CHARACTERISTICS OF PROGESTERONE, LUTEINIZING HORMONE, AND CORTISOL CONCENTRATIONS AND OVARIAN ACTIVITY IN EXERCISING MARES

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2016

Major Subject: Physiology of Reproduction

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ABSTRACT

Over the course of two breeding seasons, the ovarian activity and concentrations of progesterone, luteinizing hormone (LH), and cortisol were evaluated and compared in six exercising (EXER) mares and six non-exercising (NOEX) mares to illuminate any potential influence of exercise on ovarian and hormonal dynamics in mares. Blood samples were collected daily and the ovaries of all mares were evaluated daily by transrectal ultrasonography to track follicular activity and ovulation. No difference between the two groups was found in regard to interovulatory interval or the maximum size of the pre-ovulatory follicle. At d -5 relative to ovulation, EXER had more <10 mm, 10-15 mm, and 15.1-25 mm follicles on their ovaries. At d -1, there were more follicles present sized 15.1-25 mm and >25 mm in the NOEX group. In regard to basal cortisol concentrations, no difference was found between the EXER and NOEX groups when comparing levels at wk 3, 6, and 9 of the study. LH concentration was greater in the EXER mares on d -4, -3, 0, 1, 2, 3, and 4 relative to ovulation. When days were grouped by d -4 and -3, -2 and -1, 0, 1 and 2, and 3 and 4, EXER LH was higher at all periods. Progesterone concentrations were determined on d 9, 15, and 21 post-ovulation. There was no difference in progesterone concentration between the two groups at d 9, but progesterone was lower at d 15 in the EXER group. At d 21, several sample values from the exercised group appeared to be below the sensitivity of the assay. With the lowest assay sensitivity concentration entered in place of the missing data, the concentration of progesterone was found to be lower in the EXER mares when compared

to their NOEX counterparts at d 21. Based on these findings, exercise-induced stress does exert an influence on the ovarian dynamics and the hormonal profiles of LH and progesterone in exercising mares at specific points during the estrous cycle.

DEDICATION

For my family and my “family.”

In loving memory of Brent Alston Dworaczyk.

ACKNOWLEDGEMENTS

They often say it "takes a village" to raise a child, and I feel like that philosophy applies to most graduate programs. I have seen the highest highs and the lowest lows of my personal life since this Ph.D. program was initiated in 2006, and I am forever grateful to my Committee Co-Chairs Dr. Martha Vogelsang and Dr. Dennis Sigler, and my Committee Members, Dr. David Forrest and Dr. Thomas Welsh, for your patience and for never giving up on me. This is especially true in the case of Dr. Martha Vogelsang, who has not only been an amazing mentor and academic advisor, but who is also the type of friend that only comes along once in a lifetime. Thank you for being there for me, fighting for me, and for always shining a light in the right direction, even when life's tunnel was really dark.

I am fortunate to have been blessed with a wonderful and extremely supportive circle of family and friends. I have the most amazing parents—Dinah Bowman and James Bowman, Jr. Thank you to them, along with Richard "Dick" Waddell, and Jimmie and Benny Dworaczyk, for their unwavering love, support, and "more-than-gentle" encouragement to reach for the stars and to never ever give up. They say that "friends are the family you get to choose for yourself"—and I would have never made it without the love from some of my very best friends— Betsy Wagner, Michelle Wolf, Amanda Woolard, Alexandra Salas, and Erin Rich. I love and am forever grateful for each you and the unique friendship that each of us have. Thank you for accepting me and loving me for who I am.

I have two loves that also deserve much recognition. The first was Brent Alston Dworaczyk, my late husband. Before he passed in 2012, Brent made me promise I would finish this program (among other things), and that promise is often what kept me going. Thank you for loving me and for giving me a reason to keep working toward this goal. Second, only because of timing and not because of a love that is any less, is my fiancée, is Thomas Lee Carey. I am forever grateful for your friendship and your unconditional love, both of which I consider to be an extra precious gift and blessing. Thank you for believing in me and always supporting me 110%, no matter what the venture or adventure.

Research is rarely the work of a single individual, and there are several others that deserve much gratitude—thank you to Dr. Gary Williams, the late Dr. Marcel Amstalden, and Research Associate Sarah Sharpton for their expertise, help, and patience in the completion of the hormonal assays for this project. Thank you also to Dr. Gary Potter for his mentorship and encouragement, and to Dave Golden and Krissy Schroeder, our Horse Center Managers at the time of this project, for their dedication to our herd and help with all the logistics that go on behind the scenes. Last, but certainly not least, thank you to my tireless and dedicated student workers and research assistants—especially the wonderful and inspiring Laura Gerik and Elissa Emmons. Not only did you guys always make sure all the "dirty work" got done, but you are both wonderful friends and I am so grateful that our paths crossed.

Thank you to all for being part of my "village".

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a dissertation committee consisting of Drs. Martha Vogelsang and Dennis Sigler, Co-Chairs, both of the Department of Animal Science, Dr. David Forrest, Committee Member, of the Department of Animal Science, and Dr. Thomas Welsh, Committee Member, of the Department of Animal Science and the College of Veterinary Medicine.

The statistics and data analyses reported in Chapter IV was conducted with the assistance of Stacy Miller, Statistical Consultant and Co-Owner of Sodville Farm Services, Inc.

All other work conducted for the dissertation was completed by the student independently.

Funding

Graduate study was supported by an Assistantship from the Department of Animal Science and the 2011 U.S. Senator Phil Gramm Doctoral Fellowship.

NOMENCLATURE

ACTH	Adrenocorticotropic Hormone
AVP	Arginine Vasopressin
B/CS	Bryan/College Station
cAMP	cyclic adenosine monophosphate
CL	Corpus Luteum
CRF	Corticotrophin Releasing Factor
CRH	Corticotrophin Releasing Hormone
d	Day
epi	Epinephrine
FSH	Follicle-Stimulating Hormone
GC	Glucocorticoid
GnRH	Gonadotrophin-Releasing Hormone
HPA	Hypothalamic-Pituitary-Adrenal
HPG	Hypothalamic-Pituitary-Gonadal
h	Hour
LH	Luteinizing Hormone
mm	Millimeter
Norepi	Norepinephrine
RIA	Radioimmunoassay
wk	Week

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CHAPTER I

INTRODUCTION

As the equine industry continues to place increasing demands on our most valuable livestock, achieving ultimate reproductive efficiency becomes a necessity. Compared to other livestock species, is an especially unique situation can arise in the case of the female equine athlete, which may be expected to continue working, training, or performing during breeding procedures and throughout the early stages of gestation. Although considered to be less than ideal, this situation is often due to breed association-imposed reproductive limitations which may limit the application of assisted reproductive technologies such as artificial insemination and embryo transfer. Therefore, the subsequent and unavoidable exercise-induced and potential heat-induced stress placed upon these mares and its influence upon their reproductive competency should be evaluated.

The influence of heat-induced stress on reproduction has been evaluated in several livestock species; however, the majority of this research has been directed at livestock species focused on meat and milk production, and not at that experienced by the athletic equine. However, a previous study by Mortensen et al. (2009), also conducted at Texas A&M University, has reported a detriment to embryo survival and quality in mares subjected to an exercise program. Researchers have suggested that this decline could be due to the elevated body temperature exhibited by these mares during exercise and at the time of breeding. This hypothesis has been supported by further

research that revealed the developmental delay of oocytes treated with heat (42°C for 2 or 4 h) in the final stages of *in vitro* maturation (IVM). In this instance, oocytes exposed to heat in the late stages of IVM protocols resulted in a significant decrease in oocyte developmental competence following intracytoplasmic sperm injection (ICSI) (Mortensen et al., 2010).

Although other livestock studies have employed intense heat-stress programs and studied the effect on female reproductive efficiency, these protocols have not necessarily been applicable to the type of stress experienced by the exercising equine female in a training program. It is of interest to evaluate the effect of an exercise program similar to that which may be implemented in the commercial horse industry on the reproductive capabilities of the mare. Researchers feel that the results of this type of study will be immediately applicable to equine industry professionals and may provide additional insight to how exercise-induced stress may influence the reproductive successes in other athletic species, such as humans.

The objectives of this study were:

1. to examine the effect of moderate exercise-induced stress (similar to what could be expected in a training program in the equine industry) on the ovarian activity of mares by recording and analyzing the interovulatory period, follicular growth rate, number of and distribution of follicles throughout the estrous cycle, and the ultimate size of the dominant follicle;

2. and to establish any potential effect of moderate exercise-induced stress on the reproductive hormones progesterone and luteinizing hormone (LH), and the stress hormone cortisol in mature mares.

CHAPTER II

LITERATURE REVIEW

Introduction

The true definition of "stress" has been debated and revised over many years by physiologists, beginning when the term was originally coined by Hans Selye in 1946. However, most agree that the basic definition of stress can be summarized as a "disruption to homeostasis" (Rivier and Rivest, 1991) and that a stressor may be simply defined as any stimuli that results in this disturbance (Tilbrook et al., 2000). Therefore, the general term and study of "stress" has evolved into three broad topics: 1) the stimuli in the environment that prompts a stress-related event, or the "stressor", 2) an organism's response to the stressor or the "stress response" and the process of allostasis, and 3) the potential diseases or disorders that can potentially develop as a result due to the prolonged exposure to stressors and their related responses.

Stressful situations are often revealed by an animal's inability to cope with its environment and can ultimately be reflected as the failure of an individual to reach its genetic potential (Dobson and Smith, 2000). This is believed to be related to the interplay of hormonal mediators and signals that can eventually compromise all major systems within an organism, beginning at the most basic cellular level. Interestingly, independent of the initial source of the stressor—whether it be physiological or psychological—the internal endocrine response is the same (Romero and Butler, 2007).

When studying the concentrations of hormones within the body, it is important to remember that the concentration is a balance between the production of and the clearance rate of the hormone of interest (Bonan et al., 1979), which can be influenced by circulatory and systemic factors. Current reproductive research and the newest technologies are becoming more capable of evaluating tissue-specific concentrations (providing more information at the actual cellular level of production) and the rate and amount of blood flow through specific organs, which may illuminate more on the clearance rate of the hormones. In the future, new technology will undoubtedly continue to answer many questions related to the production, presence, and clearance of hormones within the human and animal body.

Endocrinology of Stress

Once a vertebrate animal or human is exposed to a stressor, an elaborate endocrine response is initiated, with the catecholamines and the glucocorticoids forming the pillars for the overall reaction to the stimuli.

