EFFECT OF KISSPEPTIN ON THE HYPOTHALAMIC-PITUITARY-GONADAL

AXIS OF THE MARE

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EFFECT OF KISSPEPTIN ON THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS OF THE MARE

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EFFECT OF KISSPEPTIN ON THE HYPOTHALAMIC-PITUITARY-GONADAL

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Robyn Rhoades Wilborn

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VITA

Robyn Rhoades Wilborn, daughter of James C. Rhoades and Myrna J. Cotton, was born on February 19, 1976 in Coffee County, Alabama. Reared in Elba, Alabama, she graduated valedictorian of Elba High School in 1994. She began her undergraduate career at Enterprise State Junior College before transferring to Auburn University in the fall of 1995. Robyn received a Bachelor of Science degree in Animal and Dairy Sciences in 1998, graduating summa cum laude. She then entered the DVM program at Auburn in the fall of 1998 and received her Doctor of Veterinary Medicine degree on May 7, 2002, graduating cum laude. On May 11, 2002, she married Barney Scott Wilborn of Birmingham and the couple relocated to Wichita, Kansas for job opportunities. After three years of small and mixed animal practice in Kansas, Robyn accepted a residency position in theriogenology at Auburn University in 2005 and entered the Graduate School to simultaneously pursue a Master of Science degree. She was awarded Diplomate status in the American College of Theriogenologists in August of 2007 and completed her residency training and advanced degree in the fall of 2008.

THESIS ABSTRACT

EFFECT OF KISSPEPTIN ON THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS OF THE MARE

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Master of Science, December 19, 2008
(DVM, Auburn University, 2002)
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Kisspeptin, a neuropeptide product of the KiSS-1 gene, has recently been shown to control the timing and release of gonadotropin releasing hormone (GnRH) from the hypothalamus in laboratory animals and move anestrus ewes to normal cyclicity. This study was designed to determine whether kisspeptin would increase plasma luteinizing hormone (LH) in mares and whether kisspeptin could induce the anestrous mare to become cyclic. Six light horse mares were confirmed to be in mid-diestrus via teasing, rectal palpation and progesterone testing. Mares were then divided into three groups of two mares each and were treated with an intravenous (iv) injection of kisspeptin (1.0 nmol/kg or 0.5 nmol/kg) or saline. Mares responded to the kisspeptin treatment with a marked rise in LH concentration ($P<0.05$), and both doses achieved a similar response.

The procedure was then repeated using the same protocol with reduced doses, and treatment groups were as follows: saline or kisspeptin (0.10 nmol/kg or 0.05 nmol/kg). There was a difference in the responses from the second experiment, with both treatments exhibiting inconsistent results. In order to determine whether kisspeptin could induce cyclicity in anestrus mares, six mares (n=3 per group) were infused for 20 hours with either saline or kisspeptin [100 μg/hr iv (77 nmol/hr)]. There was no effect of kisspeptin on plasma LH or on ovulation. These data suggest that mares, unlike ewes, may be unable to respond to kisspeptin during seasonal anestrus. Thus, a bolus iv treatment (0.5 nmol/kg) of kisspeptin or saline was administered to five seasonally acyclic mares in a cross-over design. Acyclic mares responded to iv kisspeptin with a rise in plasma LH ($P<0.05$), but despite a 7-fold increase, this level failed to exceed 1 mg/ml. These data suggest a regulatory role for kisspeptin in cyclicity and seasonality of horses, but suggest that effects in long-day breeders like the mare differ from those in short-day breeders like the ewe.

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I. INTRODUCTION

The hypothalamic-pituitary-gonadal (HPG) axis has been well-studied in the equid, although the methods for clinically manipulating the HPG axis have remained somewhat limited. One critical area involves the synchronized and sustained release of LH from the anterior pituitary to begin the ovulation cascade during the breeding season. The release of LH is particularly important when it initiates the mare's first ovulation of the season, signaling the transition from winter anestrus to the period of reproductive cyclicity.

Several factors play a role in signaling the initiation and perpetuation of reproductive cyclicity in the seasonal female. Of these, photoperiod [1-8] and body condition [2, 6, 9-11] are undisputed. Other factors, such as temperature and environmental stressors, are gaining attention and appear to play a significant role in the events surrounding cyclicity [6, 12-16]. Regardless of the initiating factor or factors, the first step in the process of cyclicity is hypothalamic release of gonadotropin releasing hormone (GnRH), and factors influencing this release of GnRH remain poorly understood. From examining models of other species, it appears that a novel neuropeptide, kisspeptin, may be the central coordinator responsible for translating these signals to induce GnRH release, thereby initiating cyclicity [17-20].

Increasing length of daylight remains the single most important factor for initiating cyclicity in the mare [1-8]. Maximum and minimum hours of day length correspond to the summer and winter solstice, respectively. Although there is some degree of variability based upon breed of mare and latitude, North American mare populations generally reach peak fertility in the days surrounding the summer solstice, or June 21 [2, 21]. Therefore the physiologic breeding season, or ovulatory season, is generally considered to be May through September, when most mares will demonstrate estrus behavior and ovulate normally [2]. Driven by numerous breed registries, the operational breeding season begins February 15, a time when most mares in the northern hemisphere remain in winter anestrus or perhaps early transition [22].

Many breed associations consider January 1 as the universal birth date of all foals for registry purposes. The two largest registries in the United States are the American Quarter Horse Association (AQHA) and the Jockey Club, which is responsible for all Thoroughbred horses registered in North America. Because foals born earlier in the year often have a competitive advantage due to size and speed, this operational breeding season necessitates breeding mares during late anestrus and early transition which presents a unique set of challenges.

Although many treatment protocols exist that attempt to hasten seasonal transition, these regimens commonly require several weeks of costly therapy and results are inconsistent. Estrus periods are often prolonged, ovulations are unpredictable, and pregnancy rates are less than desirable compared to the physiologic breeding season [22].

The ability to establish an early breeding season is of clinical interest, hence the need to further explore seasonal changes and develop more reliable alternatives for

inducing cyclicity outside of the physiologic breeding season. Understanding the role of kisspeptin in the mare will likely prove to be a critical control point in our ability to regulate cyclicity and seasonal transition and increase reproductive efficiency. The potential to improve efficiency has obvious benefits to veterinarians, owners and managers, including more timely breeding, more efficient stallion use, increased pregnancy rates, and a higher percentage of live foals born earlier in the calendar year.

The objectives of this study were 1) to determine if there is an LH response to intravenous (iv) kisspeptin administration in the mare during the physiologic breeding season, 2) to determine if a constant rate infusion of kisspeptin can stimulate LH release sufficient to induce ovulation outside of the natural breeding season, and 3) to determine if the mare is capable of responding to an iv bolus dose of kisspeptin during seasonal anestrus. This work has shown that diestrus mares are capable of responding to iv kisspeptin with a subsequent rise in plasma LH concentrations. Constant rate iv infusion during winter anestrus did not alter plasma LH concentrations and was not associated with induction of ovulation or luteinization of any ovarian follicles. Acyclic mares responded to an iv bolus dose of kisspeptin with a rise in plasma LH concentration, but the response was greatly diminished compared to that seen during the natural breeding season.

II. LITERATURE REVIEW

Industry Basis for Early Breeding

In 1833, the English Jockey Club adopted the universal birth date of January 1 for all Thoroughbreds in the Northern Hemisphere, replacing the original birth date of May 1 [23]. This resolution was apparently made to alleviate administrative encumbrances, without regard for the seasonal physiology of the mare. In hindsight, this decision can easily be seen as unfortunate, but in a sport rich in history, this time-honored practice is difficult to overcome. Since its formation in New York City in 1894, The Jockey Club has followed English tradition and considers January 1 the universal birth date for all Thoroughbred foals born within a calendar year in the United States [24].

The American Quarter Horse Association, the largest breed registry in the United States, reported 117, 830 new registrations for 2007, the majority of which were foals [25]. Responsible for all Thoroughbred records in the United States, The Jockey Club boasts that their foal crop has remained steady over the last several years, averaging just over 37,000 new foals per year [24]. These are only two breed registries that recognize January 1 as the universal birth date for all foals, but they are representative of the financial impact on the industry in terms of number of foals per year. Many other

breed associations have since followed suit, placing intense economic pressure on the equine breeding industry to produce foals born early in the calendar year.

The majority of breeding attempts occur very early in the year with a goal of producing a foal that is born as early as possible after January 1. Because foals born in January, February and March are usually larger, stronger, and faster than those born later in the year, they are generally considered to be more competitive and therefore more desirable. These early-born foals command a premium, thereby encouraging the industry to produce foals earlier than the physiologic breeding season of the mare would ordinarily allow. Most clinicians see this universal birth date as an unfortunate administrative decision that has become the unrealistic basis for the equine breeding season [21].

Reproductive Physiology and Seasonality of the Mare

During winter anestrus, concentrations of sex steroids and gonadotropins in the peripheral circulation of the mare are low. Because the stimulatory effects of increasing day length on the reproductive cycle of the mare were well-documented [1-8], it was hypothesized that the pineal gland, known for processing photoperiodic information in many species, was integral to the hormonal feedback mechanisms responsible for initiating cyclicity. Pineal activity was found to be inversely related to the number of mares that were ovulatory at the time of sampling, confirming the antigonadal activity of the pineal gland and melatonin in the mare [5]. Released during hours of darkness, melatonin has an inhibitory effect on the HPG axis of the mare. This inhibition is

released as day length increases, and follicle stimulating hormone (FSH) is secreted which stimulates ovarian follicular development. As follicles grow, increasing amounts of estrogen are produced by the developing follicle, leading to manifestation of behavioral estrus [2].

