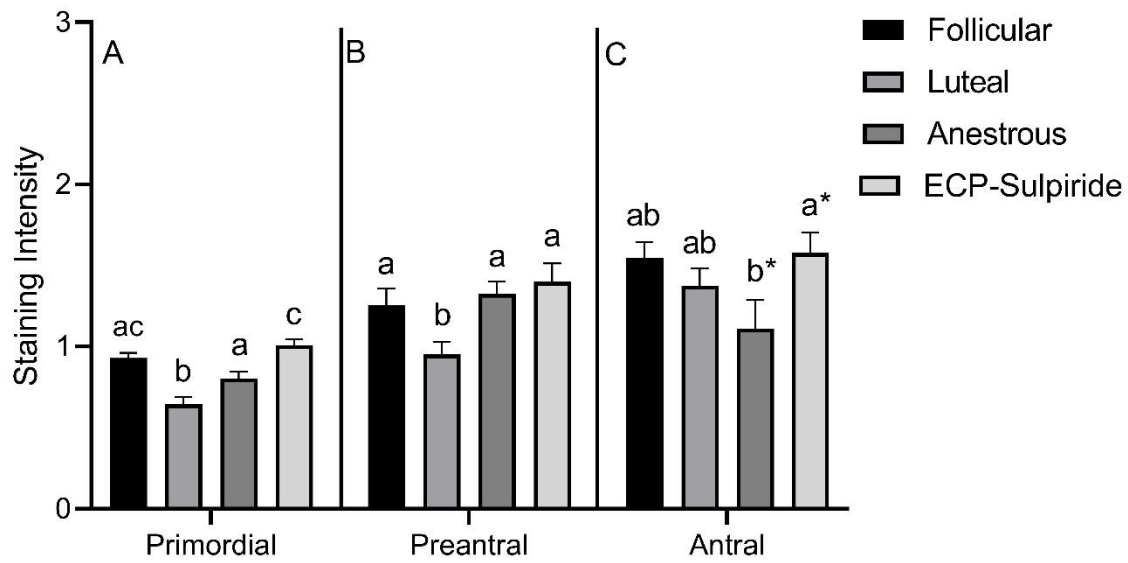


Figure 4.2. Mean staining intensity for kisspeptin in primordial (panel A), preantral (panel B) and antral (panel C) follicles within different reproductive states. Means with different superscripts within a panel differ at  $P \leq 0.05$ . Superscripts with \* denotes differences at  $P < 0.1$ .

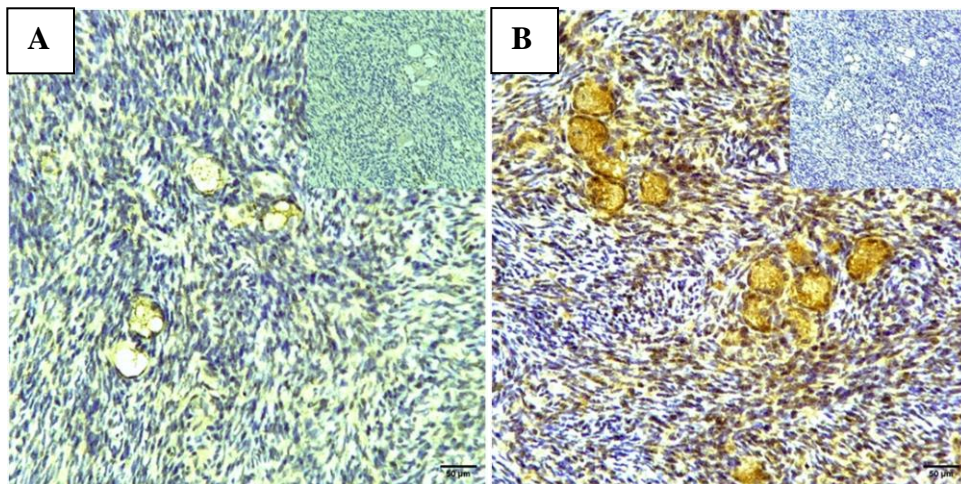


Figure 4.3. Kisspeptin immunostaining in primordial follicles of anestrus ovaries (panel A) and ovaries from mares stimulated with estradiol cypionate and sulpiride (panel B). Omission of primary antibody served as a negative control (inserts). Scale bars are 50  $\mu\text{m}$ .



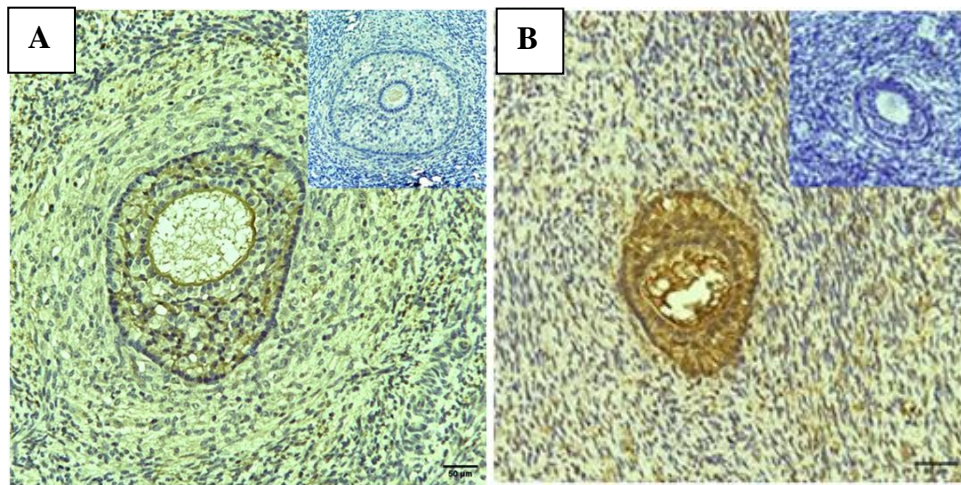


Figure 4.4. Kisspeptin immunostaining in preantral follicles of anestrus ovaries (panel A) and ovaries from mares stimulated with estradiol cypionate and sulpiride (panel B). Omission of primary antibody served as a negative control (inserts). Scale bars are 50  $\mu\text{m}$ .