The catecholamines, epinephrine or epi (also called adrenalin) and norepinephrine or norepi (also called noradrenalin) are both 6-carbon ring hormones with carbon side chains, which determines the biological specificity. Epi and norepi are both produced prior to any perceived stressor and are stored in secretory vesicles within the adrenal medulla and within the nerve terminals of the sympathetic nervous system (Romero and Butler, 2007). On the cellular level, once the sympathetic nervous system is activated, the catecholamines are released into the bloodstream to be bound by

specific membrane-bound G-protein receptors on cells. Once bound, a cascade of events beginning with an intracellular cAMP pathway lead to cellular reactions and onward to systemic responses. Again, because catecholamines are produced and stored prior to demand, both epi and norepi can be immediately introduced in to the bloodstream within seconds (Romero and Butler, 2007), resulting in their characteristic systemic "Fight or Flight" responses, such as increased heart rate, a heightened sense of visual and mental acuity, an increase in gas exchange efficiency in the respiratory system, an increase in brain blood flow coupled with the decrease of blood flow to the extremities, an increase in circulating glucose (by increasing glycogen-breakdown), and the shutdown of non-emergency systems such as digestion and reproduction (Goldstein, 1987; Romero and Butler, 2007).

The glucocorticoids (GCs) form the other pillar of the endocrine response to stressors. Structurally, GCs are steroid hormones that have a 4-ring carbon backbone with a variety of different hydroxyl groups and carbon side chains oriented around the rings. Like the catecholamines, the side chains projections determine the individuality of the hormone and establish its specificity for its respective receptor. The primary GCs released in response to a stress-related situation are cortisol and corticosterone. Both of these GCs can be found in some concentration in most all species, but different species tend to produce and rely more upon one than the other. For example, fish and most mammals, including humans, rely more upon cortisol; and birds, reptiles, amphibians, and some rodents utilize corticosterone more to mediate their responses to stress (Romero and Butler, 2007).

Like all steroids, cortisol and corticosterone are not stored prior to demand, unlike the catecholamines. Therefore, their origination and delivery is different, and their overall effect is more delayed. Once a stressor is detected, the amygdala and hippocampus regions of the brain send neurological signals to the hypothalamus (especially the paraventricular nucleus). The cells of the hypothalamus then send axon projections to the median eminence where they terminate along the capillaries of the localized portal blood system affiliated with the anterior pituitary. Once these hypothalamic cells are stimulated by a stress response, they release the hormones corticotrophin-releasing factor (CRF) (also called corticotrophin-releasing hormone or CRH) and arginine vasopressin (AVP) into the portal blood system to expedite their delivery to the anterior pituitary (Romero and Butler, 2007). Corticotrophs are the cells of the anterior pituitary are responsible for the production of a variety of peptides involved in the stress response, including adrenocorticotrophic hormone (ACTH), β-endorphin (involved in pain modulation), and α-melanocyte-stimulating hormone (Engler et al., 1989). Once CRF and AVP are delivered to the anterior pituitary, they bind to their receptors and the release of ACTH is stimulated. Once released into general circulation, ACTH travels to the adrenal cortex, where it binds with its receptors and stimulates the release of the GCs. This cascade of events originating at the hypothalamus and ending with the adrenal gland is call the Hypothalamic-Pituitary-Adrenal (HPA) axis (Romero and Butler, 2007).

Once released, the GCs travel throughout the peripheral circulation to their target tissues. When in transit, GCs are usually bound to corticosteroid binding globulins

(CBGs), but unbound or free GCs also exist and are increased during a stress response. Both cortisol and corticosterone bind to the same receptors and have identical functions in their respective species. Once bound, GCs pass through the cell membrane and bind to an intracellular cytoplasmic receptor, which then enters the cell nucleus and acts as a transcription factor. These activated receptors bind to stretches of DNA sequences called "glucocorticoid response elements" and then proceed to act as either as a promoter or an inhibitor of gene transcription. Therefore, the ultimate response to GC production is the production or inhibition of proteins (Romero and Butler, 2007). Eventually, the circulating levels of GCs will exert a negative feedback mechanism on the hypothalamus and pituitary to regulate and decrease the secretion of CRF, AVP, and ACTH (Plotsky et al., 1989). Due to the time required to ultimately result in the production or inhibition of active proteins the influence of GCs is greatly delayed when compared to the response time of the catecholamines.

Overall, the organism-level of the effect of GCs can be seen as an increase in blood glucose concentration, altered behavior, inhibited growth, inhibited reproduction, and a limited immune system. The overall goal of this cascade is to help an individual recover from a stressor by shutting down systems not immediately needed for survival and reallocating energies to those systems that are necessary (Romero and Butler, 2007)

The coupled actions of catecholamines and GCs work together to combat most stressful situations, and their partnership to regulate the stress response is necessary for an individual's survival (Tilbrook et al., 2000). However, the negative effect of prolonged exposure to stressful situations is known to impact each of the major systems

of the body. The following represents a summary of information related to the acute or prolonged exposure to stress and its influence on different aspects of the reproductive system in the female.

Stress and Ovarian Dynamics

The effect of stress on ovarian activity and follicular dynamics has become an area of interest in past years— particularly studying the distribution of different sizes of follicles present on the ovary, the qualities and dominance of the ovulatory or Graafian follicle, and the interovulatory period.

The process of follicular selection and deviation in monovular mammals (such as cattle, horses, and humans) is the event where the largest follicle establishes dominance over the second-largest (before it can reach a similar diameter) follicle and other smaller follicles and thereby develops the capacity to ovulate. This event is noted by a transient elevation in LH in heifers and the long preovulatory LH surge observed in mares (Ginther, et al., 2000). This process appears to be regulated by the different follicles' stages of development and the presence or absence of LH or FSH receptors. For example, in heifers, LH receptors appear in the granulosa cells of the future dominant follicle approximately 8 h prior to deviation. At this point, LH stimulates the production of estradiol and insulin-like growth factor-1 (IGF-1), which is believed to be responsible for the response of the largest follicle to the lower concentrations of FSH. Conversely, the smaller follicles do not establish LH receptors at this time of the cycle and maintain a

closer dependency on FSH and exhibit a slower growth rate or regress following the selection of the dominant follicle (Ginther et al., 2000).

Although research pertaining to the ovarian dynamics of the mare, and particularly how those dynamics may be affected by exercise or other stressors, has been limited until recently, it has become a topic of recent investigation. Also in this laboratory, and as the impetus for the current study, Mortensen et al. (2009) observed a longer interovulatory interval after $PGF_{2\alpha}$ administration, the ovulation of smaller Graafian follicles, and a subsequent reduction in successful embryo collection from exercised mares and a subsequent tendency for poorer quality embryos from mares that had been subjected to 30 min of moderate daily exercise when compared to their non-exercised counterparts.

Continued research by Mortensen with Kelley et al. (2008, 2009, and 2011) began to more closely evaluate the effects of exercise on ovarian activity and follicular development in the exercising mare. The reported potential influence of exercise on the interovulatory interval in the equine has been contradictory. Kelley et al. (2009) reported an increased interovulatory period in the first and fourth of four consecutively observed estrous cycles in moderately exercised mares. Although the second and third interovulatory interval of these cycles tended to be longer, the results were not significant. In a 2011 publication, these researchers also reported a longer mean interovulatory period when four cycles were combined for analysis. However, these researchers also reported no difference in the overall length of the estrous cycle (2008). In regard to the ovulatory follicle, results are also contradictory. At the reading

immediately before confirmed ovulation, Kelley at al. (2011) found no difference in the follicle diameters of the ovulatory follicles between the two groups, but at d -5 relative to ovulation, Kelley et al. (2008) reported the ovulatory follicles to have a larger mean diameter in exercised mares.

The growth and development of the largest subordinate follicle (the second largest follicle present) was also evaluated in several studies. In 2008, Kelley et al. found the subordinate follicle to demonstrate an accelerated growth rate and overall greater size -5 d relative to ovulation. This increased diameter of the subordinate follicle was confirmed in their 2011 study. Researchers suggested that this increased activity in the subordinate follicle may be an indication of delayed deviation, being when the growth rate of the preovulatory follicle changes and accelerates compared to the subordinate follicle. Regarding deviation, these researchers (2008) also reported the less-clear notable deviation in exercised mares, with definite deviation only noticeable in 11 of 16 cycles of exercised mares (compared to 13 of 14 cycles in non-exercised mares). This may also indicate a reduced dominance of the ovulatory follicle over its subordinate or other cohort members, which is supported by the presence of fewer smaller 6-20 mm follicles and more larger >20 mm follicles in exercising mares (Kelley et al., 2011). Further supporting this idea, Kelley et al. (2008) reported a tendency for exercised mares to have two or more dominant follicles present on the ovary (2011) and also observed an increase in double-ovulations (2008) in exercised mares.

In a unique study by Smith et al. (2012), researchers observed changes in ovarian blood flow and the vascular perfusion of the preovulatory follicle in exercised mares.

Blood flow through both ovarian arteries was greater in fully (30 min of moderate exercise administered daily) and partially (moderate exercise administered 30 min per d during the periovulatory period and rested after ovulation) exercised mares when compared to non-exercised mares. However, the measured vascular perfusion of the wall of the preovulatory follicle was less in both groups of exercised individuals. Contradictory to the findings of Kelley et al. (2011), Smith et al. did not find any difference in the mean diameter of the dominate follicle among either exercised group of mares compared to the rested mares.

Even short term exposure to a stressor has been shown to influence ovarian dynamics. In recent unpublished 2015 study by Mesa et al., reproductively synchronized gilts were subjected to 6 min of exercise twice daily. The gilts were harvested 2 d after the onset of estrus, and their ovaries were evaluated. No difference was found in regard to the total number of follicles present at evaluations, corpora hemorrhagica, or corpora lutea, but the exercised gilts did exhibit more medium (3-6 mm) and small (<2 mm) follicles present on the ovaries at the time of harvest when compared to the non-exercised control group.

Considering the dairy cow, a comprehensive review by Rensis and Scaramuzzi (2003) also reports an effect of stress (heat stress, specifically) on dairy cows. Researchers have reported that heat stress delays ovulatory follicle selection in cows and lengthens the follicular wave. The decreased dominance and functionality of the preovulatory follicle in heat-stressed cows has also been noted, seen even on the hormonal level through the reduced steroidogenic capacity of the dominant follicle's

theca and granulosa cells (resulting also in decreased estradiol concentrations). This decreased dominance can also be seen on a large scale like that observed by Kelley et al., whereas more medium-size subordinate follicles survive and develop (Rensis and Scaramuzzi, 2003; Roth et al., 2000). Rensis and Scaramuzzi also suggest that this could be why there is an increased incidence of twinning in the summer months, when dairy cattle are subjected to higher environmental temperatures. Jordan (2007) supports this summary with another, suggesting that heat stress influences nearly every aspect of reproduction in the dairy cow, including the ovarian dynamics of follicular growth and development (Jordan, 2007).

A 1973 human study by Peyser et al. also reported the delay of ovulation in response to a stressful situation. These researchers attempting to characterize the LH surge in women by hospitalizing the women for a period of 7 to 10 d around the expected time of ovulation to enable blood collections at 4 h intervals. However, in both subjects, the surge was delayed, and ovulation attenuated, until 48 h post-discharge from the hospital.