As mares transition from winter anestrus towards cyclicity, they enter a period often referred to as anovulatory receptivity. This term is used to describe the receptive, or estrus, behavior of the mare towards the teaser stallion, associated with a lack of ovulation during this estrus period [21, 26, 27]. Rather than lasting for 5-7 days, as expected during the physiologic breeding season, estrus behavior during the transition period often persists for more than 10 days, and sometimes up to several weeks. Follicles develop, but often persist, luteinize, or undergo atresia more commonly than they proceed through normal ovulation. It is not possible to predict with any degree of accuracy which follicles will culminate in ovulation, and when ovulation might occur during this time resulting in immense frustration for owners and veterinarians and a great deal of wasted effort [21, 26, 27]. In the Thoroughbred industry, where breeding must be accomplished via live cover, this unpredictable transition period also necessitates a great deal of inefficient stallion use.

Without intervention during vernal transition, mares typically develop two to three follicular waves prior to ovulation of a dominant follicle [2]. It is generally thought this occurs because of inadequate stores of pituitary LH necessary to induce ovulation [28]. Both FSH and LH concentrations in the peripheral circulation are influenced by photoperiod, with greater activity of both gonadotropins occurring during the physiologic breeding season [29]. While concentrations of FSH in the anterior pituitary did not

appear to be affected by season, pituitary LH concentrations vary greatly with respect to season [28]. Many groups have shown that both peripheral and pituitary concentrations of LH are much lower in seasonally acyclic mares [28-31]. Concentrations of GnRH in the equine hypothalamus also vary significantly and were lowest during winter anestrus. Interestingly, there was no seasonal change in the concentration or number of GnRH receptors in the anterior pituitary [28].

Current Therapies for Seasonal Transition

Existing therapies to hasten the onset of cyclicity in the mare are aimed at altering photoperiod or targeting the effects of photoperiod on the HPG axis. Common therapies include artificial lighting, dopamine receptor antagonists, and treatment with GnRH [22]. Artificial lighting can effectively truncate winter anestrus and shift the process of vernal transition to an earlier time of year [8]. This was accomplished as early as 1947 by Burkhardt et al, and remains a widely used method [8]. However, providing added day length is costly in materials, labor and electricity and must be continued for several weeks to achieve desired results. One of the most popular protocols for artificial lighting begins at least eight weeks prior to the desired, or operational, breeding season by increasing the hours of light by 30 minutes per week until a total of 15 hours of light per day is provided [27].

Dopamine receptor antagonists such as sulpiride and domperidone are effective at shortening the transition period, and appear to be more effective when used in combination with artificial lighting. These treatments are both similar in cost and

recommended treatment regimen, i.e. daily administration until ovulation occurs. When used in combination with photostimulation, typical treatment regimens for dopamine antagonists range from 2-4 weeks [22]. Sulpiride is available through compounding pharmacies in the United States and is administered as an intramuscular (im) injection once or twice daily, whereas domperidone is available in a commercial preparation for once daily oral administration (Equidone®, Equi-Tox Pharma, Central, SC). The mechanism for the efficacy of dopamine antagonists in hastening the onset of cyclicity is not completely understood, but an interaction with prolactin is likely. In mares, seasonal prolactin concentrations fluctuate and are directly correlated with photoperiod, temperature, and changes in hair coat [3, 30, 32]. Endogenous prolactin concentrations can be increased in the mare by artificially extending day length during naturally short photoperiods [32]. Both sulpiride and domperidone increase prolactin concentrations in peripheral circulation [33-36]. It is not clear whether this prolactin increase primarily impacts the HPG axis on a central level or alters sensitivity of gonadotropin receptors on the ovary [37]. Dopamine appears to provide tonic inhibition to the HPG axis during anestrus and it is possible to hasten cyclicity by the removal of this inhibition through the use of dopamine antagonists [33].

A third treatment to shorten the transition period in mares utilizes multiple injections of GnRH. Protocols of repeated small doses of GnRH in hypothalamo-pituitary-disconnected sheep increased pituitary LH stores by increasing both synthesis and accumulation of LH in the anterior pituitary [38]. Also, low-dose GnRH infusion to luteal phase women caused a significant increase in their LH-responsiveness to a subsequent GnRH bolus [39]. Twice daily im injections of native GnRH to anestrous or

transitional mares achieved ovulation in 79% of mares in 13.7 +/- 7.4 days [40]. It should be noted that many confounding variables existed, as this was a retrospective study based upon mares on seven farms that had received the GnRH treatment both with and without ovulation induction agents. Collectively, the current protocols to hasten seasonal transition are costly in time, labor, and medication and produce inconsistent, unreliable results.

Discovery of Kisspeptin

Kisspeptins were originally discovered in 1997 by Lee and Welch while conducting oncology research at the Pennsylvania State University College of Medicine. The goal of the project was to examine metastasis suppression in melanoma and breast cancer cells. Lee and Welch observed that certain cell lines metastasized more readily than others, and that these cell lines had decreased expression of a metastasis suppressor gene, later designated as KiSS-1 [41, 42]. The gene was given its name because of its location on chromosome 1 and because the college is located in Hershey, PA, home to the popular chocolate treat, the Hershey's Kiss. Peptide products of the KiSS-1 gene are referred to as kisspeptins and the endogenous circulating form, kisspeptin-54, is also known as metastin because of its anti-metastatic properties [41, 42]. This exciting discovery fostered great interest in the field of oncology, but it was six years before the field of reproduction learned of this peptide.

Reproductive research leading to the discovery of kisspeptin initially focused on its ligand, the G protein-coupled receptor 54 (GPR54). The model for these studies was a

condition called Idiopathic Hypogonadotropic Hypogonadism (IHH) [43]. In 2003, three independent teams described that this condition was associated with pathology of the GPR54 receptor [43-45]. Seminara et al [43] observed that patients with IHH had a receptor mutation that directly affected GPR54. In humans and mice lacking function of GPR54 there seemed to be an inability to drive LH and FSH release. However, when IHH patients were given exogenous GnRH, a normal gonadotropin response followed [43]. Furthermore, the hypothalamic production of GnRH in the knock-out mice was unaffected by the GPR54 mutation, ruling out a mechanism directly linked to GnRH synthesis. This study led researchers to postulate that the GPR54 receptor is a key player in regulating the HPG axis and may function as a direct releasing mechanism for GnRH [43].

Prior to Seminara's work with IHH, the GPR54 receptor had been labeled an "orphan receptor" because, like many G protein-coupled receptors, its ligand and function were not known. In 2001, three independent laboratories identified the ligand for this receptor and kisspeptins were recognized as natural, high-affinity ligands for GPR54 [46-48]. Therefore, when Seminara's group discovered the function of GPR54 in the HPG axis in 2003, the ligand was already known and the reproductive world was introduced to kisspeptin and its receptor GPR54.

Role of Kisspeptin in the Placenta

During 2003, Bilban et al identified kisspeptin as a key regulator of placental trophoblast cells in humans [49]. The following year, Terao et al published work identifying expression of the KiSS-1 gene in rodent placenta [50]. The anti-metastatic properties of kisspeptin are thought to limit the invasion of placental trophoblasts into uterine tissues during pregnancy. Concentrations of kisspeptin, or metastin, in peripheral circulation of women were measured and increased dramatically during pregnancy, reaching third-trimester levels that were increased 7,000-fold compared to non-pregnant women, and then returned to non-pregnant levels within 5 days following parturition [51]. More recently, Smets et al examined the relationship of plasma metastin concentrations during the first trimester to delivery of small for gestational age (SGA) neonates. Peripheral metastin levels were significantly lower for SGA pregnancies than for those progressing normally [52]. Although our current study with mares does not examine kisspeptin during pregnancy, it is an important aspect of this literature review and relates to several areas of potential investigation in the equine species.

Localization of Kisspeptin in the HPG Axis

With regard to its role in the HPG axis, there is clear evidence to support the argument that the kisspeptin-GPR54 system is responsible for signaling GnRH release. First, the receptor has been co-localized on GnRH neurons [53, 54] and the presence of

kisspeptin in GnRH cells has been demonstrated [55]. Second, and equally as important, administration of kisspeptin has been shown to have a direct releasing effect on GnRH [54].

Co-localization of the GPR54 receptor on GnRH neurons was documented in the brain of the rat as recently as 2004. Irwig et al found that up to 77% of rat GnRH neurons co-expressed GPR54 mRNA [53]. This important piece of information supports the idea that GPR54 stimulation signaled GnRH release. In 2006, Pompolo et al. demonstrated the presence of kisspeptin in GnRH cells in the ovine brain [55], adding another piece to the puzzle. As recently as 2008, the hypothalamus of the mare was examined for the presence of this peptide and kisspeptin immunoreactive cell bodies were located in the arcuate nucleus (ARC) of the equine hypothalamus [56]. Close appositions of kisspeptin fibers and GnRH cell bodies were also found in this area, but only in small numbers. However, close appositions of kisspeptin fibers and GnRH fibers were found in large numbers in the median eminence (ME) of the equine brain [56]. To date, this remains the only peer-reviewed journal article examining the role of kisspeptin in the equine species.