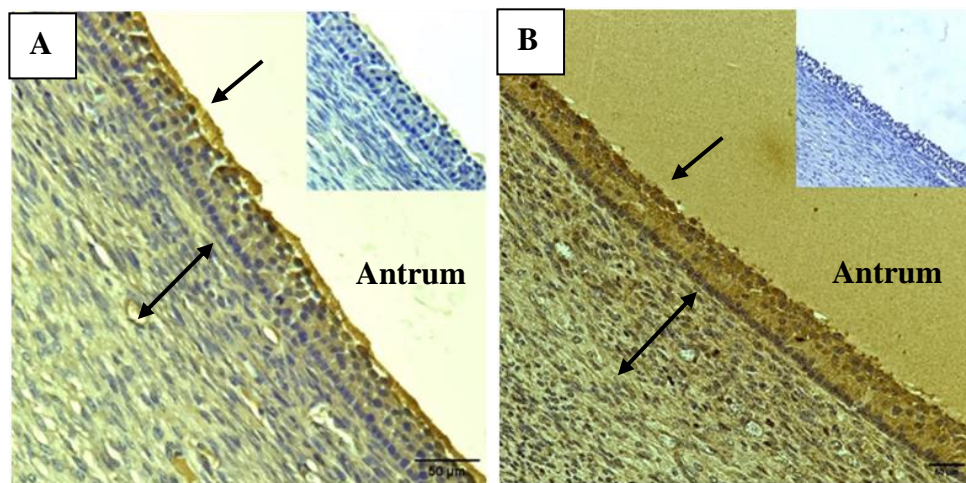


Figure 4.5. Kisspeptin immunostaining in antral follicles of luteal phase ovaries (panel A) and follicular phase ovaries (panel B). Single arrow illustrates staining in granulosa cells while double arrow illustrates staining in theca cells. Omission of primary antibody served as a negative control (inserts). Scale bars are 50  $\mu\text{m}$ .

### 4.3. Kisspeptin receptor staining intensity between follicle sizes within different reproductive states

Mean kisspeptin receptor staining intensity between follicle sizes within different reproductive states is presented in Figure 4.6. In primordial follicles, staining was most intense in ovaries from mares stimulated with ECP-sulpiride and was greater ( $P < 0.01$ ) compared to all other reproductive states. Figure 4.7 illustrates staining observed in primordial follicles within anestrus ovaries and ovaries from mares stimulated with ECP-sulpiride.

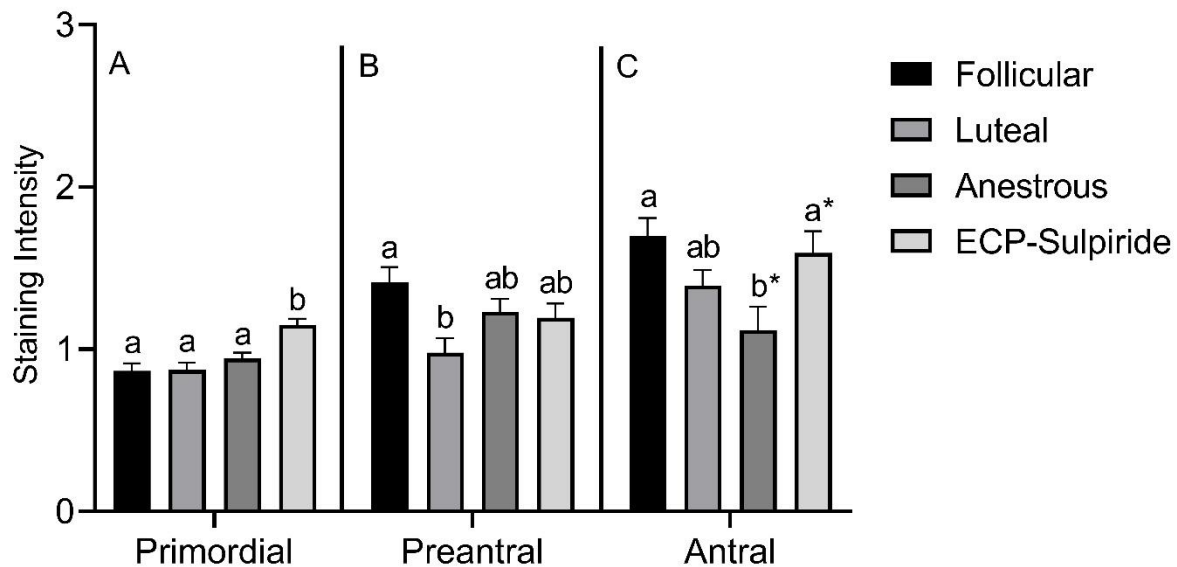


Figure 4.6. Mean staining intensity for kisspeptin receptors in primordial (panel A), preantral (panel B) and antral (panel C) follicles within different reproductive states. Means with different superscripts within a panel differ at  $P \leq 0.05$ . Superscripts with \* denotes differences at  $P < 0.1$ .

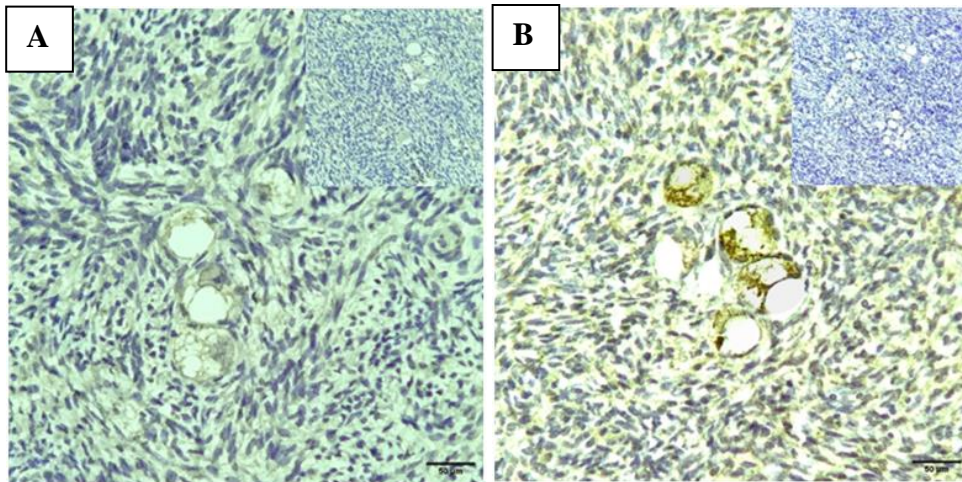


Figure 4.7. Kisspeptin receptor immunostaining in primordial follicles of anestrus ovaries (panel A) and ovaries from mares stimulated with estradiol cypionate and sulpiride (panel B). Omission of primary antibody served as a negative control (inserts). Scale bars are 50  $\mu$ m.

In preantral follicles, staining was similar between follicular, luteal and ECP-sulpiride stimulated ovaries. Staining was less ( $P < 0.01$ ) in luteal phase ovaries compared to follicular phase ovaries but did not differ from anestrus or ECP-sulpiride stimulated ovaries.

Kisspeptin receptor staining intensity in antral follicles was less ( $P \leq 0.01$ ) in anestrus ovaries compared to follicular phase ovaries and tended ( $P = 0.07$ ) to be less than ECP-sulpiride stimulated ovaries. Staining in antral follicles was similar between follicular, luteal and ECP-sulpiride stimulated ovaries. Figure 4.8 illustrates kisspeptin receptor staining observed in an antral follicle of luteal and follicular phase ovaries.