Stress and Cortisol

Cortisol concentration is often evaluated to determine the effect of a stressor on an individual, and the production and release of cortisol in response to a stressor is well documented in many species. However, it is interesting to consider the wide range of activities and events that may seem to be unlikely stressors that result in a stress response and cortisol production.

Nearly every aspect of horse ownership or management impacts cortisol concentration in the equine, including stimuli such as exercise (Williams et al, 2002; Nagata et al., 1999; Desmecht et al., 1996; Lassourd et al., 1996; Kelley et al., 2011), conditioning or training (Marc et al., 2000), transport (Clark et al. 1993), feeding, social interactions, and the daily management routine (Irvine and Alexander, 1994), and heat (Williams et al., 2002). Facility design in cattle-handling facilities have also demonstrated differences in cortisol concentration in cattle (Kasimanickam et al., 2014) and could be applicable to equine facilities also. However, most of these activities result in the acute rise is cortisol following exposure to a stressor, and not a chronic elevated increase in basal cortisol levels for any length of time. However, and as already reviewed, chronic cortisol production and bioactivity can negatively affect every system of the body, through the manipulation of protein production within the cell.

It is important to remember that cortisol release is not just a stress-related response. It is present in small amounts even in non-stressed individuals, and a daily circadian rhythm has been reported (Irvine and Alexander, 1994). Irvine and Alexander (1994) found a circadian rhythm to exist in untrained and undisturbed mares housed on a plentiful pasture. This rhythm showed a peak in cortisol concentration at 0600 to 0900 h, and a nadir at 1800 to 2100 h daily. However, this rhythm was easily obliterated by changing the mares' routines to include stall confinement or housing in a holding pen for sample collection. Irvine and Alexander (1994) also evaluated racehorses in training to detect the presence of any circadian rhythm. Interesting, a rhythm similar to that seen in the pastured mares was detected, leading researchers to conclude that horses in training

could adapt to their environment thereby allowing the rhythm to emerge or that the rhythm could be entrained into a horse's daily routine. This study is important because it also reveals that cortisol analysis can be compromised by normal circadian rhythms if time-of-day is ignored, and that a basal cortisol concentration can be difficult to determine due to the short-term fluctuations of the hormone related to normal management procedures and how well a horse is accustomed to its environment (Irvine and Alexander, 1994).

In regard to reproduction, it is believed that cortisol and other stress-related hormones impede reproduction on the endocrine level by altering the HPG axis at the hypothalamus and pituitary levels first, then leading to secondary effects at the level of the gonad. However, although reproductive hormones and activities are often irregular in stressed animals, reproduction is not typically completely attenuated. Researchers believe that there are processes to limit the over-stimulation of the HPA axis and control its effects. These safeties are believed to include a cortisol negative feedback mechanism on the HPA axis to restrict the amplification of on-going stress responses (Dobson and Smith, 2000). Unfortunately, the quantification of cortisol, coupled with a correlation to negative effects upon an organism's livelihood, is difficult to ascertain.

In regard to the exercising equine and cortisol concentrations, several studies have been reported. Nagata et al. (1999) evaluated cortisol concentrations in Thoroughbred horses (n=5) subjected to incremental exercise protocols or relative workload exercises (relative to maximum oxygen consumption) on a treadmill until exhaustion. As one would expect, plasma cortisol concentrations increased with the

onset of exercise, reaching maximal concentrations between 5 and 30 min after the conclusion of exercise, and the response appeared to be correlated to the duration of the exercise regimen (Nagata et al., 1999).

To evaluate the concentration of cortisol in response to different equine disciplines, Desmecht et al. (1996) studied horses engaged in show-jumping, cross-country in three-day eventing competitions, trotting races, galloping races, and endurance riding. Similar to the findings of Nagata et al. (1999), Desmechet et al. found that the increase in cortisol concentration was related to the intensity and duration of exercise for all sporting events. However, the endurance protocol resulted in the greatest elevation of cortisol while show-jumping demonstrated the least. Lassourd et al. (1996) also evaluated endurance horses' cortisol concentrations. This group found that endurance-type exercise could very quickly results in a two- to three-fold increase in plasma cortisol concentrations, with values quickly returning to pre-exercise levels after the conclusion of exercise.

Kelley et al. (2011) evaluated cortisol concentrations in exercising and non-exercising mares over a period of several months. These researchers collected blood samples for analysis before and after exercise on a daily basis. These researchers found no difference in the concentration of cortisol between the two groups when analyzing the samples collected each morning prior to exercise. However, as one would expect, the samples collected post-exercise exhibited a higher mean concentration of cortisol in the exercised mares (6.29 ± 0.22 ug/dL) compared to their rested counterparts (5.62 ± 0.16 ug/dL). Interestingly, the published pre- and post- exercise cortisol means were only

slightly different— being 6.23 ± 0.19 ug/dL pre-exercise and 6.29 ± 0.22 ug/dL post-exercise for the exercised group of mares. It is unknown if these values were compared for any statistical difference.

Dobson and Smith (2000) evaluated the stress response of ewes subjected to 2 h of transport in a vehicle. They found that this stimuli resulted in the immediate and constant increase in AVP and CRH into the hypophyseal-portal blood of ewes. However, the ACTH response did not reach a maximum until approximately 15 min after the initiation of transport, and cortisol did not reach its maximum concentration until the beginning of the second hour of transport. Researchers concluded that the stimulus acting upon the hypothalamus constantly throughout the period of stimulation, even though the final output of cortisol was delayed until the second hour of the transport.

Several researchers have worked to determine if the cortisol response lessens in response to repeated exposures to a stimulus or training. With the ewe, Dobson and Smith (2000) reported that increasing the duration of transport did not prolong the cortisol response at the same magnitude, and that repeating the incident of transport on a weekly or longer basis did not reduce the cortisol response. However, a general observation reported by researchers stated that daily transport did result in a reduced response after four trips (Dobson and Smith, 2000). In the horse, Marc et al. (2000) evaluated the use of cortisol concentrations (and any potential adaptation due to training) as a potential tool to evaluate training status. This study consisted of three phases. During Phase 1, the exercised animals were subjected to 20 wk of training on an equine

treadmill (n=5) and the control group of counterparts were confined to stalls (n=5). During Phase 2, the horses were switched and the exercised group was subjected to 10 wk of training and the other five served as controls and were rested. During Phase 3, all horses were subjected to schooling under saddle. However, during Phase 3, five of the horses were enrolled in dressage and jumping training, and the other five were enrolled in the same training program plus an additional endurance component. Horses were subjected to regular standardized exercise tests (SETs) during all phases, which included blood collections for analysis of ACTH and cortisol concentrations (during and after the conclusion exercise). Treadmill exercise was found to increase both ACTH and cortisol concentrations. Maximum ACTH concentrations were recorded at the end of exercise and maximum cortisol levels were found to occur 20 to 30 min following the conclusion of activity. Researchers found no significant difference between the cortisol concentrations of the exercised or control groups during Phase 1 consisting of 20 wk of training on the treadmill. However, during Phase 2, higher cortisol concentrations were seen in the control horses once subjected to exercise when compared to the trained horses. At Phase 3, cortisol responses to the SET were also greater in the more highly trained horses when the incline of the treadmill was set to 5% grade, but not at the 3% grade of the SET. Interestingly, the researchers also subjected the experimental horses to an ACTH challenge after 24 wk of training, and the control exhibited a higher concentration of cortisol following the ACTH administration. Marc et al. (2000) concluded that either a treadmill SET or an ACTH challenge could potentially be used as a physiological marker for training levels and performance. A potential for cortisol

adaptation to a stressor has also been found in human athletes. Mastorakos et al. (2005) found that elevated concentrations of CRF and the downstream increase of ACH and cortisol is diminished in highly trained human athletes, however, the same individuals did exhibit a chronic mild hypercortisolism at their baseline concentration.

Stress and the Gonadotrophins

For review, the production and release of the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are regulated by a hormonal cascade similar to the previously described HPA axis, but that is expressed between the hypothalamus, pituitary, and gonads (instead of the adrenal glands), and is referred to as the HPG axis.

To stimulate this release of LH and FSH, the hypothalamus releases gonadotrophin releasing hormone (GnRH) into a localized blood portal system, which acts on the anterior pituitary to release LH and FSH into general circulation. LH is a glycoprotein hormone known to be expressed in elevating concentrations throughout proestrus (in response to increasing GnRH stimulation from the hypothalamus) and estrus, ultimately stimulating ovulation. However, the reported peak of LH concentration may not been realize until after ovulations occurs. The act of ovulation then leads to the formation of the subsequent corpus luteum (CL) on the ovary, which is responsible for early progesterone production and exerts negative feedback on the HPG pathway at the level of the hypothalamus. In the absence of the maternal recognition of pregnancy, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is released from the uterine endometrium to lyse

the CL and cease the production of progesterone. This decline in progesterone is the negative feedback on the release of GnRH and downstream LH and FSH, and thereby the cycle is permitted to begin again.

This production portion of this pathway is most activated following the luteolysis of the corpus luteum (CL) of the previous cycle and the associated reduction of circulating progesterone. This reduction in negative feedback leads to the reactivation of the HPG pathway during the follicular phase of the estrous cycle— characterized by the release of LH and FSH from the ovary, which are responsible for follicular growth, development, and preparation for ovulation, sexual receptivity, and ultimately, the actual event of ovulation (Senger, 1997).

FSH, also a glycoprotein, and LH secretion mimic each other throughout proestrus, but FSH does not have the dramatic peak like that of LH near ovulation. FSH is responsible for supporting the growth and development of the follicular cohort, and the associated production of estradiol produced and released by the growing dominant follicle prior to ovulation (Senger, 1997).

The stress-induced secretion of GCs has been shown to negatively affect the production and secretion of several hormones related to reproductive efficiency, and most agree that the exposure to a stressor can disrupt the expression and concentration of LH. A thorough review by Romero and Butler (2007) reviews the common ideals of how the GCs act upon the production and secretion of gonadotrophins. Glucocorticoids have been shown to inhibit GnRH from the hypothalamus and reduce the pituitary's sensitivity to GnRH, thereby limiting the downstream release of LH and FSH and the

reproductive activities controlled by each (Romero and Butler, 2007). Olive (2010) specifically reports that this is traceable back to the release of CRH and its negative feedback on the GnRH neurons (Olive 2010) in the hypothalamus. GCs have also been shown to reduce the sensitivity to LH at the target level of the gonad. Acute and brief exposure to stressors and the resulting GCs have shown to have limited effects on general reproductive capacities, but prolonged exposure to a stressful situation can result in sub- or infertility (Romero and Butler, 2007).

The effect of stress upon reproductive efficiency has been specifically studied in a variety of livestock species, but most of these studies have focused on production animals, such as dairy cattle, beef cattle, sheep, and goats. Several studies have also been published to evaluate the specific concerns of heat stress on reproduction, which is a great concern to many producers in hot and humid climates. This has especially become a topic of concern in dairy cattle were milk production can decline significantly when animals succumb to heat stress.