Response of HPG Axis to Kisspeptin Administration

More interesting than demonstrating its presence in reproductive tissues is research which demonstrates the response to administration of kisspeptin. Response to exogenous administration of kisspeptin was determined in most experiments by measuring LH levels pre and post-treatment. Administration of this peptide has been

shown to induce release of GnRH and thus induce a significant rise in LH in mice [54, 57], rats [53, 58-62], hamsters [20, 63-65], monkeys [66, 67], ewes [68, 69], humans [70, 71], gilts [72], and cattle [73, 74]. Males as well as females respond to kisspeptin injection, and the response is rapid regardless of the route of administration, less than 15 minutes in most cases [17]. Investigating the dose-response further, a number of studies report that acyline, a GnRH antagonist, administered prior to kisspeptin blocked these stimulatory effects on LH, thereby isolating the action of kisspeptin to the level of the GnRH neurons [53, 57, 60, 63, 66, 67].

The fact that kisspeptin has been proven to induce an LH surge begs the question: Is kisspeptin capable of producing an LH surge sufficient to induce ovulation in cycling females? In studies currently published in peer-reviewed journal articles, ovulation has been successfully induced by the administration of kisspeptin to rats [60] and ewes [68].

In 2004, Matsui et al induced ovulation in female rats via subcutaneous administration of kisspeptin. This study utilized a group pre-treated with a GnRH antagonist and achieved results consistent with previous work (i.e. blockade of gonadotropin release) [60]. Matsui also examined c-Fos immunoreactivity in GnRH neurons and further confirmed that kisspeptin acts via this pathway. Subsequent studies by Kinoshita et al in 2005 confirmed that kisspeptin administration induces a GnRH/LH surge in female rats in the presence of estrogen. This stimulation of GnRH was isolated to the preoptic area (POA) in the rat and was suggestive of an endogenous release in this same location. Based on these results, Kinoshita hypothesizes that the arcuate nucleus

may be the target tissue for response to increasing levels of estrogen during proestrus. This would in turn stimulate kisspeptin release from the POA and the subsequent GnRH/LH surge that precedes ovulation [59].

Caraty et al successfully used kisspeptin to synchronize pre-ovulatory LH surges and ovulation in cyclic ewes [68]. They administered a constant rate iv infusion (0.48 μmol/hr for 8 hours), with LH beginning to rise within 2 hours following the start of the treatment. This infusion culminated in synchronized pre-ovulatory LH surges among the treated animals, whereas the LH responses in control animals were variable in magnitude and more widely dispersed with respect to time [68].

In addition to its role in GnRH/gonadotropin release, a local role for kisspeptin in the control of ovulation has been proposed. Castellano et al showed evidence of KiSS-1 mRNA expression on the ovary which fluctuates in a cyclic manner, increasing substantially at proestrus. This rise in expression was prevented by blocking the preovulatory LH surge and expression was restored by administration of exogenous LH in the form of hCG [75].

During the course of the current project, at least two groups attempted to induce ovulation in the mare using iv administration of kisspeptin and published these results as abstracts within conference proceedings. The first group used Welsh pony mares and administered a single iv dose of 10 mg/mare of human KP-10 (NeoMPS S.A., Strasbourg, France) and reported a reduced time to ovulation (2 +/- 1.3 days) compared to controls (4 +/- 1.5 days) [76]. Because 50% of the kisspeptin mares in this study ovulated within 24 hours of treatment, these researchers proposed the response might be partially due to a local mechanism at the level of the ovary as described by Castellano

[75]. In contrast, however, Magee et al saw no difference in the use of saline versus 1.0 mg rat KP-10 (KiSS-10, AnaSpec, Inc., San Jose, CA, USA) in the time to ovulation in normal light horse mares [77]. It was beyond the scope of our present study to examine ovulation induction in the mare.

The discovery of kisspeptin provides new opportunities for manipulation of the HPG axis in the mare, specifically as it relates to LH release. The period of seasonal transition is one of the most difficult times of the year for mares to be successfully bred, yet industry registration standards encourage breeding during this period.

Role of Kisspeptin in Seasonality

In 2007, Caraty et al examined how kisspeptin might be used to alter the reproductive seasonality of ewes [68]. They determined that kisspeptin infusion in anestrous ewes initiated LH release and subsequent ovulation outside of the normal physiologic breeding season. Acyclic ewes were administered kisspeptin as an iv infusion of 12.4 nmol/hr for either 30 hours (total of 0.38 μmol) or 48 hours (total of 0.6 μmol), and ovulation rates were 80% and 86% respectively for the two experiments [68]. It is important to recognize that constant rate infusion protocols were adopted following unsuccessful attempts utilizing multiple iv injections of kisspeptin (12.6 nmol every 12 hours for 5 days). The pulsatile injections of kisspeptin were sufficient to transiently elevate peripheral LH concentrations, but no subsequent LH surge was elicited from the pulsatile treatment [68].

During the past two years, and concurrent with our project, studies have been published examining the role of kisspeptin in the seasonality of hamsters. Like the mare, both Syrian and Siberian hamsters are seasonally polyestrous and resume cyclicity as photoperiod increases. Recent work by Mason et al examining kisspeptin-induced LH response in female Siberian hamsters showed a disparity in LH response between cyclic and acyclic females as well as a reversal in hypothalamic kisspeptin expression between long and short photoperiods [63]. This group challenged animals housed in long-day and short-day conditions with human KP-10, and the magnitude of response varied greatly between the two groups. Females exposed to extended photoperiod exhibited a marked LH rise, while females housed in short photoperiod failed to exhibit an LH rise. This is an interesting finding considering that this group reported different results for the male hamster of this species [20]. In 2007, Greives et al showed that male Siberian hamsters responded to human KP-10 with a subsequent rise in LH, and that this response was independent of photoperiod. Males of a different hamster species (Syrian) housed in short photoperiod responded to constant infusion of KP-10 as evidenced by a recrudescence in reproductive activity (testosterone and testicular weight) despite the decreased day length [65]. However, this attempt at reproductive recrudescence in males was repeated using the previous species of hamster (Siberian) and achieved negative results [64]. Aside from species, other differences exist between the positive and negative results in the two experiments including dose and route of administration of CRI [1 mM intracerebroventricularly (icv) and 10,000 mM subcutaneously (sc)], making it difficult to discern whether the results are truly the result of a species-specific response

[64, 65]. This initial research using the hamster model will serve as a potential model for comparison of future equine studies examining the role of kisspeptin in seasonality of this species.

Objectives of Current Study

It is reasonable to hypothesize that kisspeptin may potentially be used to release LH in the equid, particularly for hastening seasonally anestrous mares into earlier cyclicity. The goals for this study were 1) to determine if the cyclic mare will respond with an LH rise following challenge with iv kisspeptin during diestrus, 2) to determine if an iv constant rate infusion during anestrus would produce an LH rise and induce ovulation, and 3) to determine if the mare will respond with an LH rise following an iv bolus dose of kisspeptin during seasonal anestrus.

III. MATERIALS AND METHODS

Experimental animals

The investigation was conducted at the J.T. Vaughan Large Animal Teaching Hospital at Auburn University in Auburn, AL (latitude 32°36'35"N, longitude 85°28'51"W) [78]. Mares were various light horse breeds selected from the Auburn University teaching herd. The mares had a history of normal cyclicity, no known reproductive pathology and ranged in age from 5 to 18 years [13.2 +/- 4.3 (SEM) years]. All mares were housed in paddocks, offered *ad libitum* coastal Bermuda hay and water, and exposed to natural temperature and photoperiod. Intravenous catheters were placed and the mares were moved from paddocks into stalls to facilitate sampling and maintain the integrity of the iv catheters. All experiments were approved by the Auburn University Institutional Animal Care and Use Committee.

Experiment 1a: LH response within the physiologic breeding season

During June and July, estrous cycles were synchronized in ten mares using an intramuscular (im) dose of 10 mg prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$, dinoprost tromethamine, Lutalyse®, Pfizer Animal Health, Exton, PA) which was repeated 14 days later. To

confirm the date of ovulation, all mares were followed by daily teasing, palpation per rectum, and ultrasonography. Six mares with the greatest synchrony in their cycles were chosen for study. These mares were sampled on a single day during mid-diestrus, and their diestral status was confirmed via teasing, palpation per rectum, ultrasonography, and progesterone concentrations both prior to and on the days of sampling.

Jugular iv catheters were placed on the day of sampling (4 hours prior to the first sample) and mares were randomly assigned to one of three treatment groups as follows, with 2 mares in each group: kisspeptin 1.0 nmol/kg (KP-10, human metastin 45-54; Peptide Institute Inc., Osaka, Japan), kisspeptin 0.5 nmol/kg, or saline control. Doses selected for mares were based upon studies performed in ewes [69]. In 2005, Caraty treated ewes with doses of KP-10 ranging from 0.02 to 22 nmol/kg body weight (BW). Doses lower than 0.5 nmol/kg produced a less than optimal response in ewes as measured by LH levels in plasma. In this study, KP-10 doses chosen for mares were based upon this early work in ewes where an iv dose of approximately 0.5 nmol/kg achieved a maximal LH response [69].

Blood samples were taken from the iv catheter every 10 minutes for a period of one hour prior to treatment. The saline control or kisspeptin treatment was then administered iv in a 3 ml volume to each mare and blood sampling followed at intervals of 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180 minutes post-treatment. Plasma was stored at -20°C until assayed to determine LH concentration. All samples were assayed to determine plasma LH concentration and statistical analysis of LH levels

was accomplished using repeated measures ANOVA considering the effects of animal, treatment (dose), time, and sampling period (cycle 1, 2, or 3) using statistical analysis software (SAS) with PROC MIXED procedures [79].

These procedures were repeated for 3 consecutive estrous cycles so that by completion, each mare received each of the different treatments and served as her own control for comparison (n=6 for each treatment). The only exception to this protocol is that following the first round of sample collection, the diestral mares were given only one injection of $PGF_{2\alpha}$ to synchronize cyclicity.