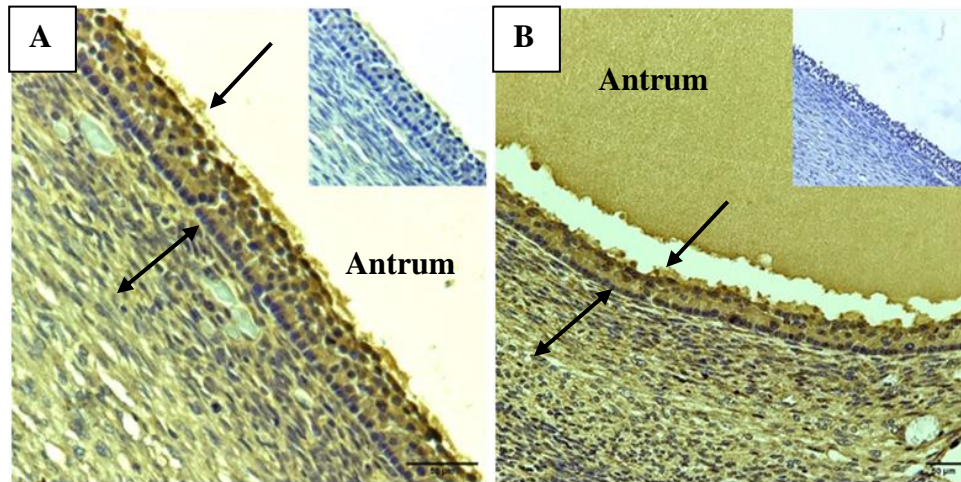


Figure 4.8. Kisspeptin receptor immunostaining in antral follicles of luteal phase ovaries (panel A) and follicular phase ovaries (panel B). Single arrow illustrates staining in granulosa cells while double arrow illustrates staining in theca cells. Omission of primary antibody served as a negative control (inserts). Scale bars are 50  $\mu$ m.

#### 4.5. Other Immunoreactive Tissues

When present, corpora lutea were assessed for presence or absence of staining but were not graded. Figure 4.9 illustrates the positive immunostaining for both Kp10 and Kiss1r. Staining was uniform throughout luteal cells and there were no subjective differences in staining pattern of CL observed.

The germinal epithelium of the ovulation fossa stained positive for kisspeptin and receptor. Oocytes also stained positive for both kisspeptin and receptor in primordial, preantral, and early antral follicles. Staining intensity of the oocyte was dependent on the staining intensity of the surrounding cells of the respective follicle. Oocytes in late antral follicles were not present in sections processed for IHC.

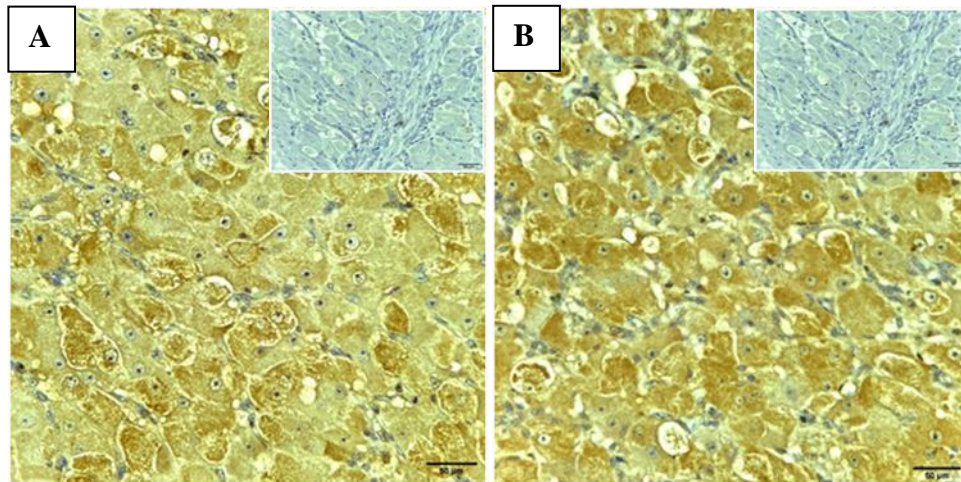


Figure 4.9. Positive immunostaining for kisspeptin-10 (panel A) and kisspeptin receptors (panel B) in luteal cells. Omission of primary antibodies served as negative controls (insert). Scale bars are 50 µm.



## CHAPTER 5. DISCUSSION

The role(s) of the kisspeptin and kisspeptin receptor system in mammalian reproduction at the level of the hypothalamus has been well established; however, extrahypothalamic actions of kisspeptin have been investigated. Peripheral actions of kisspeptin/Kiss1r have been explored as regulators of uterine and placental function, particularly in trophoblast migration, implantation, and placentation as reviewed by Gomes and Sones [85]. Kisspeptin and its cognate receptor have been identified in the gonads of several species including the ovaries of rats [79], hamsters [45], humans and marmosets [78], cats [76], dogs [77], pigs [80], and buffalos [81–83]. Pattern and intensity of staining for both peptides varies between studies and appears to be species dependent. In the horse, kisspeptin and Kiss1r were immunolocalized in Leydig cells of the testes [2]. Corresponding transcripts were also found, indicating the possibility of de novo synthesis [2]. Furthermore, kisspeptin expression has been found in the endometrium and embryonic tissues of pregnant mares (unpublished).

The present study is the first to compare immunostaining for kisspeptin and its receptor between follicle sizes and reproductive states. McGrath [86] observed kisspeptin immunoreactive staining in the cells of some preantral follicles, as well as in the largest antral follicle in an ovary from a mare in vernal transition, but no comparisons were made in that study as immunostaining was not the primary objective. In the present study, regardless of reproductive state, intensity of staining for both peptides increased as follicle size increased. This may indicate a role for kisspeptin/Kiss1r in late-stage follicular growth, maturation, and/or ovulation. In mares, immunostaining for Kp10 was least intense in primordial follicles of luteal and anestrus phase ovaries and was most intense in antral follicles of follicular phase ovaries and ovaries from mares treated with ECP-sulpiride. In rats and hamsters, expression of

kisspeptin peaks in the afternoon during proestrus, also providing evidence that kisspeptin may be involved in ovulatory events [45,79]. Immunostaining for Kiss1r was least intense in primordial follicles of follicular and luteal phase ovaries and was most intense in antral follicles of follicular phase ovaries and ovaries from mares treated with ECP-sulpiride.

These findings are consistent with other findings in swine ovarian follicles [80], in which immunostaining for kisspeptin and receptor were present in preantral and antral follicles with intense staining in granulosa cells and less intense, but still present, staining in theca cells. This contrasts with human and marmoset [78] and rat [79] studies in which immunostaining for kisspeptin and its receptor were minimal or absent in granulosa cells but were present in theca cells and the luteal cells, similar to the current findings and other species, such as the buffalo [81].