In a review of heat stress and its effects on reproduction published by Rensis and Scaramuzzi (2003), the endocrine changes in heat-stressed dairy cattle are explained. According to this review, reported changes in LH concentrations in circulation following a stress event have been contradictory. Some studies have reported an increase (Roman-Ponce, et al., 1981) in LH, while others have reported a decline (Madan et al., 1973; Wise et al., 1988; Gilad et al., 1993). However, decreases in LH pulse amplitude (Gilad et al., 1993) and pulse frequency (Wise et al., 1988) associated with the preovulatory surge have been consistently reported in dairy cattle subjected to heat stress. Also in

heat-stressed dairy cattle, Dobson and Smith (2000) reported an activation of the HPA axis leading to LH deprivation, resulting in reduced estradiol production and attenuated ovulation.

Similar results in ewes were reported by Tilbrook et al., where a shorter-term period of stress induced by several hours of isolation and restraint decreased LH pulse frequency in ewes (1999). Conversely, in goats, Ozawa et al. (2005) found no change in the peak concentration of LH between a heat-stressed treatment group and control group. However, these researchers did note that the ovulatory surge of LH was delayed by approximately 21 h in the heat-stressed group (Ozawa et al., 2005).

In addition to heat stress, livestock are often exposed to other relatively unique stimuli such as transportation, sheering, and other forms of management and handling not often realized by other organisms. These stimuli can also be seen as stressors, and researchers have evaluated several individually to determine their influence on gonadotrophin activity and reproduction. Like that seen in heat stressed cows, transportation and sheering stress has been shown to suppress LH secretion in cows (Nanda et al., 1990) and ewes (Dobson, 1987 and Smart et al., 1994) respectively.

The establishment of transportation as a stressor is well understood and transportation-related stress has been studied in the equine field. However, few have gone so far as to evaluate the effect of transportation specifically on the gonadotrophins. Nambo et al. (1995) subjected a small group of three of mares to transportation-related stress during their luteal phase (n=2) or the transition between the luteal phase to the follicular phase (n=1). Although it is important to consider the small sample size

utilized and that the study was apparently observation-based in nature, these researchers did conclude that the stress of transport could suppress the basal concentration of LH in cyclic mares. After evaluating their experimental subjects over the course of ten 5-hr transportation trials, separated by 1 h of rest between trials, the two mares determined to be in the luteal phase of their estrous cycle exhibited suppressed concentrations of LH. However, that decline may be somewhat relative to the stage of the estrous cycle, as the other mare in the transitional state to the follicular phase showed an increase LH during the first two 5-hr transportation period and then a decline in LH stretching until the end of the 60 h trial. These researchers did not find any changes in the levels of circulating FSH (Nambo et al., 1996). In dairy cattle, Dobson and Smith (2000) also reported that that transport was shown to reduce the frequency and amplitude of GnRH and LH pulses and the LH surge.

Exercise has also been indicated at a stressor, and research has been published relating its implication in reproduction. Although human reproductive research is very limited, likely due to ethical concerns and limited availability to willing research subjects, several relatively non-invasive studies have been reported. A human study by Bonen et al. (1979), evaluated the concentrations of the gonadotrophins in ten women subjected to an acute bout of 30 min of intense bicycle exercise. Researchers found that this bout of exercise did not result in any changes in FSH or LH concentration, and similar results have also been reported by Jurkowski (1978). However, seven of the ten subjects agreed to train for 30 min per d, 3 d per wk, or 8 wk. All but one subject suffered from irregular cycles following the onset of this training program, and it

became impossible to retest subjects in the same phase of their estrous cycles, even with extending the trial to 11 wk. Researchers fully acknowledged the limitations of this study given the inconsistency of the cycles. However, they did ultimately report that LH concentrations were not altered by training, but a decline in FSH production was noted. This decline in FSH was supported by another study by Bonen et al. (1978) where the hormone was reduced throughout the menstrual cycle of teenage athletic swimmers.

Another unique human study evaluated the stress of medical hospitalization on the human LH profile. A 1973 study by Peyser et al., evaluated the LH surge in response to hospitalization-induced stress. In the two subjects observed, both demonstrated a delayed LH surge when hospitalized in the period 7 to 10 d prior to expected ovulation. Interestingly, the LH surge occurred within 48 hr following discharge from the hospital (Peyser, et al., 1973).

Similar to the current study, Kelley et al. (2011) researched the exercising equine and the potential for alterations in gonadotrophin concentration. In this study, mares were exercised at a moderate rate of 30 min, 6 d per wk, and blood samples were collected in the first and fourth of four observed estrous cycles. When comparing these two cycles, researchers found and reported that there were no difference in FSH concentration when comparing the exercised and non-exercised mares, but that there were significant differences in LH profiles. In the combined cycles, the exercised mares demonstrated lower LH mean and lower LH peak concentrations compared to the non-exercised mares.

Although the irregularity of the LH profile coupled with stressful events has been widely reported and accepted, less decisive information is available on FSH. Rensis and Scaramuzzi (2003) provide information to suggest that FSH is elevated in dairy cattle subjected to heat-stress situations, which is possibly related to decreased levels of inhibin. In humans, changes in FSH concentration in response to exercise-induced stress have been conflicting, as several studies have reported no significant increase of FSH following exercise-induced stress (Sutton et al., 1973; Bonen et al., 1979), but Jurkowski et al (1978) found a significant increase in FSH following exhaustive exercise during the follicular phase of the ovary. In goats, Ozawa et al. (2003) found no difference in the patterns or levels of FSH concentration in heat-stressed goats compared to non-stressed animal, and in the equine study by Kelley et al. (2011) there was no significant difference in mean FSH concentrations in moderately exercised horses compared to non-exercised individuals.

Stress and Progesterone and Estradiol

Changes in progesterone concentration resulting from heat-induced stress in dairy cattle have been widely debated. As reported by a comprehensive review by Rensis and Scaramuzzi (2003), several studies have demonstrated an increase (Trout et al., 1998; Abilay et al., 1975; and Vaught et al., 1977), while other have observed no change (Roth et al., 2000 and Guzeloglu et al., 2001) or a decline (Rosenberg et al., 1977; Younas et al., 1993; Jonsson et al., 1997; Ronchi et al., 2001) in circulating progesterone concentrations following exposure to stressful environment. Rensis and

Scaramuzzi (2003) hypothesize that these discrepancies are possibly due to type of stressors (acute vs. chronic) and (or) variations in dry matter intake, which has been shown to influence both the rate of luteal production and the rate of hepatic metabolism of progesterone. Conversely, in goats, Ozawa et al. (2005) observed no change in progesterone concentrations in females subjected to 48 h of heat stress compared to non-stressed counterparts.

In the human study by Bonen et al. (1979), researchers reported an increase in progesterone following the acute exercise bout of 30 min in untrained individuals during the luteal phase and menses of their estrous cycle. Following a training period of 8 to 11 wk (variation due to irregularity of estrous cycle following initiation of exercise protocol), it was reported that the same individuals experienced erratic estrous cycles, making it impossible to test the same subjects in the same phase of estrous cycle, but no changes in progesterone concentrations were reported. However, another study by Bonen et al. (1978) reported an overall shortened luteal phase in teenage swimmers (when progesterone is the primary hormone involved in the menstrual cycle).

Although not directly related to the current topic of the cycling mares, a 2014 study by Anton et al., evaluated the progesterone concentration of exercising pregnant mares. This is of interest due to the similar origin of progesterone in both cycling and pregnant mares during first month of gestation (the CL). In this study, pregnant mares were subjected to moderate exercise on d 16 through 80 of gestation and blood samples were collected every 2 d to evaluate cortisol and progesterone concentrations. Progesterone concentrations were found to be less in the exercised mares, but not until

after d 60 of gestation when compared to the control group. Peak progesterone concentration also peaked earlier in the exercised mares (at d 52 of gestation compared to d 68 for the control group). Although all mares went on to execute normal parturitions and deliver healthy foals (Anton et al., 2014), the progesterone production differences between the two groups is worth noting. However, in contrast to the evaluation of progesterone concentration in the current study, it is important to keep in mind that the progesterone found in circulation at this stage of pregnancy is originating from the formation of secondary corpora lutea (CL) in response to the formation of endometrial cups between d 36 and 38 of gestation and their production of equine chorionic gonadotrophin or eCG (Allen et al., 1972 and Allen et al., 2001), and not the original CL formed at ovulation during estrus.

Although not evaluated in the current study, the effect of stress on estradiol concentration is worth mentioning due to its important role in the estrous cycle and reproduction. There are several studies in dairy cattle which report a decline in plasma estradiol concentrations following subjection to heat stress (Wolfenson et al., 1997; Wolfenson et al., 1995; and Wilson et al., 1998). Rensis and Scarmuzzi (2003) support these results and suggest the decrease in estradiol is associated with a decrease in LH concentrations and the reduced dominance of the ovulatory follicle. This change in estradiol is also supported by research reported by Ozawa et al. (2005), where a decline in the plasma concentration of estradiol was reported in goats subjected to 48 h of heat stress.

In the human study by Bonen et al. (1979), an increase in estradiol concentrations was noted following the initial acute exercise bout of 30 min in untrained women, but no difference in estradiol concentrations was seen in the same people following the training period of 8 to 11 wk. However, others have reported that continued intensive training can lead to states of hypoestrogenism. In a review of the effect of exercise and the female human reproductive system by Warren and Perlroth (2001), the authors discuss the state of hypoestrogenism in women engaged in intense exercise. They report that the negative energy state (energy expenditure of exercise is greater than dietary intake of energy) incurred by women appears to be the primary factor responsible for the reduction of GnRH pulsatility (resulting in delayed menarche and irregular cyclicity) and the overall suppression of GnRH (which compromises fertility and bone density) seen in women particularly engaged in sports that emphasis leanness. Thus, the nutritional restriction necessary to attain their goals of extremely low body fat may be a ultimate factor in the onset of hypoestrogenism observed in these athletes (Warren and Perlroth, 2001) and explain their higher prevalence of menstrual irregularities compared to athletes engaged in activities such as cycling or swimming (Olive, 2010). However, those engaged in the non-weight bearing exercise (example: swimming) were still found to be twice as likely to experience menstrual dysfunction when compared to the normal population (Olive, 2010). Olive (2010) also summarized the same sentiments, reporting the highest incidence of amenorrhea to be found in elite athletes that participate in sports that emphasize thin physiques and low body mass index (BMI). In sports were power is emphasized, the HPA axis is likely to become activated and researchers believe the

resulting increased concentration of circulating androgens may be the cause of impaired follicular growth overall ovulatory impairment. (Olive, 2010).

Conclusion

Undoubtedly, livestock producers should aim to achieve the highest reproductive efficiency within their herds or individual animals to realize the greatest financial profit margin and gains. The existing body of research has demonstrated a need to understand sources and impact of stressors and how they may influence the reproductive capacities of the female. However, it is also necessary to consider how the current body of research related to animal research could be applied to human medicine.