Experiment 1b: LH response within the physiologic breeding season

There were no differences in LH concentrations between the mares treated with the KP-10 doses utilized in Experiment 1a, with both doses achieving what appeared to be a maximal LH response. Therefore, a second dose effect study was conducted. In Experiment 1b, the doses were lowered and mares were more closely synchronized to increase efficiency of sampling. Doses for Experiment 1b were as follows: 0, 0.1 nmol/kg, and 0.05 nmol/kg of KP-10. During August, nine mares were synchronized with daily im treatments of 150 mg progesterone and 10 mg estradiol 17-ß in oil (P&E; Progesterone 50 mg/ml and Estradiol 17-ß 3.33 mg/ml; Hagyard Pharmacy, Lexington, KY) for a period of 10 days [80]. A single im treatment of 10 mg $PGF_{2\alpha}$ (Lutalyse®, Pfizer Animal Health, Exton, PA) was administered on the last day of treatment and mares were examined daily with teasing, palpation per rectum, and ultrasonography beginning three days later. An ovulation induction agent, 3000 IU of human chorionic

gonadotropin (hCG; Novarel®, Ferring Pharmaceuticals, Inc., Suffern, NY), was administered iv to all mares nine days after removal of P&E treatment in order to achieve greater synchrony. Mares were examined daily until ovulation was detected. Six mares with the greatest synchrony were then sampled over three consecutive days in diestrus, starting on day 8 or 9 post-ovulation and continuing through day 10 or 11 post-ovulation. Mares were confirmed to be in diestrus via daily progesterone concentrations as outlined for the previous experiment. Sampling and statistical analysis then proceeded as described for Experiment 1a.

Experiment 2: Effect of kisspeptin infusion outside of the physiologic breeding season

This experiment was designed to determine whether kisspeptin infusion could stimulate increased LH concentration in peripheral blood and ovulation in seasonally anestrous mares. Six healthy light horse mares with no known reproductive pathology were confirmed to be in winter anestrus or early transition. This status was based upon lack of response to teasing, quiescent uterine tone and minimal ovarian activity on palpation per rectum, no detectable luteal tissue on ultrasonography, and plasma progesterone consistently less than 1.0 ng/ml prior to and on the day of sampling. Mares received iv catheters and were randomly assigned to 2 groups of 3 mares each, with one group receiving the treatment and the other group serving as the control. Mares in the treatment group received an iv constant rate infusion (CRI) of KP-10 in saline delivered at the rate of 100 μg/hr (77 nmol/hr) for a period of 20 hours. Mares in the control group received a CRI of saline for the same time period. This model was extrapolated based

upon studies which induced ovulation in seasonally anestrous ewes [68]. Blood samples were collected at 1, 2, 3, 4, 8, 12, 16, 20 hours after beginning the CRI. Once the CRI was discontinued at 20 hours, samples were taken every 30 minutes for the next 13 hours. Plasma samples were assayed to determine LH concentrations during and following the infusion. This data was analyzed using repeated measures ANOVA considering the effects of animal, treatment, and time using SAS software with PROC MIXED procedures.

Following 20 hours of infusion all animals were monitored with daily teasing, palpation per rectum, and ultrasonography for a period of 5 days to detect subsequent follicular activity and ovulation.

Experiment 3: Dose-response outside of the physiologic breeding season

Based upon the lack of LH response from Experiment 2, an experiment was designed to determine if seasonally acyclic mares were capable of responding to a single iv bolus of kisspeptin, similar to cyclic mares described in Experiment 1a. Five healthy light horse mares with no known reproductive pathology were confirmed to be in winter anestrus or early transition as described previously. These mares received iv catheters as outlined above and baseline samples were obtained every 10 minutes for 30 minutes prior to treatment. These five mares were randomly divided into two treatment groups, one group receiving KP-10 at a dose of 0.5 nmol/kg and the other group receiving a saline control. This dose was chosen based upon the LH response achieved in Exp 1 as the 0.5 nmol/kg iv dose elicited what appeared to be a maximal response. Sampling times post-

administration were the same as for Experiment 1 above. Mares were sampled on two consecutive days with the treatment groups being reversed the second day in a cross-over design and each mare serving as her own control.

A positive control was also used to insure that KP-10 from the same lot number was functional in this protocol. Cows (n=3), which are not seasonal breeders, were sampled during this same time period using both saline and 100 pmol/kg (0.1 nmol/kg) of KP-10, a dose previously shown to stimulate an LH response [74]. In addition, a healthy mare from the same herd (n=1), demonstrated to have normal cycles despite the short photoperiod, was sampled on the same days using both saline and 0.5 nmol/kg of KP-10.

All LH values were analyzed using repeated measures ANOVA as described for Experiment 1.

LH and Progesterone Assays

All blood samples were collected using EDTA and plasma was harvested, stored frozen at -20°C, and thawed immediately prior to assay to determine LH concentration (ng/ml). This assay was performed according to a published protocol [81] using reagents kindly provided by Dr. Don Thompson with equine LH standards provided by Dr. Janet Roser. The assay was validated for parallelism and quantitative recovery. Intra- and interassay coefficients of variation (CVs) are listed in the results for each experiment.

To confirm cyclicity status of mares, blood was collected as described and plasma harvested to determine progesterone concentrations using the Coat-a-Count®

Progesterone Radioimmunoassay (RIA) kit; Siemens, Los Angeles, CA. This kit has been proven as a reliable method of progesterone quantification in the mare and used often for this purpose [36, 82-84].

IV. RESULTS

Experiment 1a: LH response within the physiologic breeding season

Normal, cyclic mares in diestrus were administered KP-10 at 0, 0.5 nmol/kg, and 1.0 nmol/kg. All treated mares appeared to respond at the time of treatment with a rapid rise in plasma LH, and both doses of KP-10 achieved a similar response (Figure 1). Statistical analysis revealed a treatment by time interaction ($P<0.03$). Analysis of individual means indicated that KP-10 (0.5 nmol/kg) increased LH at 20, 25, 30, 40 and 50 minutes post-treatment ($P<0.05$) compared to saline-treated mares. The 0.5 nmol/kg dose also induced a significant change ($P<0.05$) for the entire sampling period post-treatment (5-180 min) when compared to the baseline sample for this dose. The 1.0 nmol/kg dose did not induce a statistically different response from control at any individual time point. This dose did, however, induce an increase ($P<0.05$) in LH concentrations from 10 min through 150 minutes post-treatment compared to its pre-injection sample. Intra- and interassay CVs for Experiment 1a were 7.7 and 11.5%, respectively.

Incremental area under the curve (IAUC) was significantly different for both doses in this experiment compared to saline-treated controls ($P=<0.05$). This data is depicted in Figure 2.

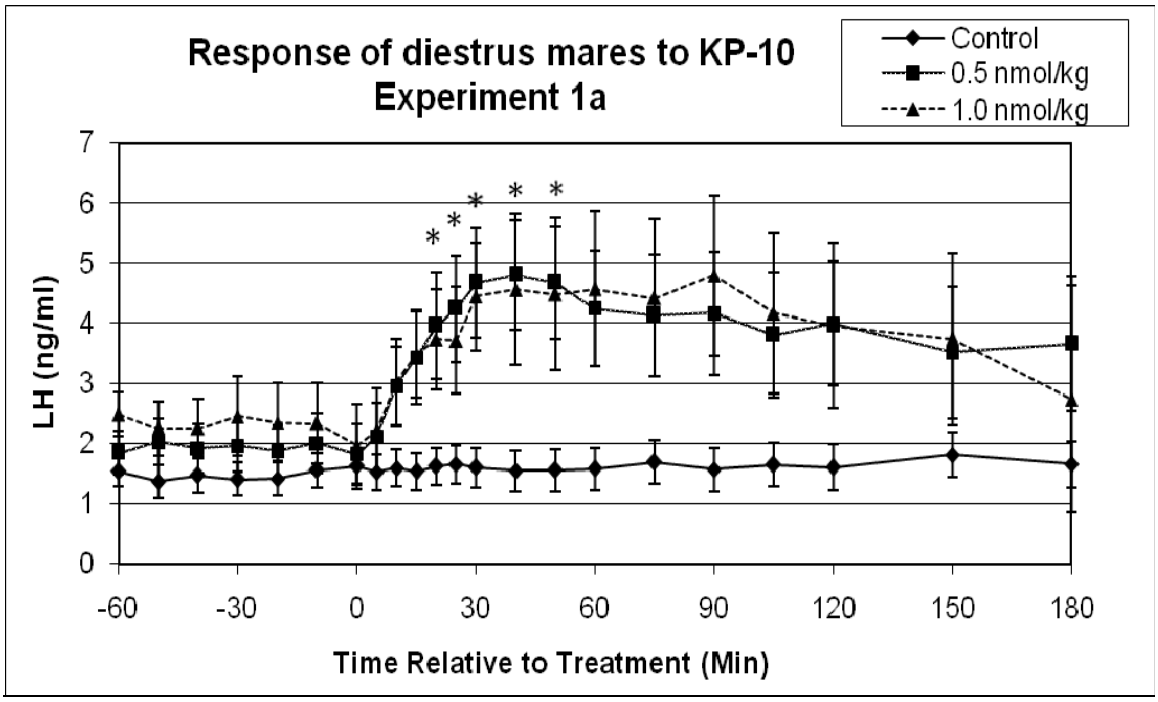


Figure 1: Depicts least squares (LS) means for mares at individual time points prior to and following treatment with KP-10 (initiated at Time 0) at doses of 0, 0.5 or 1.0 nmol/kg. Asterisks represent individual time points of significant difference ($p<0.05$) between 0.5 nmol/kg dose and control. Data represent LS means ± standard error of the mean (SEM).