In the canine ovary [77], immunoreactive staining pattern is consistent with the findings of the current study except for the absence of staining for kisspeptin and its receptor in theca cells of follicles. In the mare, staining for both peptides was present in theca cells, albeit weaker than neighboring granulosa cells. No kisspeptin immunosignal was detected in granulosa cells of anestrus bitches; however, Kiss1r immunosignal was consistent across all reproductive states, including anestrus [77]. This differs from the present study where kisspeptin was detected in granulosa cells of anestrus ovaries, although weaker than cycling or stimulated granulosa cells. Canine oocytes consistently stained for kisspeptin and its receptor, regardless of reproductive state [77]. This may indicate a role for kisspeptin in the bidirectional communication between developing oocytes and surrounding granulosa cells.

Kisspeptin as an oocyte maturation factor has been largely explored [87–92]. In rats, Kiss1r expression is predominantly in oocytes, while kisspeptin expression is largely expressed

in granulosa cells [92]. Chakravarthi et al. [89] further identified expression of Kiss1r in oocytes, but not in granulosa cells. Furthermore, oocytes derived from wild-type and estrogen receptor  $\beta$  (Er $\beta$ ) null rats were supplemented in vitro with Kp10. Expression of both growth and differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), two proteins secreted by oocytes and highly correlated with oocyte maturation, were upregulated [89]. Interestingly, bone morphogenetic protein 7 (BMP7), a protein implicated in facilitating the transition from primordial to primary follicle, was downregulated by Kp10 [89]. Upregulation of c-mos, an oncoprotein required for meiosis, has also been observed in oocytes matured in the presence of kisspeptin; however, FSH was critical to the permissive actions of kisspeptin as it did induce a significant increase in oocyte expression of Kiss1r [91]. Resumption of meiosis is a critical event in oocyte maturation. A remarkable increase in kisspeptin expression has been observed in mouse oocytes around the time of meiotic resumption [91], suggesting that perhaps kisspeptin is involved in the ability to undergo meiosis. Taken together, a great case can be made for a role for kisspeptin and its receptor in oocyte maturation. Further gene expression studies are needed to determine expression levels in equine ovarian cells.

In the domestic cat ovary [76], immunoreactive staining patterns of kisspeptin and receptor are consistent with the findings of the current study except for the absence of staining for kisspeptin receptor in the granulosa cells of antral follicles but presence of stain in theca cells of the same follicles. In the present study, there were no instances of kisspeptin or receptor staining present in only theca cells of follicles and absent in granulosa cells of the same follicles, although some antral follicles exhibited greater staining intensity in granulosa cells compared to theca cells of the same follicle, similar to the findings of Tanyapanachon et al. [76] and Santos et al. [93]. Increased staining intensity in granulosa cells for kisspeptin and receptor indicate the

potential for granulosa cells to be a site for kisspeptin synthesis in the follicle. In this study, preliminary gene expression analysis revealed Kp10 transcripts in granulosa cells, luteal cells, and ovarian stroma of mares (data not presented). Staining for kisspeptin and receptor was present in all follicle types in the domestic cat ovary [76], similar to current findings, suggesting a potential role for kisspeptin in follicular development throughout gonadotropin-independent and gonadotropin-dependent stages of follicular growth.

The hamster and ewe provide models for assessing the role of kisspeptin in mediating photoperiodic signals to the HPO axis [41,89,91,94]. The Siberian hamster, a long-day breeder like the horse, provided the first model for characterizing kisspeptin immunoreactivity in a seasonally breeding species at the ovarian level [45]. Kisspeptin and receptor immunostaining was characterized throughout the estrous cycle as well as during photoperiod induced recrudescence. Staining intensity varied depending on stage of the cycle, similar to current findings, with a noticeable increase in staining intensity during the period of recrudescence compared to the anestrus period, implicating potential roles for kisspeptin in the resumption of ovarian activity [46]. Seasonally anestrus mares induced to cycle early with ECP-sulpiride provided an opportunity to assess immunosignals in a recrudescenced state. Obvious increases in Kp10 staining intensity were observed in primordial and antral follicles of ECP-sulpiride treated mares compared to seasonally anestrus mares. Kisspeptin receptor staining was also greater in primordial and antral follicles of ECP-sulpiride treated mares. Staining intensities for both peptides was similar between follicular phase ovaries and ovaries from ECP-stimulated mares suggesting that treatment with ECP-sulpiride restored ovarian kisspeptin activity to a follicular phase-like state. Without more quantitative analyses, such as gene expression, it cannot be definitely concluded that this is the case. In a recent study by Bailey et al. [3], incorporation of

Kp10 into treatment with EB-sulpiride resulted in more mares ovulating early compared to mares receiving EB-sulpiride only. Whether the action of Kp10 was at the level of the ovary or via enhanced endocrine responses from HP axis could not be concluded from that study. The finding that staining intensity for both peptides was greater in ECP-sulpiride stimulated mares compared to seasonally anestrous mares was significant as research continues to explore the mechanisms by which the mare transitions into and out of cyclicity. Kisspeptin/Kiss1r may be involved in the recrudescence of ovarian activity in the seasonally anestrous mare.

Immunopositive signals for both Kp10 and Kiss1r were observed in luteal cells with no observed differences in intensities between large and small luteal cells. This is similar to findings in the buffalo [81] and Siberian hamster [45]. However, further studies are needed to assess the differences in kisspeptin and receptor immunoreactivity throughout different stages of luteal development, such as CL formation, maintenance, and regression to determine relative significance of kisspeptin in the mare CL. The presence of kisspeptin transcripts and kisspeptin immunoreactive staining in the CL of most mammalian species studied to date indicates that kisspeptin may have potential roles in CL formation and/or regression by similar means of the proposed pathways of ovarian tissue remodeling leading up to ovulation, as proposed by Castellano et al. [79]. The kisspeptinergic system may also play a role in the stimulation of progesterone secretion as evidenced by an *in vitro* study of cultured rat luteal cells treated with kisspeptin [95].

*In vitro* culture of rat luteal cells treated with kisspeptin resulted in stimulation of progesterone secretion [95]. Granulosa cells cultured from swine ovarian follicles demonstrated increased progesterone secretion and decreased/inhibited estradiol secretion when treated with kisspeptin *in vitro* [80]. Both human [94] and porcine [80] cultured granulosa cells treated with

Kp10 and FSH demonstrated that kisspeptin supports the stimulatory actions of FSH on cell viability, proliferation, and estradiol and progesterone output. Transcripts of key steroidogenic enzymes such as StAR, CYP11A and 3 $\beta$ -HSD are also upregulated in rat luteal cells [95] and chicken granulosa cells [84] cultured with Kp10, providing further evidence of the steroidogenic promoting activity of kisspeptin.