In a review of exercise and human fertility by Olive in 2010, the increased prevalence of reproductive dysfunction and infertility in female athletes is discussed. Included in this update is a summary of a health survey conducted in Norway in 1984-1986 that included 3,887 women and focused on exercise and infertility. Results from this study reported that athletic women (independent of age, smoking, and body mass index or BMI) had a 3.2-fold greater chance of being infertile, and exercising to exhaustion increase the risk of infertility 2.3 times (Olive, 2010). However, in today's world, it is not uncommon for medical doctors to allow or encourage women to exercise during pregnancy (Zavorsky, et al., 2011 from Anton, et al., 2014). Stress-related endocrine changes within the future or expectant mother, along with other changes related to exercise such as internal heat production and elevated body temperature, potentially excessive energy expenditures, and physical stressors or injury, may

necessitate the reconsideration of exercise for women or animals trying to achieve pregnancy or those already pregnant.

Without a doubt, more research is needed to help the advancement of both the livestock industries and human medicine to fully illuminate the effect of stress, specifically exercise-induced stress and heat stress, on fertility. After reviewing the body of literature currently available on the topic, the objectives of the current study have been created to:

1. examine the effect of moderate exercise-induced stress (similar to what could be expected in a training program in the equine industry) on the ovarian activity of mares by recording and analyzing the interovulatory period, follicular growth rate, number of and distribution of follicles throughout the estrous cycle, and the ultimate size of the dominant follicle; and
2. to establish any potential effect of moderate exercise-induced stress on the reproductive hormones progesterone and luteinizing hormone (LH), and the stress hormone cortisol in mature mares.

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

Mature stock-type mares of American Quarter Horse (AQHA) or American Paint Horse (APHA) breeding and varying ages were utilized during two consecutive physiologic breeding seasons (May-September of 2008 and 2009) for this study. All mares were owned by Texas A&M University Department of Animal Science or Texas A&M University Parsons' Mounted Cavalry. A total of 15 unique mares were utilized over the two-year data collection period. During each data collection or breeding season, 12 mares were utilized (6 per treatment group). However, during the second season of the study, three mares from the previous year were unavailable and were substituted. At the onset of this study, all mares were believed to be of normal fertility, having demonstrated normal cyclic activity in previous breeding seasons. Although the exact ages of several mares were not known, the oldest mare was born in 1987 and the youngest was born in 2004, thus aged 21-22 and 4-5 year of age, respectively, at the time of the study. Based only on the known years of birth, the average age of the utilized mares during the 2008 breeding season was 12 years, and the average age of mares during the 2009 breeding season was 11 years.

Collection Period

The data collection portion of this study was conducted during the physiological equine breeding season of the Northern Hemisphere, which includes the months of May through September. Due to facility confinements, personnel, and animal limitations, it was necessary to split the study into two consecutive summers (years 2008 and 2009) of data collection.

Treatment Groups

During each breeding season, 6 of the 12 mares were assigned to one of the two groups: 1) those subjected to exercise-induced heat stress (EXER), and 2) those that were not exercised (NONEX). All mares but 2 were randomly assigned to their respective treatments. Two mares with known histories of slight lameness were maintained on the NONEX treatment during both summers of study. This project was approved by the Institutional Animal Care and Use Committee and can be referenced by Animal Use Protocol 2006-246.

Exercised Group Details

The treatment group (EXER) was exercised by mechanical free walker 5 d each week: Monday, Wednesday, Thursday, Friday, and Sunday (See Figure 1 and Figure 2). This allowed for two days rest each week, without the days being consecutive. Exercise was initiated at 1300 h on each of the described exercise days.

At the onset of data collection, the exercise regimen was as follows: mandatory warm-up period consisting of 10 min at a brisk walk, more intense exercise period at a jog or lope (depending on each individual's comfortable stride length) for 15 min, mandatory cool-down period of 15 min at a walk. It was expected that cardiovascular and muscular fitness would increase throughout the data collection period, therefore, the more intense exercise segment of this program was increased in intensity and duration to ensure a consistent stress stimulus. The fitness of the mares was evaluated bi-weekly by comparing the length of time necessary for the post-exercise heart rate to return to resting levels (recovery heart rate). As this period shortened (as the mares become more physically fit), the exercise program was intensified such that, on average, the mares' post-exercise heart rate would return to resting levels within 30 min post-cool down. Following completion of the daily exercise regimen, all mares were turned out separately or within small groups to exercise freely and socialize.

Figure 1. EXER mares were exercised per mechanical free-walker beginning at 1300 h 5 d each week at the Texas A&M University Horse Center. This image taken at the Texas A&M University Horse Center by author.

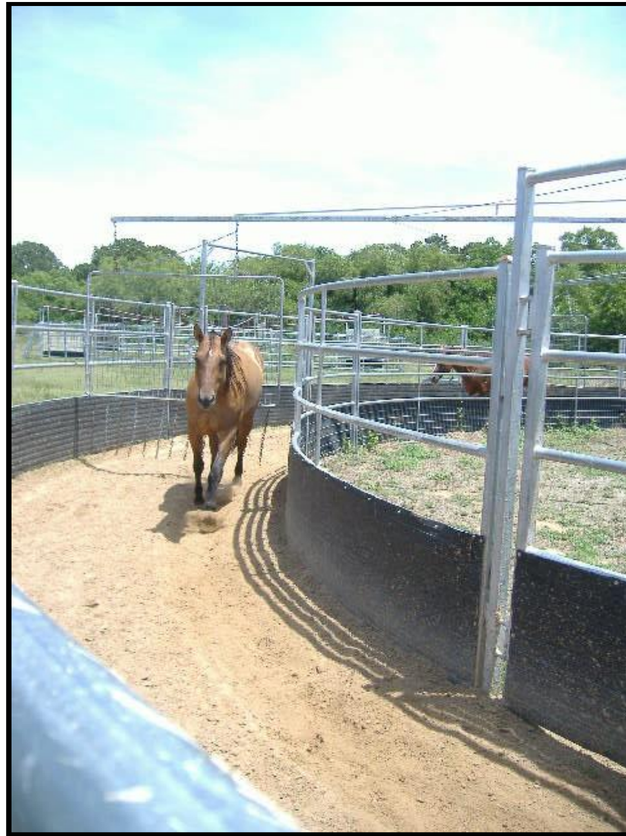

Figure 2. EXER mares were exercised daily in a mechanical free walker. This image shows the interior track of walker. This image taken at the Texas A&M University Horse Center by author.

Housing

Housing limitations at both of the Department of Animal Science facilities necessitated the two treatment groups being maintained at separate locations. The EXER group was maintained and exercised at the Department of Animal Science Horse Center (See Figure 3) and the NONEX group was housed at the Department of Animal Science Equestrian Center. The goal was to house both groups similarly, even though

mares were maintained at two separate facilities approximately 5 km apart. The stalls at both facilities were a minimum of 4 m by 4 m.

Figure 3. Mares were housed in stalls bedded with commercially-available cedar shavings. This image was shows the EXER mares and their housing at the Texas A&M University Horse Center by author.

Air temperature and relative humidity data were recorded by a mechanical HOBO™ system every hour (See Figure 4), on the hour, at both locations throughout the duration of the study for comparison. The HOBO™ devices were suspended at approximately 2.5 m above the ground at both locations.

Figure 4. HOBO environmental data collection device. Image provided courtesy of manufacturer, Onset Computer Corporation; Bourne, MA.

Mares in both groups were turned-out every day except Tuesdays or Saturdays, individually or in small groups, for at least 15 min of free exercise. The paddocks available at both facilities are large enough to allow simultaneous exercise at all gaits by multiple horses without risk of crowding or injury.

Diet

All mares were fed a commercially available concentrate of 14% crude protein (Producer's Cooperative Association, Bryan, Texas) and Coastal Bermudagrass hay twice daily at a minimum of 1% BW/d to ensure normal gut motility and to provide nutritional value. The body condition of each mare was evaluated once every 2 wk and concentrate and hay were adjusted as needed on an individual basis to maintain a Body Condition Score of 5-6 as described by Henneke et al., 1981. Mares were provided a minimum of 19 L water daily.

Adaptation Period

At the onset of both trials, all mares were subjected to a 2-wk adaptation period to acclimate them to their new housing and routine. During this period, mares were subjected to an exercise program or stall confinement similar to what was experienced during the data collection portion of this study. The purpose of this adaptation period was to acclimatize the mares to and limit any extraneous stressors from the mechanical exerciser (EXER group only), stall confinement, herd separation, normal farm activities, and the change in surrounding environment.

Collection Period

Data collection (including blood samples, ultrasonography, and environmental data from both facilities) began following the 2-wk adaption period, and concluded on the third day following the third ultrasound-confirmed ovulation from each mare or the end of study. This established collection period ensured that follicular data and blood samples would be collected for two complete estrous cycles for each mare.

Heart Rate and Temperature Sampling

The heart rate of each EXER mare was obtained once per week, on the same day throughout the study. For example, Mare 1A's heart rate was recorded every Monday. Heart rates were collected by digital palpation of the mandibular artery and were collected at rest (before leaving the stall), at the conclusion of the warm-up and exercise protocol, following the cool-down period, and then every 5 min until the heart rate

returned to near-resting levels (those documented before leaving the stall). Rectal temperature was also obtained via digital thermometer, and was also recorded on each day of exercise at the same intervals.

Non-exercised Group Details

The six NONEX mares' activities and management mimicked that of the EXER group, with the exception of exercise, heart rate collection, and rectal temperature collection. These mares were also turned out separately or in small groups every day except Tuesday and Saturday in large paddocks to allow for free-exercise and socialization.

Blood Collection

Two 10-ml blood samples were collected daily via jugular venipuncture between 1200 and 1300 (See Figure 5). Other than morning feeding typically between 0700 and 0830, these samples were collected apart from or before as much handling, palpation, exercising, turnout, or stall-cleaning as possible.

The NOEX mares' samples were collected between 1200 and 1230, and the EXER mares' samples were collected between 1230 and 1300 daily. Samples from which plasma would be obtained were collected in BD Vacutainer™ chemistry tubes containing heparin to prevent the blood from clotting. These samples were immediately placed on ice for one h prior to centrifugation to ensure proper separation of formed elements of blood and plasma. Samples from which serum was to be obtained were

collected in BD Vacutainer™ chemistry tubes without any additional treatment or anticoagulant. These serum samples remained at environmental temperature for one h prior to centrifugation to ensure proper clotting. Following one h on ice or at environmental temperature, all samples were centrifuged at 2500 rpm for 30 min. Serum or plasma were transferred by disposable polyethylene pipette to permanently labeled polypropylene microcentrifuge tubes and frozen for later endocrinological analysis.