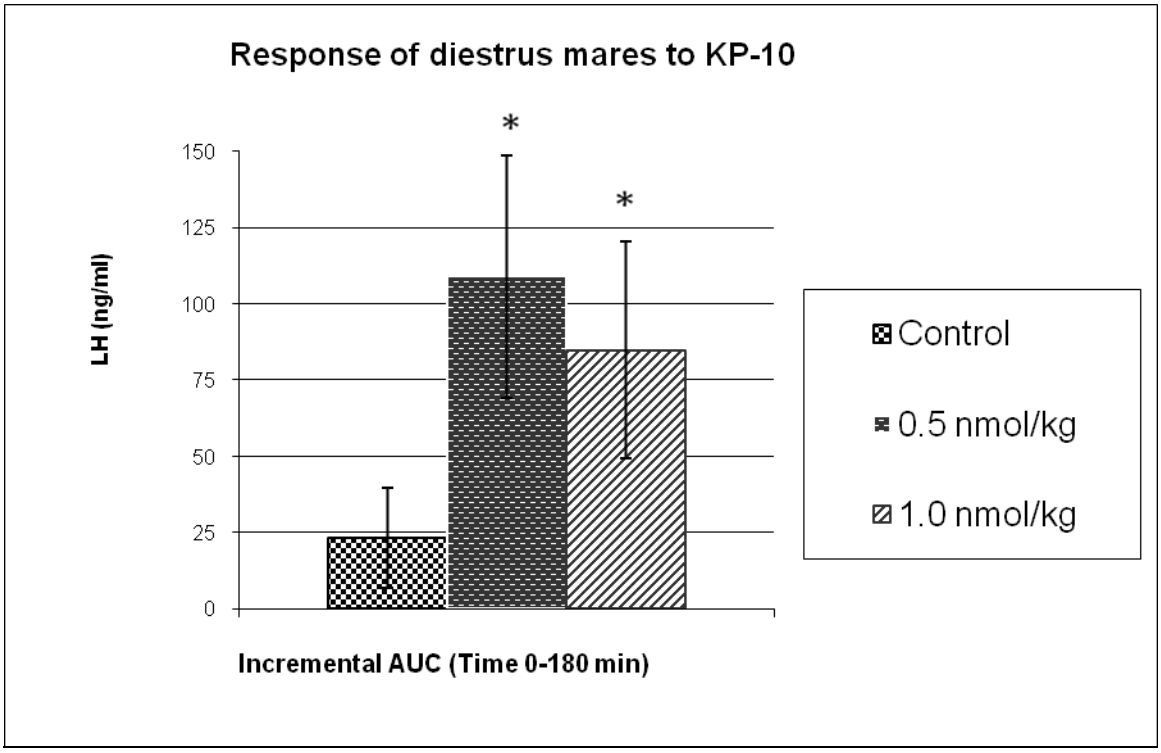


Figure 2: Incremental AUC for all 3 treatments from time 0 (treatment) to 180 minutes post-treatment. Asterisks represent difference ($P<0.05$) between treatment and control.

Experiment 1b: LH response within the physiologic breeding season

Because there was no effect of dose in the previous experiment, the procedure was repeated using lower doses of 0, 0.1 nmol/kg and 0.05 nmol/kg of KP-10. Although there was a rise in plasma LH beginning at the time of treatment, the response was inconsistent compared to the doses used previously in Experiment 1a. Statistical analysis revealed an effect of treatment by time (P=0.01). There was an individual difference between the control and 0.05 nmol/kg treatment at 25 minutes post-treatment (P<0.04), and a trend was present (P<0.1) between these treatments with the 0.05 nmol/kg dose increasing LH concentrations from 30 to 75 minutes post-treatment. No individual differences occurred between the 0.1 nmol/kg treatment and controls, indicating that this dose was unable to elevate LH levels more significantly than controls at any individual time point. However, with respect to its baseline sample, the 0.1 nnmol/kg dose was significantly different and was increased for all points from 10 to 60 minutes post-treatment. Similarly, the 0.05 nmol/kg dose was significantly different at all points from 15 to 75 minutes post-treatment with respect to its baseline sample (Figure 3). Intra- and interassay CVs for Experiment 1b were 7.4 and 14.3% respectively.

Area under the curve analysis for Experiment 1b also revealed differences between controls and the 0.05 nmol/kg treatment. Incremental AUC (IAUC) for the 0.05 nmol/kg dose from 0-180 minutes post-treatment was significantly increased (P=0.02) compared to saline-treated controls, as depicted in Figure 4. Despite this difference, it appears that iv doses of KP-10 less than 0.5 nmol/kg produced an inconsistent response.

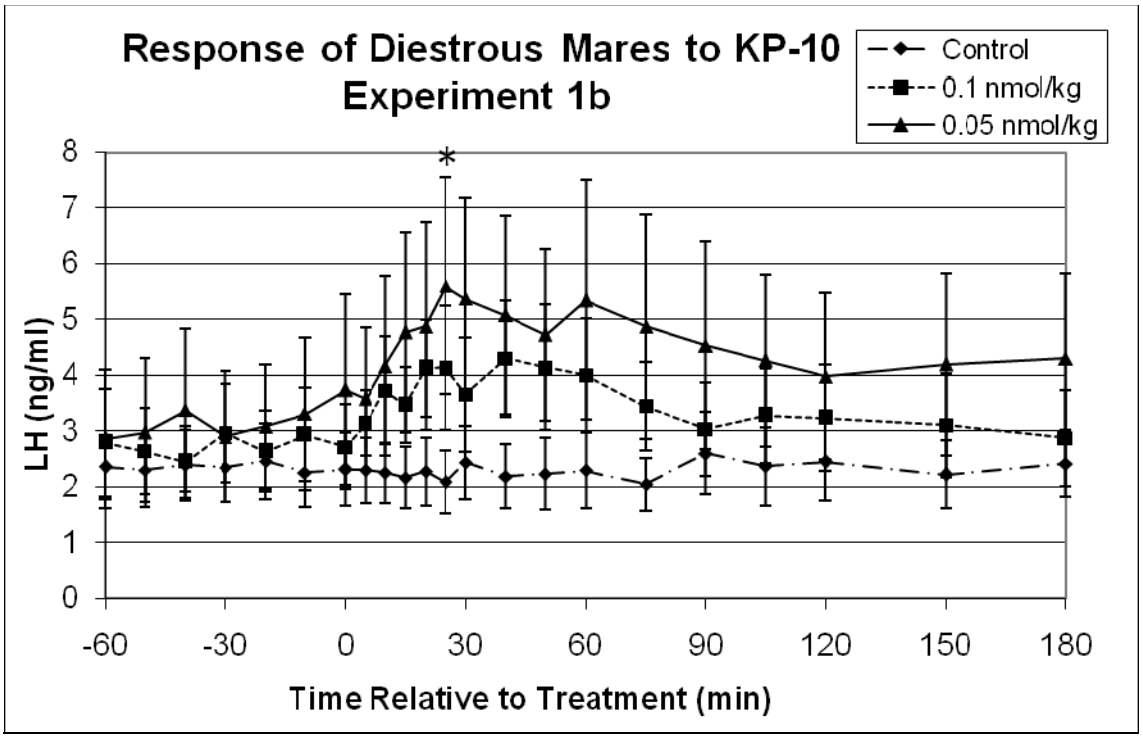


Figure 3: Depicts LS means for mares at individual time points prior to and following treatment with KP-10 (initiated at Time 0) at doses of 0, 0.05 or 0.1 nmol/kg. Asterisks represent individual time points of significant difference ($p<0.05$) between 0.05 nmol/kg dose and control. Data represent LS means ± SEM.

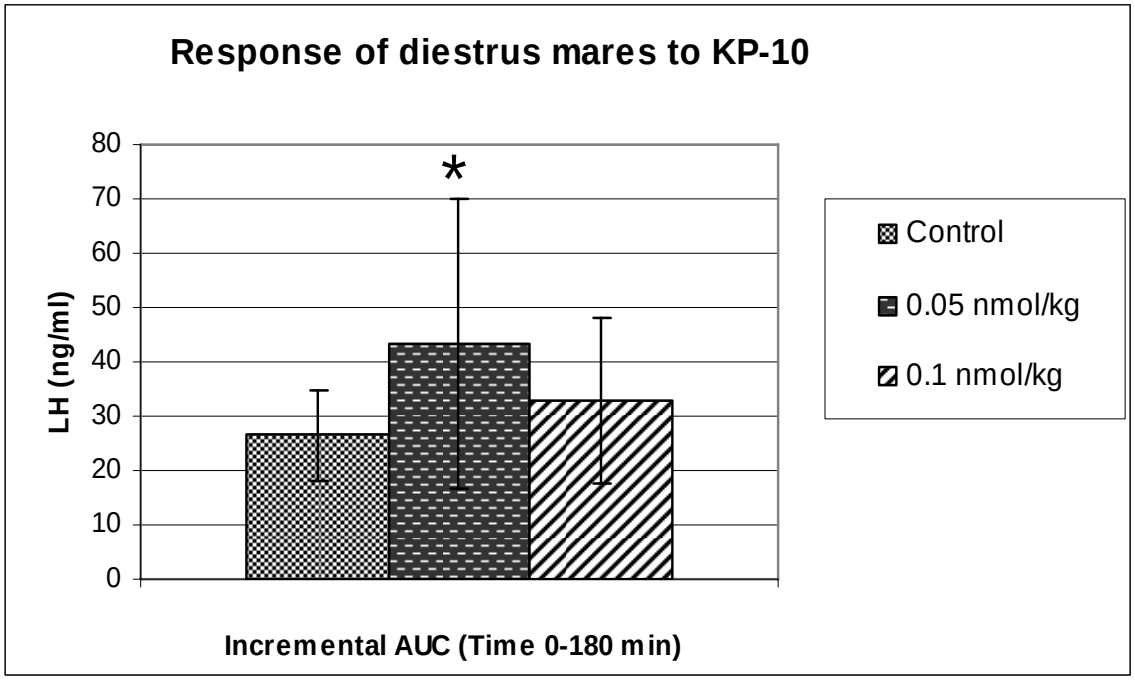


Figure 4: Incremental AUC for all 3 treatments from time 0 (treatment) to 180 minutes post-treatment. Asterisk represents difference ($P<0.05$) between 0.05 nmol/kg treatment and control.