Follicular fluid kisspeptin has been measured in some species [4,91] and positively correlates with number of oocytes in women undergoing assisted reproductive technologies [4]. In this study, follicular fluid of antral follicles demonstrated positive immunostaining for kisspeptin; however, without quantitative measurement, these results are tenuous. Additional staining was observed in the ovulation fossa, although no semi-quantitative assessments were attempted.

The findings of the present study provide evidence that the kisspeptinergic system exists within the equine ovary and changes in relation to follicle size as well as reproductive state. Based on semi-quantitative staining intensity, kisspeptin and its cognate receptor are more abundant in antral follicles of cycling ovaries. The presence of kisspeptin and its receptor in the oocyte and steroidogenic cells of follicles and CL indicate that kisspeptin may have roles in oocyte maturation, follicular development, steroidogenesis, and/or ovulation. Variations in staining patterns indicate potential roles for the kisspeptin/receptor system in ovulation, as reported in other species. Further functional studies are needed in the horse to assess regulatory roles of kisspeptin in ovarian steroidogenesis, as well as follicular development and oocyte maturation.

## **SUMMARY AND CONCLUSIONS**

In conclusion, the present study is the first to localize Kp-10 and Kiss1r in equine ovaries. Staining was present for both Kp-10 and Kiss1r in all follicle types across all stages, as well as oocytes and CL. The most intense staining for Kp-10 and Kiss1r occurred in antral follicles of ovaries from mares treated with ECP-sulpiride and follicular phase ovaries. Compared to the decreased staining intensities in anestrus ovaries, this suggests that the kisspeptin/receptor system may have potential roles in follicular growth leading up to ovulation. Further research is needed to elucidate the modulatory mechanisms of kisspeptin on ovarian steroidogenesis and follicular development in the horse model.

## REFERENCES

- [1] American Horse Council Foundation. Economic impact of the U.S. horse industry 2017. <https://www.horsecouncil.org/resources/economics/> (accessed June 29, 2021).
- [2] Petrucci L, Maranesi M, Verini Supplizi A, Dall’Aglia C, Mandara MT, Quassinti L, et al. Kisspeptin/GnRH1 system in Leydig cells of horse (*Equus caballus*): Presence and function. *Theriogenology* 2020;152:1–7. <https://doi.org/10.1016/j.theriogenology.2020.04.006>.
- [3] Bailey VN, Sones JL, Camp CM, Gomes VCL, Oberhaus EL. Endocrine and ovarian responses to combined estradiol benzoate-sulpiride in seasonally anovulatory mares treated with kisspeptin. *Anim Reprod Sci* 2022;107087. <https://doi.org/10.1016/J.ANIREPROSCI.2022.107087>.
- [4] Taniguchi Y, Kuwahara A, Tachibana A, Yano Y, Yano K, Yamamoto Y, et al. Intra-follicular kisspeptin levels are related to oocyte maturation and gonadal hormones in patients who are undergoing assisted reproductive technology. *Reprod Med Biol* 2017;16. <https://doi.org/10.1002/rmb2.12056>.
- [5] Hadley ME, Levine JE. *Endocrinology*. 6th ed. Upper Saddle River, NJ: Pearson Education, Inc.; 2007.
- [6] McKinnon AO, Squires EL, Vaala WE, Varner DD. *Equine Reproduction*. 2nd ed. Chichester, West Sussex, United Kingdom : Wiley-Blackwell; 2011.
- [7] Senger PL. *Pathways to Pregnancy & Parturition* . 3rd ed. Redmond, OR: Current Conceptions, Inc.; 2012.
- [8] Mossman HW, Duke KL. *Comparative Morphology of the Mammalian Ovary*. Madison, Wisconsin: University of Wisconsin Press; 1973.
- [9] Walt ML, Stabenfeldt GH, Hughes JP, Neely DP, Bradbury R. Development of the equine ovary and ovulation fossa. *J Reprod Fertil* 1979;Suppl. 27:471–7.
- [10] Ginther OJ, Beg MA, Gastal MO, Gastal EL. Follicle dynamics and selection in mares. *Anim Reprod*, v 2004;11.
- [11] Aurich C. Reproductive cycles of horses. *Anim Reprod Sci* 2011;124:220–8. <https://doi.org/10.1016/J.ANIREPROSCI.2011.02.005>.
- [12] Meyers-Brown GA, Loud MC, Hyland JC, Roser JF. Deep anestrous mares under natural photoperiod treated with recombinant equine FSH (reFSH) and LH (reLH) have fertile ovulations and become pregnant. *Theriogenology* 2017;98:108–15. <https://doi.org/10.1016/J.THERIOGENOLOGY.2017.05.001>.
- [13] Nagy P, Guillaume D, Daels P. Seasonality in mares. *Anim Reprod Sci* 2000;60:245–62. [https://doi.org/10.1016/S0378-4320\(00\)00133-0](https://doi.org/10.1016/S0378-4320(00)00133-0).