Figure 5. Whole blood was collected from each mare between 1200 and 1300 daily. This image taken with one of the NOEX mares at the N.W. "Dick" Freeman Arena facility, managed by Texas A&M University- Department of Animal Science. Image courtesy of Deana Flak.

Ultrasonography

Each mare's reproductive tract was palpated per rectum and evaluated by ultrasound daily (Monday-Sunday) to track ovarian activity. During each procedure, all ovarian follicles greater than 10 mm in size were measured, all visually recognizable follicles were counted, and all ovulations were recorded (See Figure 6 and Figure 7).

Figure 6. The ovarian activity of each mare was evaluated via ultrasound on a daily basis. This image was taken with one of the EXER mares in the Breeding Barn at the Texas A&M University Horse Center. This image courtesy of Deana Flak.

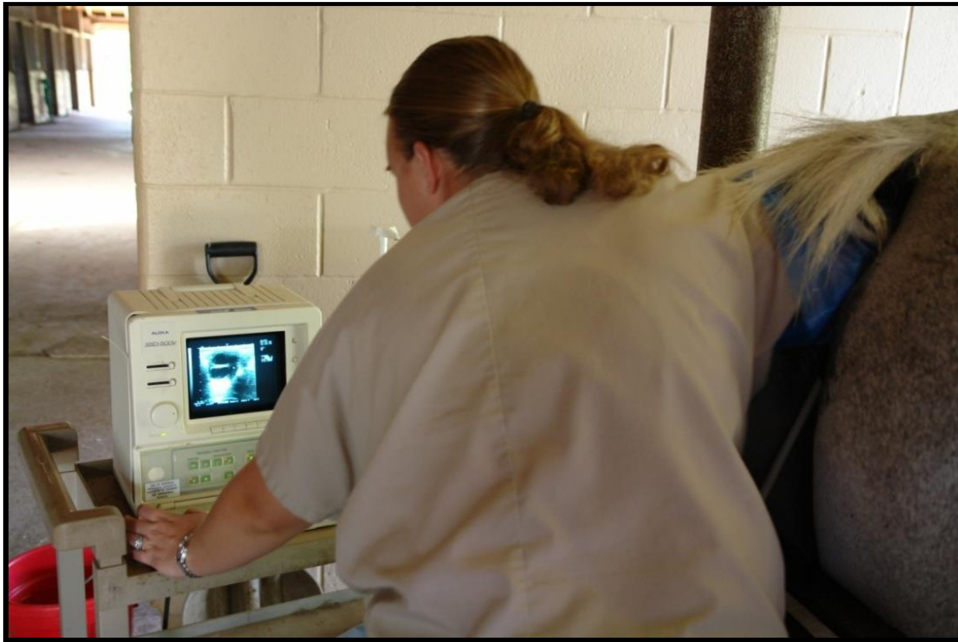

Figure 7. The ovarian activity of each mare was evaluated via ultrasound daily. In this image, the ultrasound image indicates a large follicle present on this mare's ovary. This image taken at the N.W. "Dick" Freeman Arena facility, managed by Texas A&M University- Department of Animal Science. This image courtesy of Deana Flak.

Hormonal Assays

Blood samples collected during the data collection portion of this study were analyzed for progesterone, luteinizing hormone (LH), and cortisol. Serum progesterone concentrations were determined by a commercially available single-antibody solid-phase radioimmunoassay (RIA) kit (Coat-A-Count Progesterone Diagnostic Kit, Siemens Healthcare Diagnostics Inc., Los Angeles, California). Plasma LH concentrations were determined via double-antibody RIA for all samples, using e-LH RIA procedures as published by Williams et al., 2007. See Appendix for summary of protocol. Cortisol

concentrations were determined using a commercially available RIA kit (Coat-A-Count, Siemens Medical Solutions Diagnostics, Deerfield, Illinois).

Statistical Analyses

To evaluate the difference in ovarian activity and follicular development between the EXER and NOEX groups, data were analyzed utilizing SPSS software, Version 17.0 (SPSS Inc., Chicago, IL, USA) univariate procedures with treatment and individual affects taken into account in the model. Interovulatory interval, maximum preovulatory follicle diameter achieved prior to ovulation, and quantities of follicles of specified diameters (<10, 10-15, 15.1-25, and >25 mm) at 5 d before and immediately prior to ovulation were evaluated.

All progesterone, LH, and cortisol data were interpreted using SAS statistical software (SAS Institute Inc., Cary, NC, USA) utilizing PROC GLM procedures. For progesterone determinations, samples collected at d 9, 15, and 21 post-ovulation (confirmed by ultrasound) were analyzed to compare the progesterone concentrations and progesterone production curve between the EXER and NOEX mares. In regard to LH, samples collected at d -5, -4, -3, -2, -1, 0, 1, 2, 3, 4 relative to ovulation were analyzed to compare LH concentrations between the EXER and NOEX groups. Cortisol concentrations were analyzed somewhat differently. Five consecutive days of samples from weeks 3, 6, and 9 of the study were hormonally evaluated for cortisol concentration. These samples were numerically pooled to compare the average concentrations of these weeks between the EXER and NOEX groups of mares.

CHAPTER IV

RESULTS AND DISCUSSION*

Ovarian Activity and Follicular Dynamics in Exercising and Non-Exercising Mares

No difference was revealed between treatments in regard to interovulatory intervals or maximum size of pre-ovulatory follicles (P>0.05). However, potential differences were noted between treatment groups related to the total number of follicles present on the ovaries at the evaluations 5 d prior to and immediately before confirmed ovulation. Significant differences and numerical trends are reported with P-values and associated mean ± standard error.

At d -5 relative to ovulation, a difference (P=.001) was noted between the EXER (17.81±1.12) and NOEX (17.43±1.78) mares when comparing the total number of antral follicles present on both ovaries. When further evaluating the sizes of these follicles at d -5, differences were revealed in the total numbers of follicles present in the <10 mm (P=0.005; EXER 5.46±0.73, NOEX 7.57±1.05), 10-15 mm (P<0.05; EXER 4.77±0.58, NOEX 2.81±0.58), and 15.1-25 mm (P<0.05, EXER 6.04±0.73, NOEX 5±0.84) size groups.

Immediately prior to ovulation, there tended (P=0.065) to be more follicles present on the ovaries of the EXER mares (18.17±1.26) compared to the NOEX mares

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(16.60±1.31). When compared in the manner illustrated above, this trend (*P*=0.062) continued in the total of 10-15 mm follicles (EXER 4.93±0.72, NOEX 3.20±0.51). However, there were significantly more follicles in the 15.1-25 mm (*P*<0.05; EXER 2.86±0.40, NOEX 3.20±0.57) and >25 mm (*P*<0.05; EXER 1.10±0.06, NOEX 1.84±0.25) follicle size categories in NOEX mares immediately before ovulation.

No difference was found between treatments in regard to interovulatory period in the current study. Other equine research is contradictory. Kelley et al (2009) reported moderately exercised mares to have a lengthened interovulatory interval during the first and fourth of four monitored estrous cycles, but the exercised mares only numerically averaged (not significant) longer interovulatory periods during the second and third cycles. In 2011, Kelley at al. again reported findings after tracking the ovarian activity of the groups of exercised and rested mares over four estrous periods. In this subsequent publication, it was reported that the exercised mares experienced a longer interovulatory interval when all four estrous cycles were combined together.

In the current study, no difference was found in the maximally achieved diameter of the preovulatory follicle before ovulation. This supports the findings reported by Kelley et al. (2009 and 2011), but contradicts the findings of Mortensen et al. (2009).

At d -5 relative to ovulation, which is at the time of approximate follicular deviation seen in exercised and rested mares (Kelley et al., 2011), it is interesting that the mares in the EXER group produced significantly more follicles in total, and within all size groupings except the > 25 mm category. As discussed in other species (Rensis and Scaramuzzi, 2003), this observed increase in follicular activity at d -5 may indicate

reduced dominance of the developing preovulatory follicle. This suggests reduced dominance of the preovulatory follicle is supported by findings also reported in the exercised female equine by Kelley et al, (2011) where they found exercised mares to have more follicles > 20 mm present on the ovary following follicular deviation and even subsequently found more double ovulations to be present in the exercised mares compared to their rested counterparts.

In the current study, after evaluating data collected closer to ovulation, a shift is notable. A numerical trend continues in a similar manner in relation to the total number of follicles present on the ovaries and follicles within the 10-15 mm category, but a significantly greater number of follicles measuring 15.1-25 mm and >25 mm were revealed in the NOEX mares immediately prior to ovulation. Kelley et al. (2011) reported similar findings with rested mares demonstrating the presence of more follicles in the 6 to 10, 11 to 15, and 16 to 20 mm categories when they groups all follicle sizes together. This increased number of large follicles in the NOEX group is interesting, and conflicts with data suggesting that stressed females exhibit more large follicles, indicating reduced dominance of the preovulatory follicle (Rensis and Scaramuzzi, 2003).

The changes found in ovarian dynamics between exercised and rested mares illustrates both the potential for a functional difference in the follicular activity between the two groups and a necessity for continued research investigating the effect of exercised-induced heat stress in the equine female athlete. Further study of the impact of exercise on follicular and oocyte development and viability will provide the equine

industry with appropriate tools for efficient reproductive management of mares involved in athletic performance activities.

Basal Cortisol Concentrations in Exercising and Non-Exercising Mares

Blood samples analyzed for the concentration of cortisol were collected prior to the administration of any exercise protocol, handling, or stall cleaning, in an effort to reveal and differences in the concentration of basal circulating cortisol in exercising (EXER) mares when compared to their non-exercised counterparts (NOEX).

The concentration of basal cortisol was evaluated in several different ways. First, and most generally, the data from the EXER treatment group and the NOEX control groups were pooled to compare the overall groups, separated only by treatment. The EXER group (n=78 samples) demonstrated a mean concentration of 43.69 ± 14.12 ng/ml. The lowest sample collected in this group was 21.36 ng/ml and the highest sample was 80.79 ng/ml. In the NOEX group (n=85 samples) the mean concentration of cortisol was 42.69 ng/ml ± 14.59 ng/ml. The lowest sample collected was 11.81 ng/ml and the highest sample was 71.41 ng/ml. There was no statistical significant found between these two groups (p=0.66) (See Table 1 and Figure 8).