Experiment 2: Effect of kisspeptin infusion outside of the physiologic breeding season

A CRI of 100 μg/hr (77 nmol/hr) of KP-10 or saline was administered iv to seasonally acyclic mares for a period of 20 hours. Plasma LH concentration did not change in any of the mares from 0 to 33 hours following the start of the 20-hour infusion (Figure 5). Intra- and interassay CVs for Experiment 2 LH assay were 9.1 and 10.9% respectively.

No pre-ovulatory follicles developed on the ovaries of any mare from 0-5 days post-infusion. In addition, there was no evidence of ovulation or luteinization of any follicle based upon these serial ultrasonographic exams and subsequent progesterone levels of <1.0 ng/ml.

Experiment 3: Dose-response outside of the physiologic breeding season

Following the lack of LH response with CRI treatment in acyclic mares, it was hypothesized that mares may not be capable of responding to KP-10 injection during this quiescent reproductive state. Acyclic mares were administered doses of 0 and 0.5 nmol/kg, a dose previously shown to elicit substantial LH release during the physiologic breeding season (Experiment 1a). Response to treatment differed by dose (P=0.04) and time (P<0.0001), with the 0.5 nmol/kg dose significantly increasing LH concentrations compared to saline-treated controls. Individual means differed from 15 to 60 minutes post-treatment (Figure 6). For the LH assay in Experiment 3, the intra- and interassay CVs were 7.4 and 13.7% respectively.

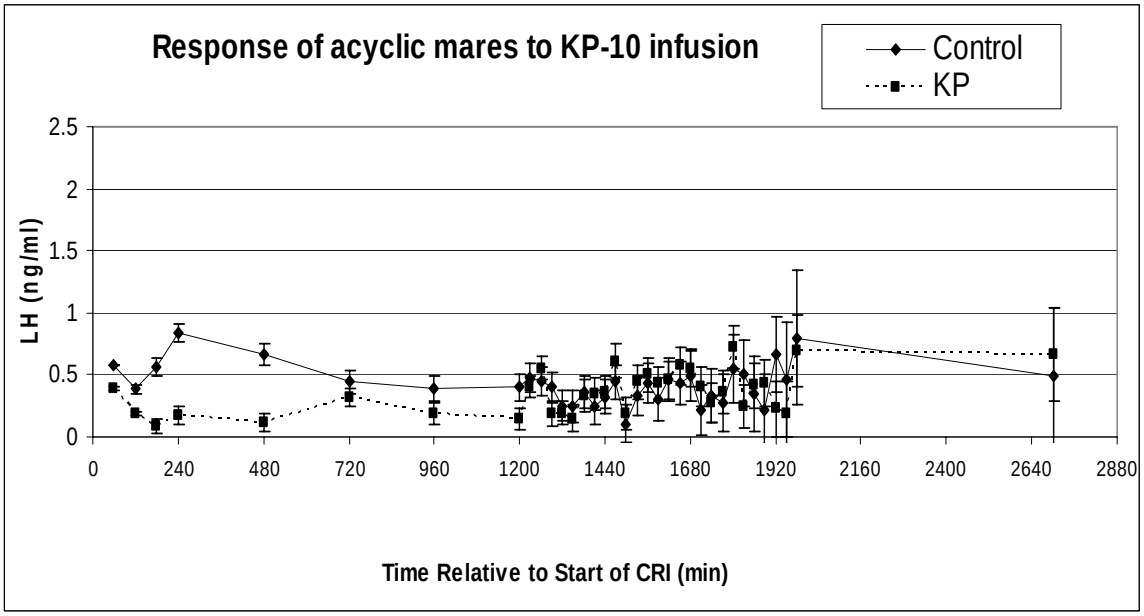


Figure 5: Effect of saline or KP-10 iv constant rate infusion on LH response in acyclic mares [100 μg/hr (77 nmol/hr) for 20 hrs] as described for Experiment 2.

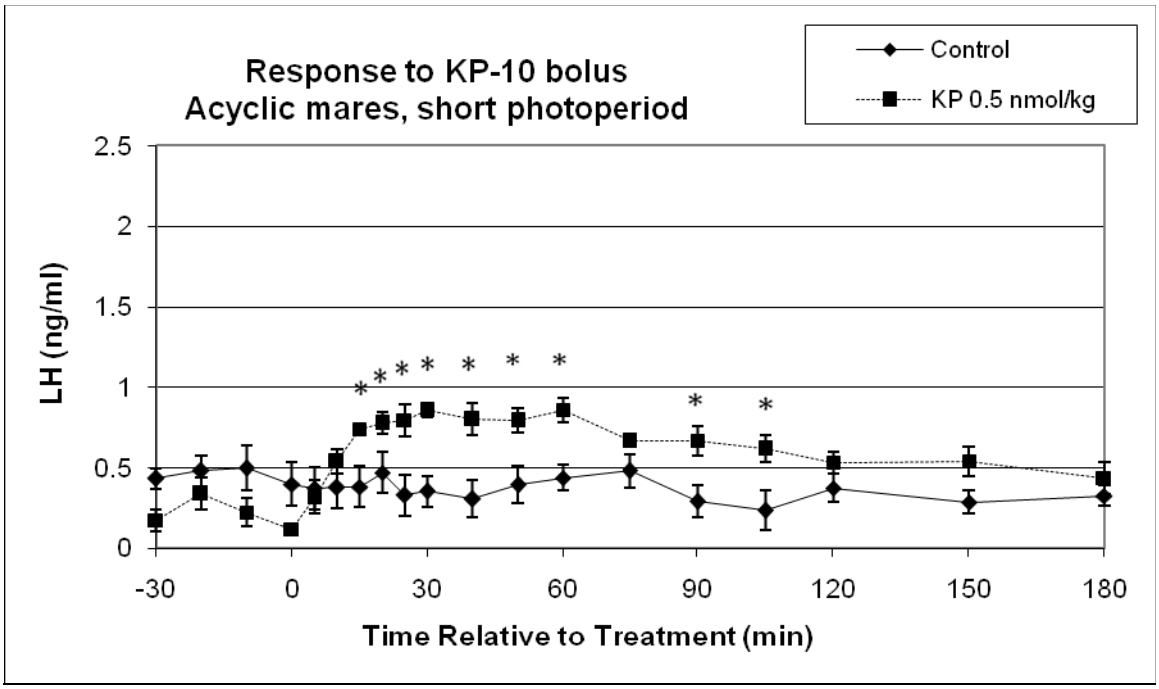
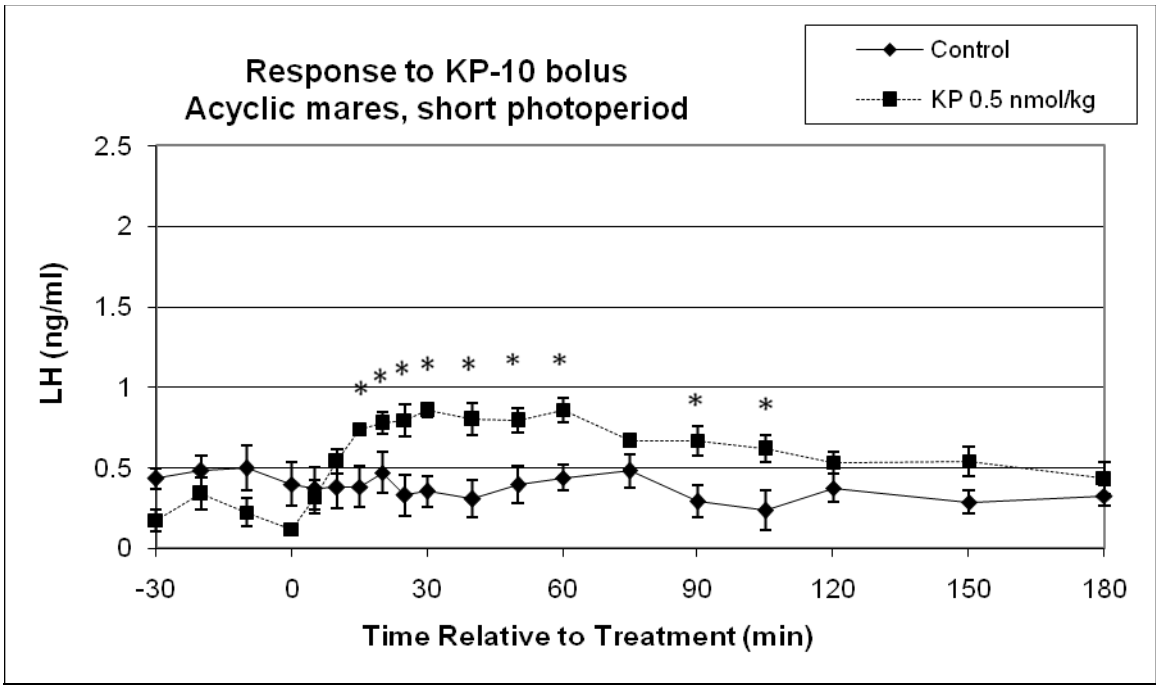


Figure 6: Depicts LS means for plasma LH response in acyclic mares (n=5) using 0.5 nmol/kg of KP-10 administered iv at Time 0. Asterisks represent individual time points of significant difference ($p<0.05$) between 0.5 nmol/kg dose and control. Data represent LS means ± standard error of the mean (SEM).