- [14] Guillaume D, Duchamp G, Nagy P, Palmer E. Determination of minimum light treatment required for photostimulation of winter anoestrous mares. *J Reprod Fertil Suppl* 2000;205–16.
- [15] Norman ST, Larsen JE, Rural Industries Research and Development Corporation (Australia). The synchronisation of oestrus and ovulation in the mare : current knowledge, further direction and a practical regimen. RIRDC; 2010.
- [16] Sharp DC, Kooistra L, Ginther OJ. Effects of artificial light on the oestrous cycle of the mare. *J Reprod Fertil Suppl* 1975;241–6.
- [17] Millar RP. GnRHs and GnRH receptors. *Anim Reprod Sci* 2005;88. <https://doi.org/10.1016/j.anireprosci.2005.05.032>.
- [18] Besognet B, Hansen BS, Daels PF. Induction of reproductive function in anestrous mares using a dopamine antagonist. *Theriogenology* 1997;47. [https://doi.org/10.1016/S0093-691X\(97\)00005-8](https://doi.org/10.1016/S0093-691X(97)00005-8).
- [19] Brendemuehl JP, Cross DL. Influence of the dopamine antagonist domperidone on the vernal transition in seasonally anoestrous mares. *J Reprod Fertil Suppl* 2000.
- [20] Panzani D, Zicchino I, Taras A, Marmorini P, Crisci A, Rota A, et al. Clinical use of dopamine antagonist sulpiride to advance first ovulation in transitional mares. *Theriogenology* 2011;75. <https://doi.org/10.1016/j.theriogenology.2010.07.019>.
- [21] Kelley KK, Thompson DL, Storer WA, Mitcham PB, Gilley RM, Burns PJ. Estradiol interactions with dopamine antagonists in mares: Prolactin secretion and reproductive traits. *J Equine Vet Sci* 2006;26. <https://doi.org/10.1016/j.jevs.2006.09.008>.
- [22] Thompson DL, Clavier SE, Mitcham PB, Earl LR. Estradiol stimulation of prolactin secretion in horses: Interaction with type of secretagogue. *J Equine Vet Sci* 2011;31. <https://doi.org/10.1016/j.jevs.2011.03.139>.
- [23] Thompson DL, Oberhaus EL. Prolactin in the Horse: Historical Perspective, Actions and Reactions, and Its Role in Reproduction. *J Equine Vet Sci* 2015;35. <https://doi.org/10.1016/j.jevs.2015.03.199>.
- [24] King SS, Roser JF, Jones KL. Follicular Fluid Prolactin and the Periovulatory Prolactin Surge in the Mare. *J Equine Vet Sci* 2008;28. <https://doi.org/10.1016/j.jevs.2008.07.007>.
- [25] Oberhaus EL, Thompson DL, Pham CK. Factors Affecting the Ovarian Response to a Combined Estradiol-Sulpiride Treatment in Seasonally Anovulatory Mares. *J Equine Vet Sci* 2017;50. <https://doi.org/10.1016/j.jevs.2016.12.001>.
- [26] Oberhaus EL, Wilson KM, Camp CM, Sones JL. Sucrose Acetate Isobutyrate (SAIB) as a Delivery Vehicle for Estradiol and Sulpiride: Evaluation of Endocrine Responses in Geldings and Ovarian Response in Seasonally Anovulatory Mares. *J Equine Vet Sci* 2022;112. <https://doi.org/10.1016/j.jevs.2022.103896>.

- [27] Oberhaus EL, Thompson DL, Foster BA, Pinto CR. Effects of Combined Estradiol-Sulpiride Treatment and Follicle Ablation on Vernal Transition in Mares: Evaluation of Plasma and Follicular Fluid Hormones and Luteinizing Hormone Receptor Gene Expression. *J Equine Vet Sci* 2018;64:69–76. <https://doi.org/10.1016/J.JEVS.2018.02.020>.
- [28] Clavier SC, Thompson DL, Caltabilota TJ, Mitcham PB. Dose-Response of Prolactin to Increasing Doses of the Dopamine Receptor Antagonist, Sulpiride, in Horses: Effect of Season in Mares and Stallions of Estradiol Pretreatment in Geldings. *J Equine Vet Sci* 2012;32. <https://doi.org/10.1016/j.jevs.2011.09.066>.
- [29] Lee J-H, Miele ME, Hicks DJ, Phillips KK, Trent J, Weissman B, et al. ARTICLES KiSS-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene. 1996.
- [30] Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor 2001.
- [31] de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* 2003;100. <https://doi.org/10.1073/pnas.1834399100>.
- [32] Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* 2009;30. <https://doi.org/10.1210/er.2009-0005>.
- [33] Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, le Poul E, et al. The Metastasis Suppressor Gene KiSS-1 Encodes Kisspeptins, the Natural Ligands of the Orphan G Protein-coupled Receptor GPR54. *Journal of Biological Chemistry* 2001;276:34631–6. <https://doi.org/10.1074/JBC.M104847200>.
- [34] Magee C, Bruemmer JE, Kirkley KS, Sylvester LA, Runyan B, Nett TM, et al. Kisspeptin has an independent and direct effect on the pituitary gland in the mare. *Theriogenology* 2020;157:199–209. <https://doi.org/10.1016/j.theriogenology.2020.07.031>.
- [35] Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, et al. Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. *Endocrinology* 2005;146. <https://doi.org/10.1210/en.2005-0195>.
- [36] Messenger S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, et al. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci U S A* 2005;102. <https://doi.org/10.1073/pnas.0409330102>.
- [37] Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T. Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. *Biochem Biophys Res Commun* 2004;320. <https://doi.org/10.1016/j.bbrc.2004.05.185>.
- [38] Roa J, Navarro VM, Tena-Sempere M. Kisspeptins in reproductive biology: Consensus knowledge and recent developments. *Biol Reprod* 2011;85. <https://doi.org/10.1095/biolreprod.111.091538>.

- [39] Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: Physiological roles and regulatory mechanisms. *Physiol Rev* 2012;92. <https://doi.org/10.1152/physrev.00037.2010>.
- [40] Smith JT, Clay CM, Caraty A, Clarke IJ. KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. *Endocrinology* 2007;148. <https://doi.org/10.1210/en.2006-1435>.
- [41] Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* 2006;401. <https://doi.org/10.1016/j.neulet.2006.03.039>.
- [42] Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CVR, Jafarzadehshirazi MR, et al. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* 2007;148. <https://doi.org/10.1210/en.2007-0961>.
- [43] Pompolo S, Pereira A, Estrada KM, Clarke IJ. Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. *Endocrinology* 2006;147. <https://doi.org/10.1210/en.2005-1123>.
- [44] Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, et al. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: A novel medium for seasonal breeding in the sheep. *Endocrinology* 2008;149. <https://doi.org/10.1210/en.2008-0581>.
- [45] Shahed A, Young KA. Differential Ovarian Expression of KiSS-1 and GPR-54 During the Estrous Cycle and Photoperiod Induced Recrudescence in Siberian Hamsters (*Phodopus sungorus*). *Mol Reprod Dev* 2009;76:444–52. <https://doi.org/10.1002/mrd.20972>.
- [46] Magee C, Foradori CD, Bruemmer JE, Arreguin-Arevalo JA, McCue PM, Handa RJ, et al. Biological and anatomical evidence for kisspeptin regulation of the hypothalamic-pituitary-gonadal axis of estrous horse mares. *Endocrinology* 2009;150. <https://doi.org/10.1210/en.2008-1698>.
- [47] Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C. Kisspeptin immunoreactive neurons in the equine hypothalamus. Interactions with GnRH neuronal system. *J Chem Neuroanat* 2008;36. <https://doi.org/10.1016/j.jchemneu.2008.07.008>.
- [48] Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, et al. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. *Endocrinology* 2005;146. <https://doi.org/10.1210/en.2004-0836>.
- [49] Lehman MN, Ebling FJP, Moenter SM, Karsch FJ. Distribution of estrogen receptor-immunoreactive cells in the sheep brain. *Endocrinology* 1993;133. <https://doi.org/10.1210/endo.133.2.8344223>.