Table 1. Data summary of overall cortisol concentrations by treatment. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : NG_ML NG_ML							
TREATMENT	N Obs	N	N Miss	Minimum	Maximum	Mean	Std Dev
EXER	78	78	0	21.3599700	80.7896800	43.6906623	14.1154964
NOEX	85	85	0	11.8082900	71.4115900	42.6918362	14.5919480

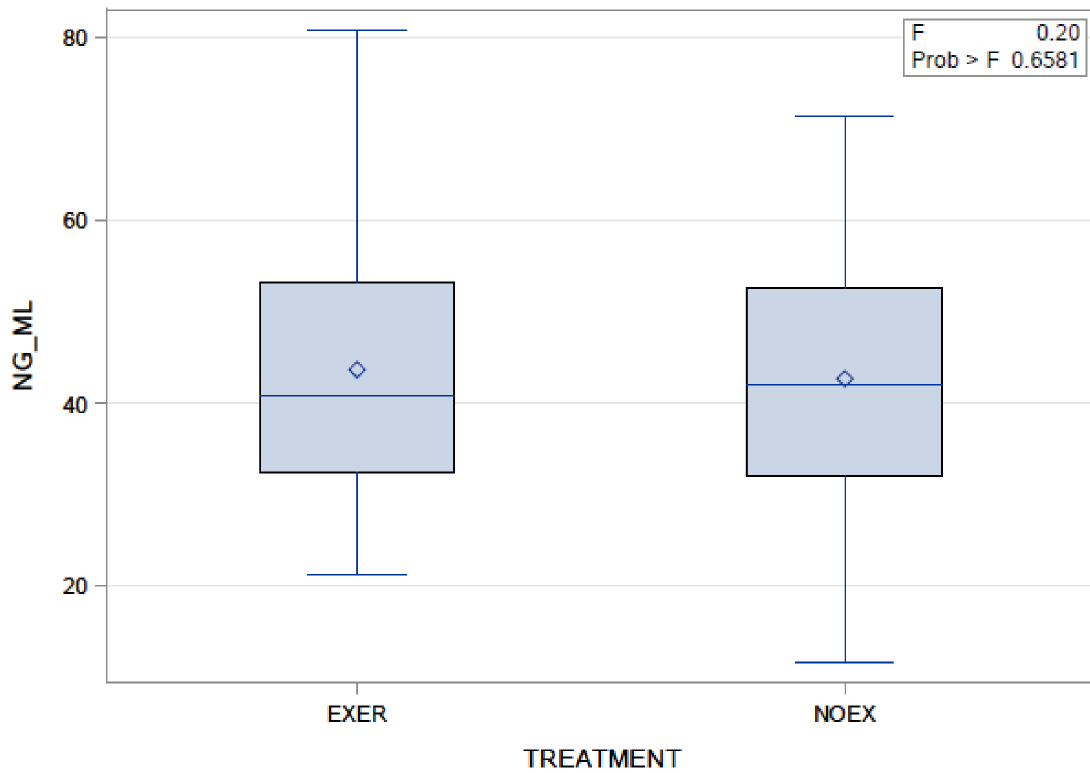

Figure 8. Overall comparison of basal cortisol concentrations between EXER and NOEX mares. Figure from SAS output, 2016

In an effort to more closely evaluate the presence of any potential changes in cortisol concentrations that may have occurred throughout the study between EXER and NOEX mares, samples collected from five days of wk 3, 6, 9 and were numerically pooled and statistically analyzed. In wk 3 of the study, the EXER cortisol mean was 45.90 ± 16.63 ng/ml (n=6) and the NOEX mean was 48.57 ± 13.65 ng/ml (n=6). There was no difference between the two groups at wk 3 of the study (*P*=0.77) (See Figure 9).

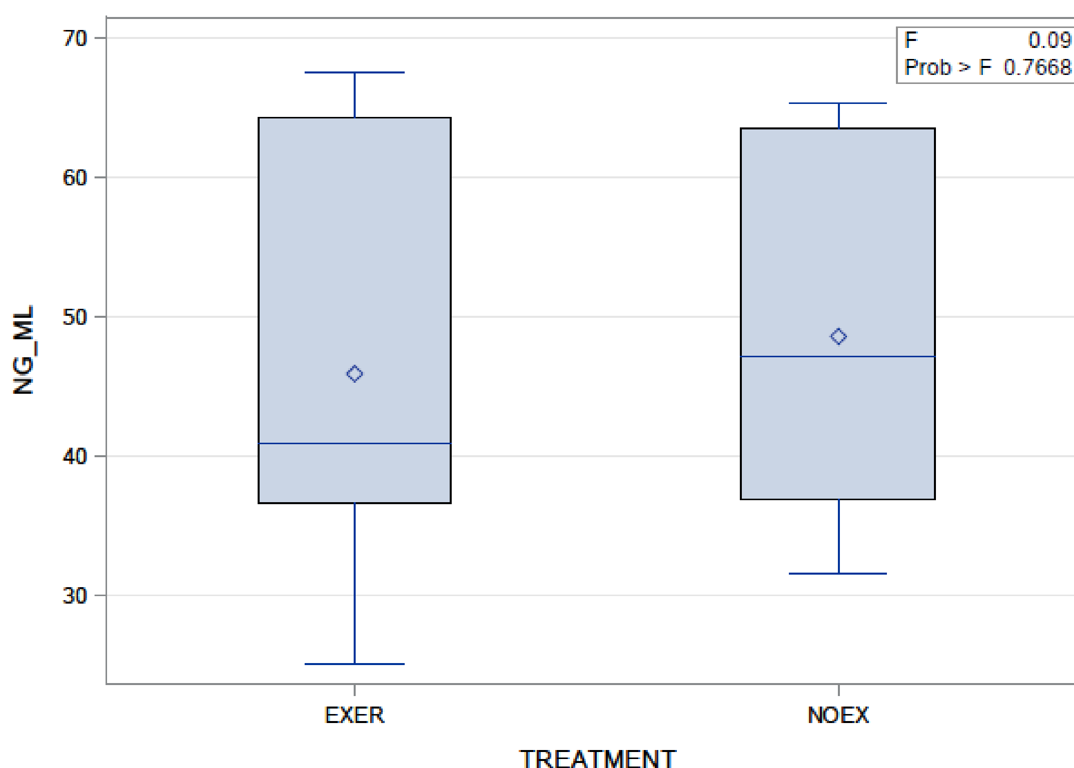

Figure 9. Comparison of pooled cortisol concentrations between EXER and NOEX mares during week 3 of study. Figure from SAS output, 2016.

Considering wk 6 of the study, the EXER group mean concentration of cortisol was 38.99 ± 13.04 ng/ml (n=6) and the NOEX group mean was 42.96 ± 11.09 ng/ml (n=6). There was no significant difference in the mean concentration of cortisol at wk 6 between the EXER and NOEX groups (*P*=0.57) (See Figure 10).

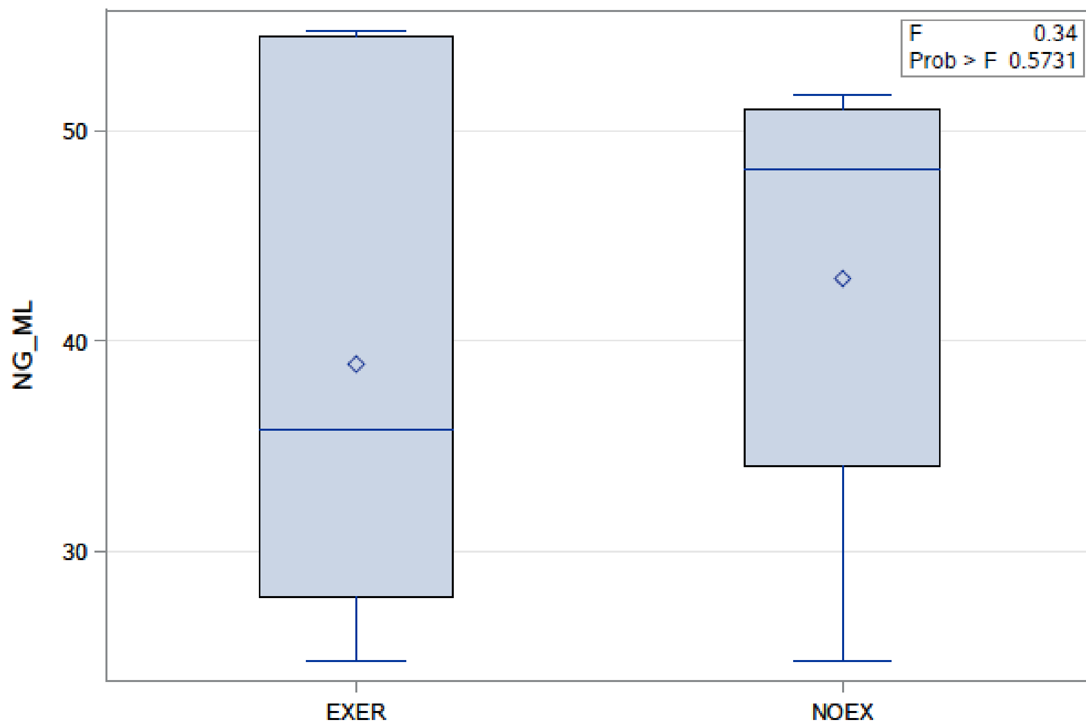

Figure 10. Comparison of pooled cortisol concentrations between EXER and NOEX mares during week 6 of study. Figure from SAS output, 2016.

At wk 9 of the study, the mean of the five pooled samples for the EXER group was 44.34 ± 11.15 ng/ml (n=4) and the mean for the NOEX group was 36.24 ± 14.40 ng/ml (n=5). Again, there was no statistical difference between the EXER and NOEX groups at this time of the study (See Figure 11).

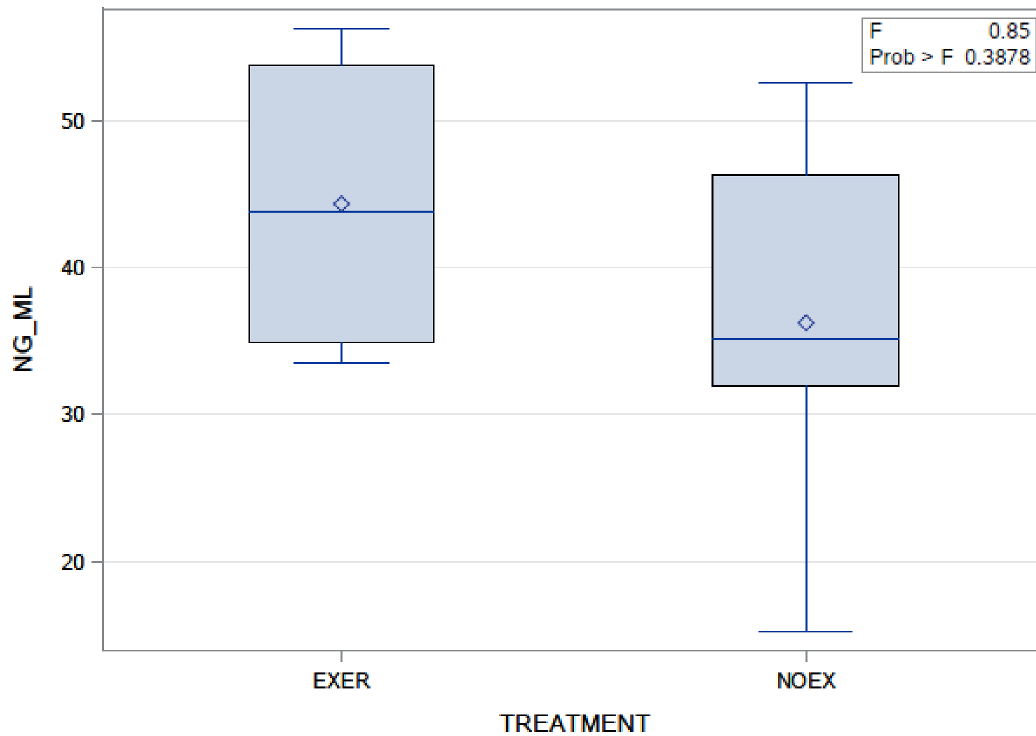

Figure 11. Comparison of pooled cortisol concentrations between EXER and NOEX mares during week 9 of study. Figure from SAS output, 2016.