Bovine controls sampled on the same day performed as expected with a predictable LH rise in response to a bolus injection of KP-10 from the same lot number (Appendix A). In addition, one normal diestrous mare confirmed to be cyclic despite the short photoperiod was also used as a positive control. This mare responded with an LH rise as expected based upon results from Experiment 1a and is depicted in (Figure 7) for comparison. (Note the change in scale of *y*-axis between upper and lower panels in Figure 7.)

Incremental AUC for the LH response to KP-10 differed in Experiment 3 as well (P=0.0092), with the dose tested (0.5 nmol/kg) showing increased IAUC compared to saline-treated controls. Though there was significant treatment effect of KP-10 outside of the physiologic breeding season, the magnitude of LH response was greatly diminished compared to that seen during the natural breeding season (Figure 8). Average LH concentrations for cyclic mares approached 5 ng/ml at peak response, which is consistent with pre-ovulatory levels preceding the first ovulation of the year. Conversely, peak LH values in acyclic mares remained less than 1.0 ng/ml. despite a 7-fold increase from baseline.



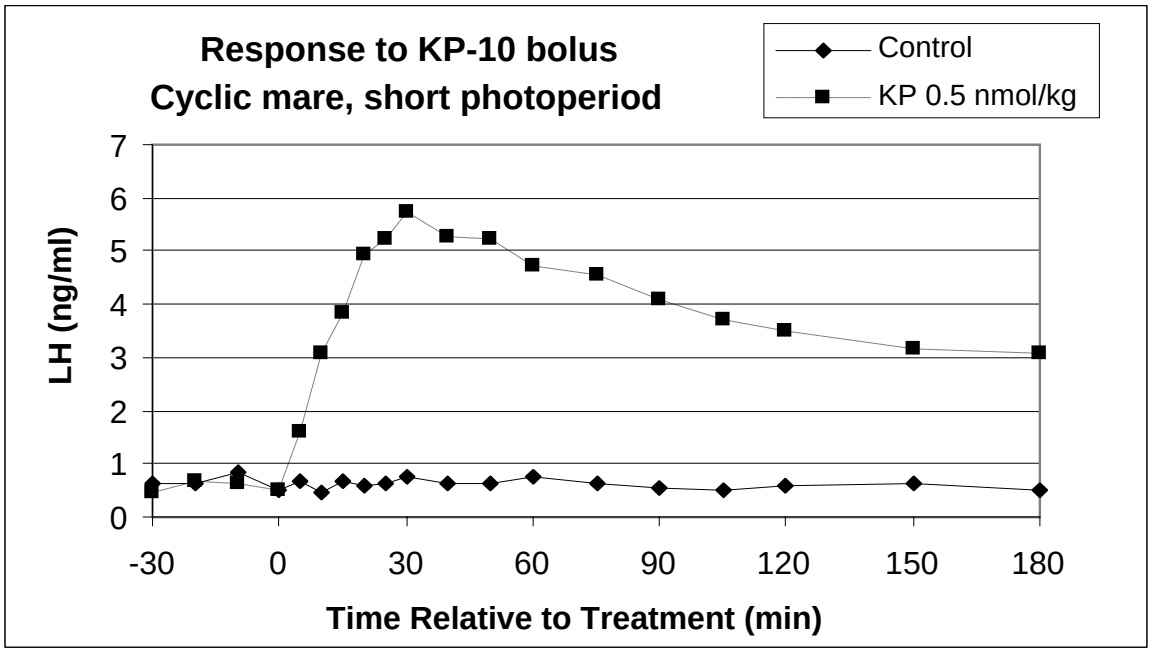


Figure 7: Upper panel depicts LH response in acyclic mares as seen in Figure 5 for comparison with positive control depicted below. Lower panel represents LH response in a cyclic mare (n=1) sampled on the same days using the same dose as described for Experiment 3.
**Note the change in scale of the y-axis between the two graphs*.

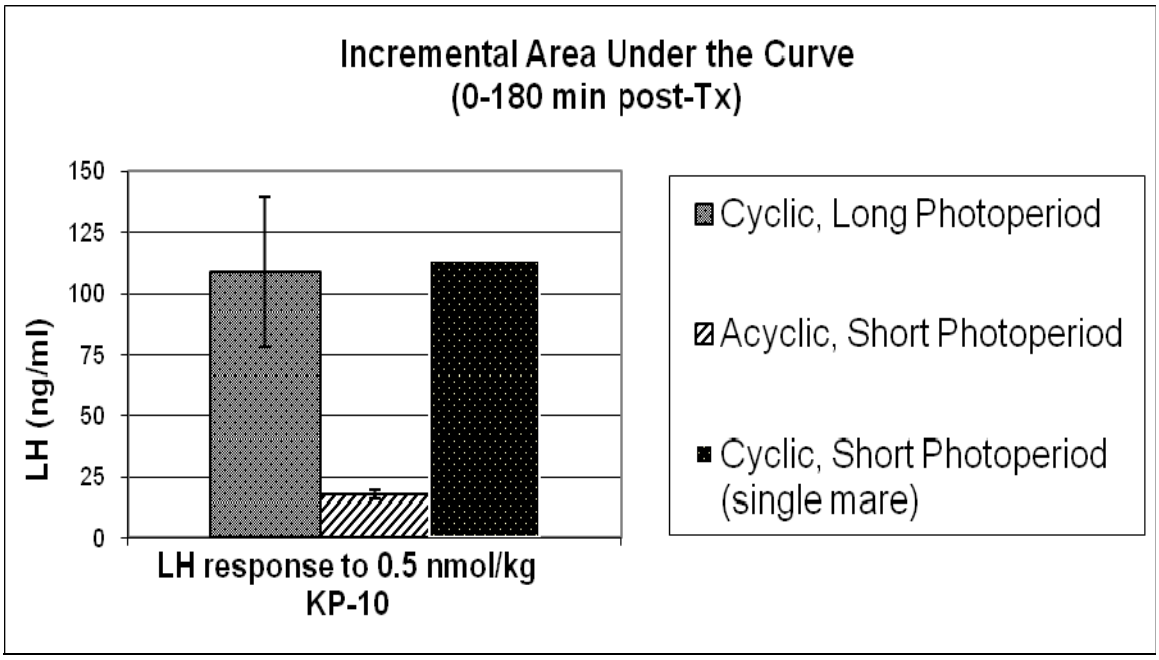


Figure 8: Incremental AUC (IAUC) for LH response following iv administration of 0.5 nmol/kg dose of KP-10 to cyclic mares (calculated from data in Figure 1) vs. acyclic mares (calculated from data in Figure 5). Also depicted is IAUC data from a single mare that was demonstrated to be cyclic despite the short photoperiod (sampled at the same time as the acyclic mares).

V. DISCUSSION

Based upon the aforementioned studies in other species, it is not surprising that the mare responded to kisspeptin administration with a subsequent rise in plasma LH. However, unlike the cow [74] or sheep [68, 69], fairly high doses of KP-10 were required to produce a repeatable LH response in the mare. Although beyond the scope of this study, this implies differing receptors, receptor density, receptor up-regulation, metabolism, half-life or sites of action within the hypothalamus compared to other species.

Induction of Ovulation

Armed with this information regarding the LH response to KP-10, it seems that a logical clinical use for this product would be as an ovulation-induction agent in the mare. The current products used for this purpose are human chorionic gonadotropin (hCG), which has LH activity in the mare, and the GnRH analog deslorelin. Although effective as ovulation induction agents, antibody formation following administration of hCG [85] and lack of commercially available deslorelin preparations in the USA make these products less than ideal. The magnitude of the LH response achieved with kisspeptin in our experiments appears comparable to endogenous pre-ovulatory LH levels, particularly

those levels that precede the first ovulation of the year [1]. Classic work by Freedman, Garcia and Ginther showed that the magnitude of the LH rise (ng/ml) continued to climb progressively higher as the season advanced, likely influenced by ovarian steroids and environmental factors [1]. Although the magnitude appears adequate, the duration of the resulting LH response will likely hamper efforts to induce ovulation in the mare with kisspeptin. The mare differs from many species in her requirement for a sustained release of LH rather than a 12 to 24-hour surge [2]. In this study, it was evident that plasma LH levels were beginning to decline by three hours post-treatment, indicating that multiple injections or perhaps a sustained release formulation may be useful to achieve ovulation induction in the mare using kisspeptin.

As mentioned previously, two groups [76, 77] have attempted ovulation induction in the mare using kisspeptin and achieved different results. Although it was beyond the focus of this study to induce ovulation in cyclic mares, using kisspeptin in this manner appears to hold a great deal of potential and certainly warrants further investigation.