- [50] Skinner DC, Caraty A, Allingham R. Unmasking the progesterone receptor in the preoptic area and hypothalamus of the ewe: No colocalization with gonadotropin-releasing neurons. *Endocrinology* 2001;142. <https://doi.org/10.1210/endo.142.2.7956>.
- [51] Goodman RL, Okhura S, Okamura H, Coolen LM, Lehman MN. KNDy Hypothesis for Generation of GnRH Pulses: Evidence from Sheep and Goats. *The GnRH Neuron and its Control*, 2018. <https://doi.org/10.1002/9781119233275.ch12>.
- [52] Goodman RL, Coolen LM, Lehman MN. Unraveling the Mechanism of Action of the GnRH Pulse Generator. A Possible Role for Kisspeptin/Neurokinin B/Dynorphin (KNDy) Neurons. *Cellular Endocrinology in Health and Disease*, 2014. <https://doi.org/10.1016/B978-0-12-408134-5.00009-3>.
- [53] Merkley CM, Coolen LM, Goodman RL, Lehman MN. Evidence for Changes in Numbers of Synaptic Inputs onto KNDy and GnRH Neurones during the Preovulatory LH Surge in the Ewe. *J Neuroendocrinol* 2015;27. <https://doi.org/10.1111/jne.12293>.
- [54] Caraty A, Smith JT, Lomet D, ben Saïd S, Morrissey A, Cognie J, et al. Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. *Endocrinology* 2007;148. <https://doi.org/10.1210/en.2007-0554>.
- [55] McGrath BM, Scott CJ, Wynn PC, Loy J, Norman ST. Kisspeptin stimulates LH secretion but not ovulation in mares during vernal transition. *Theriogenology* 2016;86. <https://doi.org/10.1016/j.theriogenology.2016.05.016>.
- [56] Magee C, Bruemmer JE, Nett TM, Squires EL, Clay CM. Kisspeptide in the estrous mare: Is it an appropriate ovulation-inducing agent? *Theriogenology* 2012;78:1987–96. <https://doi.org/10.1016/J.THERIOGENOLOGY.2012.07.012>.
- [57] Decourt CV, Merzouki Y, Duchamp G, Bruneau B, Catary A, Briant C. Effects of constant intravenous kisspeptin administration on luteinizing hormone release in cyclic mares. *Anim Reprod Sci* 2010;121:65–7. <https://doi.org/10.1016/J.ANIREPROSCI.2010.04.071>.
- [58] Decourt C, Caraty A, Briant C, Guillaume D, Lomet D, Chesneau D, et al. Acute injection and chronic perfusion of kisspeptin elicit gonadotropins release but fail to trigger ovulation in the mare. *Biol Reprod* 2014;90. <https://doi.org/10.1095/biolreprod.113.114157>.
- [59] Nett TM, Pickett BW, Seidel GE, Voss JL. Levels of luteinizing hormone and progesterone during the estrous cycle and early pregnancy in mares. *Biol Reprod* 1976;14. <https://doi.org/10.1095/biolreprod14.4.412>.
- [60] Briant C, Schneider J, Guillaume D, Ottogalli M, Duchamp G, Bruneau B, et al. Kisspeptin induces ovulation in cycling Welsh pony mares. *Anim Reprod Sci* 2006;94:217–9. <https://doi.org/10.1016/J.ANIREPROSCI.2006.04.021>.

- [61] Williams GL, Thorson JF, Prezotto LD, Velez IC, Cardoso RC, Amstalden M. Reproductive seasonality in the mare: Neuroendocrine basis and pharmacologic control. *Domest Anim Endocrinol* 2012;43. <https://doi.org/10.1016/j.domaniend.2012.04.001>.
- [62] Tomita K, Niida A, Oishi S, Ohno H, Cluzeau J, Navenot JM, et al. Structure-activity relationship study on small peptidic GPR54 agonists. *Bioorg Med Chem* 2006;14. <https://doi.org/10.1016/j.bmc.2006.07.009>.
- [63] Oishi S, Misu R, Tomita K, Setsuda S, Masuda R, Ohno H, et al. Activation of neuropeptide FF receptors by kisspeptin receptor ligands. *ACS Med Chem Lett* 2011;2. <https://doi.org/10.1021/ml1002053>.
- [64] Asami T, Nishizawa N, Matsui H, Nishibori K, Ishibashi Y, Horikoshi Y, et al. Design, synthesis, and biological evaluation of novel investigational nonapeptide KISS1R agonists with testosterone-suppressive activity. *J Med Chem* 2013;56. <https://doi.org/10.1021/jm401056w>.
- [65] Matsui H, Asami T. Effects and therapeutic potentials of kisspeptin analogs: Regulation of the hypothalamic-pituitary-gonadal axis. *Neuroendocrinology* 2014;99. <https://doi.org/10.1159/000357809>.
- [66] Beltramo M, Robert V, Galibert M, Madinier JB, Marceau P, Dardente H, et al. Rational design of triazololipeptides analogs of kisspeptin inducing a long-lasting increase of gonadotropins. *J Med Chem* 2015;58. <https://doi.org/10.1021/jm5019675>.
- [67] Decourt C, Robert V, Anger K, Galibert M, Madinier JB, Liu X, et al. A synthetic kisspeptin analog that triggers ovulation and advances puberty. *Sci Rep* 2016;6. <https://doi.org/10.1038/srep26908>.
- [68] Salzano G, Robert V, Lomet D, Decourt C, Hommet E, Derouin-Tochon F, et al. A customized long acting formulation of the kisspeptin analog C6 triggers ovulation in anestrus ewe. *J Neuroendocrinol* 2022;34. <https://doi.org/10.1111/jne.13121>.
- [69] Decourt C, Robert V, Lomet D, Anger K, Georgelin M, Poissenot K, et al. The kisspeptin analog C6 is a possible alternative to PMSG (pregnant mare serum gonadotropin) for triggering synchronized and fertile ovulations in the Alpine goat. *PLoS One* 2019;14. <https://doi.org/10.1371/journal.pone.0214424>.
- [70] Fanelli D, Beltramo M, Conte G, Cerretini B, Lomet D, Rota A, et al. The Kisspeptin analogue C6 induces ovulation in jennies. *Theriogenology* 2022;189:107–12. <https://doi.org/10.1016/j.theriogenology.2022.06.014>.
- [71] Lehman MN, Coolen LM, Goodman RL. Minireview: Kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: A central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* 2010;151. <https://doi.org/10.1210/en.2010-0022>.