Disparity of Response Between Seasonal Females

Most interesting in the present study is the change in the magnitude of LH response associated with reproductive status (cyclic vs. acyclic) illustrated in Figure 6. Although the acyclic mares in Experiment 3 responded to the 0.5 nmol/kg iv dose of KP-10 with a significant rise in LH, the degree of the response was much less than that seen in diestrous mares during the physiologic breeding season, as previously contrasted in Figure 7. This is consistent with classic mare cyclicity studies which showed that LH in

the periphery and pituitary is present in much lower concentrations in seasonally acyclic mares [28-31]. Although several factors are involved, many clinicians and researchers believe that it is inadequate pituitary stores of LH which prevent the mare's progression from transition to cyclicity any earlier in the year. It has been suggested that kisspeptin given at optimal doses exhausts pituitary stores of LH, as evidenced by the inability of a subsequent GnRH injection to further stimulate LH levels [72]. Pituitary exhaustion induced by kisspeptin seems to be a reasonable explanation for the differences seen between the cyclic and acyclic responses in our experiments. These results support the previous work demonstrating that pituitary stores of LH are higher in summer and lower in winter [28, 30, 31].

In 2007, Caraty et al were able to induce ovulation in acyclic ewes utilizing a similar CRI protocol to that employed in Experiment 2 [68]. Our experiment with mares achieved different results. Moreover, there was no significant LH release and no subsequent ovarian activity following CRI treatment. In comparing these results, it is important to examine the distinct differences between these two species. Although ewes and mares are both seasonally polyestrous, they respond very differently to changes in day length and melatonin release. Released during periods of darkness, or decreasing day length, melatonin is stimulatory to the HPG axis in the ewe. Cyclicity outside of the natural breeding season can be induced in this species by administration of exogenous melatonin [86]. In the mare, however, melatonin serves an inhibitory function with respect to the HPG axis. The ability to receive and interpret photoperiod cues in order to reduce melatonin release is critical to vernal transition in the mare [87, 88]. Interestingly, seasonal prolactin profiles are similar for both sheep and horses, presumably due to

photoperiod [3, 89]. This information suggests that like melatonin, prolactin may also have opposite effects on the HPG axes of these two species.

As evidenced by the work in ewes, kisspeptin certainly functions in seasonal regulation of the HPG axis, but from our negative results with the CRI experiment, it is unclear at this point how kisspeptin regulates seasonality of the mare. The mare transitions into cyclicity in response to increasing photoperiod. Similarly, both the Siberian and Syrian hamsters are seasonally polyestrous and respond to increasing day length. These characteristics make them a useful model for comparison of our results with regard to kisspeptin and seasonality.

Our decreased LH response in acyclic mares is consistent with kisspeptin work by Mason et al which observed a decreased LH response in female Siberian hamsters held in short photoperiod [63]. Interestingly, this group also observed a reversal of kisspeptin expression in the hypothalamus between long and short photoperiods. Low expression was observed in the ARC during long photoperiods (cyclicity), but kisspeptin was highly expressed in the anteroventral periventricular nucleus (AVPV) during this time, with the reverse holding true during short photoperiods [63]. Critical to reproduction, the AVPV has been confirmed as a site for estrogen positive feedback necessary to regulate ovulation [90]. Mason et al suggest that the seasonal control mediated by kisspeptin is two-fold and includes both decreased expression in the AVPV as well as a decrease in sensitivity of the HPG axis to the peptide [63]. However, as mentioned previously, at least two groups saw differing results regarding seasonality between males of two different hamster species. Revel at al [65] were able to induce a reproductive recrudescence of males held in short photoperiod, whereas Greives et al [64] were

unsuccessful. Although both Syrian and Siberian hamsters are long-day breeders, it is evident from these equivocal results that there are likely species differences, and certainly sex differences, in the hamster's ability to respond to kisspeptin with regard to season.

While somewhat divided regarding the exact pathway and mechanisms involved, research groups agree that kisspeptin plays a key role in many aspects of reproduction, including seasonality. What remains unclear is how this messaging system functions with regard to species, seasonality, and sex of the animals examined. It appears from our work with mares that the magnitude of the kisspeptin-induced LH response is directly related to cyclicity status rather than photoperiod, leading us to speculate that sex steroids play an integral role in this feedback system. This was confirmed in recent work by Whitlock et al which demonstrated that sex steroids appear to increase the sensitivity of the HPG axis to KP-10 [74]. In addition to steroid feedback, body condition is also known to contribute to reproductive cyclicity in mares [2, 6, 9-11], and leptin receptors have been identified on kisspeptin neurons [19]. Many investigators have proposed that kisspeptin is the central coordinator of these multiple signals which drive the HPG axis.

Several potential explanations exist which warrant further investigation in the mare, including kisspeptin availability, receptor (GPR54) availability, and receptor up-regulation, particularly with regard to sex steroids and season. More useful information is certainly on the horizon with regard to the exact role of kisspeptin in regulating the HPG axis of the mare. Based on research in other species, functions in equine reproduction could extend into areas such as regulation of puberty, maternal recognition

of pregnancy, maintenance of pregnancy, and identification of high risk pregnancies. The potential clinical applications for kisspeptin in the mare are wide-reaching and likely extend well beyond cyclicity and seasonal transition.

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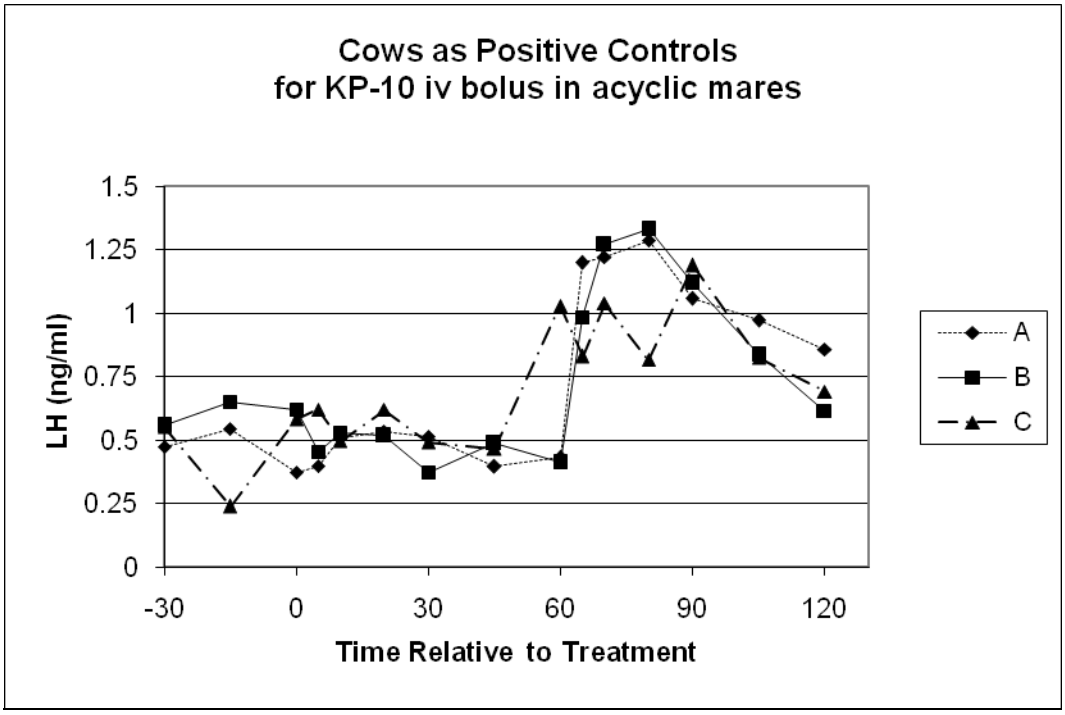
APPENDICES

APPENDIX A

Bovine Controls for Experiment 3



Appendix A: Graph depicting LH response in diestrous cows (n=3; A,B,C) sampled on the same day as the acyclic mares and using the same lot number of KP-10. Treatments were given iv at time 0 (saline treatment) and 60 minutes (0.1 nmol/kg KP-10).

APPENDIX B

Equine LH Assay Reagents and Protocol

Assay Buffer: 11.7 g EDTA

0.9 g Na Phosphate (monobasic)

4.8 g Na Phosphate (dibasic)

8.6 g NaCl

10.0 g BSA

Dissolve in 700 ml distilled H_2O, pH to 7.4, q.s. to 1 L with distilled H_2O

PBS-EDTA: 19 g EDTA

0.9 g Na Phosphate (monobasic)

4.8 g Na Phosphate (dibasic)

8.6 g NaCl

Dissolve in 700 ml distilled H_2O, pH to 7.4, q.s. to 1 L with distilled H_2O

NRS-PBS-EDTA: Add 100 uL of normal rabbit serum to 40 ml PBS-EDTA

LH Standard: Equine LH (eLH) provided by Dr. Janet Roser of UC Davis

Primary antibody: Provided by Dr. Don Thompson of LSU (LSU Ab^1)

Dilution 1:3000

Secondary antibody: Sheep anti-rabbit IgG (oLH Ab^2)

Add 1 ml of sheep-anti-rabbit to 39 ml PBS-EDTA

Trace: Dilute iodinated oLH in assay buffer to 30,000-35,000 cpm per 100 µL

Equine LH Assay Protocol

Day 1: Set up standards and curve as specified on sheet (16.0-0.25). Add 100 µL of primary antibody to tubes 5 through end of assay. Add 100 µL of trace to all tubes. Vortex and store at room temperature.

Day 2: Add 200 µL of secondary antibody to tubes 3 through end of assay. Vortex and store at room temperature.

Day 3: Spin tubes at 3300 rpm for 20 min. Pour off fluid and blot tubes dry. Count for one minute on gamma counter.