- [72] Goubillon ML, Forsdike RA, Robinson JE, Ciofi P, Caraty A, Herbison AE. Identification of neurokinin B-expressing neurons as an highly estrogen-receptive, sexually dimorphic cell group in the ovine arcuate nucleus. *Endocrinology* 2000;141:4218–25. <https://doi.org/10.1210/endo.141.11.7743>.
- [73] Foradori CD, Coolen LM, Fitzgerald ME, Skinner DC, Goodman RL, Lehman MN. Colocalization of progesterone receptors in parvicellular dynorphin neurons of the ovine preoptic area and hypothalamus. *Endocrinology* 2002;143. <https://doi.org/10.1210/en.2002-220586>.
- [74] Foradori CD, Amstalden M, Goodman RL, Lehman MN. Colocalisation of dynorphin A and neurokinin B immunoreactivity in the arcuate nucleus and median eminence of the sheep. *J Neuroendocrinol* 2006;18. <https://doi.org/10.1111/j.1365-2826.2006.01445.x>.
- [75] Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA. Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res* 2010;1364. <https://doi.org/10.1016/j.brainres.2010.08.059>.
- [76] Tanyapanyachon P, Amelkina O, Chatdarong K. The expression of kisspeptin and its receptor in the domestic cat ovary and uterus in different stages of the ovarian cycle 2018. <https://doi.org/10.1016/j.theriogenology.2018.05.019>.
- [77] Cieleish ME, McGrath BM, Scott CJ, Norman ST, Stephen CP. The localization of kisspeptin and kisspeptin receptor in the canine ovary during different stages of the reproductive cycle. *Reproduction in Domestic Animals* 2017;52:24–8. <https://doi.org/10.1111/rda.12841>.
- [78] Gaytán F, Gaytán M, Castellano JM, Romero M, Roa J, Aparicio B, et al. KiSS-1 in the mammalian ovary: Distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. *Am J Physiol Endocrinol Metab* 2009;296. <https://doi.org/10.1152/ajpendo.90895.2008>.
- [79] Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, et al. Expression of KiSS-1 in rat ovary: Putative local regulator of ovulation? *Endocrinology* 2006;147. <https://doi.org/10.1210/en.2006-0117>.
- [80] Basini G, Grasselli F, Bussolati S, Ciccimarra R, Maranesi M, Bufalari A, et al. Presence and function of kisspeptin/KISS1R system in swine ovarian follicles. *Theriogenology* 2018;115:1–8. <https://doi.org/10.1016/J.THERIOGENOLOGY.2018.04.006>.
- [81] Rajin TR, Patra MK, Sheikh PA, Singh AK, Mishra GK, Karikalan M, et al. Expression of kisspeptin and its receptor in different functional classes of ovarian follicle in the buffalo (*Bubalus bubalis*). *Theriogenology* 2022;179:87–96. <https://doi.org/10.1016/j.theriogenology.2021.11.017>.
- [82] Mishra GK, Patra MK, Singh LK, Upmanyu V, Chakravarti S, Karikalan M, et al. Expression and functional role of kisspeptin and its receptor in the cyclic corpus luteum of

- buffalo (*Bubalus bubalis*). *Theriogenology* 2019;130:71–8.  
<https://doi.org/10.1016/J.THERIOGENOLOGY.2019.02.037>.
- [83] Mishra GK, Patra MK, Singh LK, Upmanyu V, Chakravarti S, Karikalan M, et al. Kiss1 and its receptor: molecular characterization and immunolocalization in the hypothalamus and corpus luteum of the buffalo. *Anim Biotechnol* 2018;30.  
<https://doi.org/10.1080/10495398.2018.1520715>.
  - [84] Xiao Y, Ni Y, Huang Y, Wu J, Grossmann R, Zhao R. Effects of kisspeptin-10 on progesterone secretion in cultured chicken ovarian granulosa cells from preovulatory (F 1-F 3) follicles. *Peptides (NY)* 2011;32. <https://doi.org/10.1016/j.peptides.2011.09.001>.
  - [85] Gomes VCL, Sones JL. From inhibition of trophoblast cell invasion to proapoptosis: what are the potential roles of kisspeptins in preeclampsia? *Am J Physiol Regul Integr Comp Physiol* 2021;321:R41–8. <https://doi.org/10.1152/AJPREGU.00258.2020>.
  - [86] McGrath B. Characterisation of the neuroanatomy of kisspeptin and RFRP-3 in the mare, and determination of the effect of kisspeptin on LH release and ovulation. Dissertation. Charles Stuart University, 2015.
  - [87] Byri P, Gangineni A, Reddy KR, Raghavender KBP. Effect of kisspeptin on in vitro maturation of sheep oocytes. *Vet World* 2017;10.  
<https://doi.org/10.14202/vetworld.2017.276-280>.
  - [88] Dorfman MD, Garcia-Rudaz C, Alderman Z, Kerr B, Lomniczi A, Dissen GA, et al. Loss of NTRK2/KISS1R signaling in oocytes causes premature Ovarian failure. *Endocrinology* 2014;155. <https://doi.org/10.1210/en.2014-1111>.
  - [89] Chakravarthi VP, Ghosh S, Housami SM, Wang H, Roby KF, Wolfe MW, et al. ER $\beta$  regulated ovarian kisspeptin plays an important role in oocyte maturation. *Mol Cell Endocrinol* 2021;527:111208. <https://doi.org/10.1016/J.MCE.2021.111208>.
  - [90] Ming H, Jun W, YuHong Y, ZiWei L, Xun L, WenFa IV. Expression and function of kisspeptin during bovine oocytes maturation in vitro. *Chinese Journal of Animal Science* 2015;51:19–22.
  - [91] Saadeldin IM, Koo OJ, Kang JT, Kwon DK, Park SJ, Kim SJ, et al. Paradoxical effects of kisspeptin: It enhances oocyte in vitro maturation but has an adverse impact on hatched blastocysts during in vitro culture. *Reprod Fertil Dev* 2012;24.  
<https://doi.org/10.1071/RD11118>.
  - [92] Chakravarthi VP, Khristi V, Ghosh S, Yerrathota S, Dai E, Roby KF, et al. ESR2 Is Essential for Gonadotropin-Induced Kiss1 Expression in Granulosa Cells. *Endocrinology* 2018;159:3860–73. <https://doi.org/10.1210/en.2018-00608>.
  - [93] Santos LC, dos Anjos Cordeiro JM, Santana L da S, Barbosa EM, Santos BR, da Silva TQM, et al. Expression profile of the Kisspeptin/Kiss1r system and angiogenic and

immunological mediators in the ovary of cyclic and pregnant cats. *Domest Anim Endocrinol* 2022;78. <https://doi.org/10.1016/j.domaniend.2021.106650>.

- [94] García-Ortega J, Pinto FM, Prados N, Bello AR, Almeida TA, Fernández-Sánchez M, et al. Expression of tachykinins and tachykinin receptors and interaction with kisspeptin in human granulosa and cumulus cells. *Biol Reprod* 2016;94. <https://doi.org/10.1095/biolreprod.116.139881>.
- [95] Peng J, Tang M, Zhang BP, Zhang P, Zhong T, Zong T, et al. Kisspeptin stimulates progesterone secretion via the Erk1/2 mitogen-activated protein kinase signaling pathway in rat luteal cells. *Fertil Steril* 2013;99. <https://doi.org/10.1016/j.fertnstert.2012.12.008>.



## **VITA**

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