The potential cortisol changes within the EXER and NOEX groups at wk 3, 6, and 9 were also analyzed (See Table 2 and Table 3). There were no significant differences found between the 3 wks evaluated in the EXER group (P=0.68) or the NOEX group (P=0.32) (See Figure 12 and Figure 13).

Table 2. Summary of weekly cortisol concentrations in EXER group. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : NG_ML NG_ML							
WEEK	N Obs	N	N Miss	Minimum	Maximum	Mean	Std Dev
3	6	6	0	25.1498160	67.4366175	45.8949806	16.6294855
6	6	6	0	24.8184280	54.7610900	38.8898600	13.0380179
9	6	4	2	33.4808575	56.2931940	44.3370684	11.1536053

Table 3. Summary of weekly cortisol concentrations in NOEX group. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : NG_ML NG_ML							
WEEK	N Obs	N	N Miss	Minimum	Maximum	Mean	Std Dev
3	6	6	0	31.6360960	65.3332375	48.5715986	13.6490953
6	6	6	0	24.8184280	51.6662820	42.9609640	11.0926974
9	6	5	1	15.2706860	52.6164160	36.2398460	14.4032251

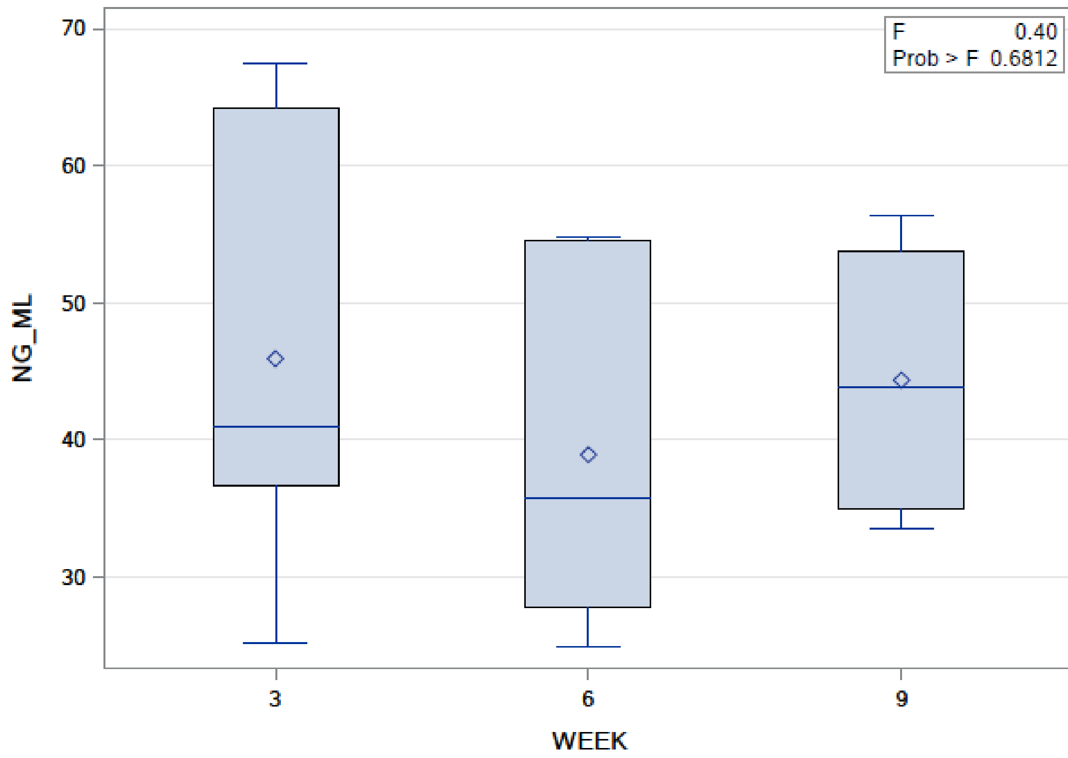

Figure 12. Comparison of pooled cortisol concentrations of EXER mares at week 3, 6, and 9 of study. Figure from SAS output, 2016.

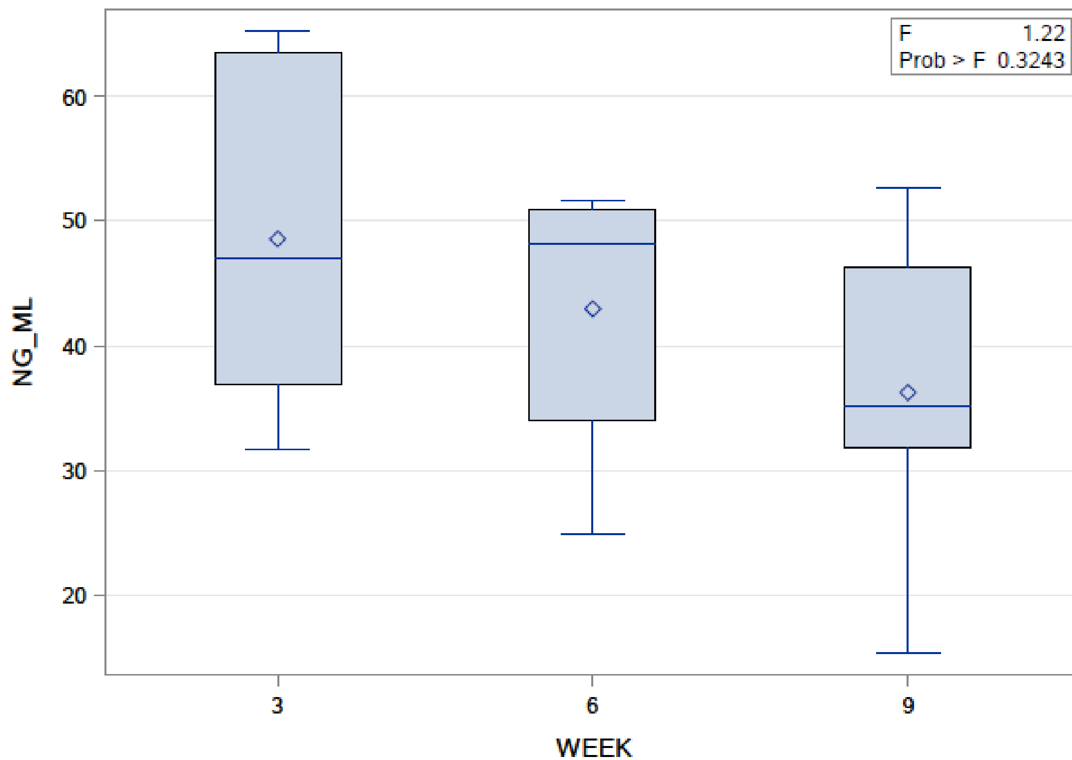

Figure 13. Comparison of pooled cortisol concentrations of NOEX mares at week 3, 6, and 9 of study. Figure from SAS output, 2016.

In the current study, no change in basal cortisol concentrations between the exercised and rested experimental groups was identified. This is supported by Kelley et al. (2011) that also reported no change in cortisol concentrations collected prior to exercise, even when daily moderate exercise was administered over the course of four estrous cycles. However, Kelley et al. did report mean cortisol concentration in exercised mares to 62.3 ± 1.9 ng/ml and mean cortisol concentration in rested mares to be 62.3 ± 1.8 ng/ml, both of which are numerically higher than what was found in the current study. It is interesting to find that in the case of both of these experimental

exercise regimens, there was no increase in basal levels of circulating cortisol. This information may be extrapolated to suggest that mares employed in a moderate training program are relatively safe from an increased level of basal cortisol concentration. Furthermore, the possible differences that were observed in the reproductive hormones and ovarian dynamics in the EXER and NOEX of the current study may not be directly related to changes in basal cortisol production. Although the basal level of cortisol does not seem to be effected by moderate exercise, the well-documented acute elevations of cortisol in response to exercise (Kelley et al, 2011) or other stressors may still have an effect on an individual's well-being and reproductive capacities.

Luteinizing Hormone Concentrations in Exercising and Non-Exercising Mares

The concentration of LH was determined by eLH RIA (protocol reported in Appendix) and statistically analyzed on a series of days leading to and following ovulation to compare values between the exercised (EXER) treatment group and the non-exercised (NOEX) control group of mares. The selected days for LH evaluation consisted of d -5, -4, -3, -2, -1, 0, 1, 2, 3, and 4 relative to ovulation, with d 0 being the day ovulation was detected (See Table 4 and Table 5).

Table 4. Summary of LH concentrations by day relative to ovulation in EXER mares. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS RELATIVE TO OVULATION	N Obs	N	N Miss	Mean	Std Dev
-5	27	26	1	1.0314642	0.6808805
-4	28	28	0	1.3857443	0.7671895
-3	28	26	2	1.6944054	0.6740197
-2	29	28	1	1.8897143	0.8604862
-1	30	30	0	2.2271077	1.0726670
0	30	30	0	3.2497510	1.3907612
1	30	30	0	3.9420403	1.9036753
2	30	27	3	5.4075037	2.6290003
3	30	28	2	4.1207311	2.4645290
4	30	30	0	3.1154187	2.5201417

Table 5. Summary of LH concentrations by day relative to ovulation in NOEX mares. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS RELATIVE TO OVULATION	N Obs	N	N Miss	Mean	Std Dev
-5	20	19	1	0.8199795	0.6543719
-4	21	20	1	0.9483325	0.7349539
-3	21	19	2	1.1309211	0.7672644
-2	25	23	2	1.4456496	1.1033094
-1	25	24	1	1.6976163	1.1511367
0	26	24	2	2.1045958	1.4395001
1	26	25	1	2.4935788	1.7551668
2	25	23	2	3.1856339	1.9220070
3	27	26	1	2.4723919	1.9769394
4	26	25	1	1.6750772	1.2344635

On d -5 relative to ovulation (or 5 d prior to ovulation) mean concentration of LH in the EXER group was 1.03 ± 0.68 ng/ml (n= 26) and in the NOEX group was 0.82 ± 0.65 ng/ml (n=19). These means were not found to be significantly different (P=0.30) (See Figure 14).

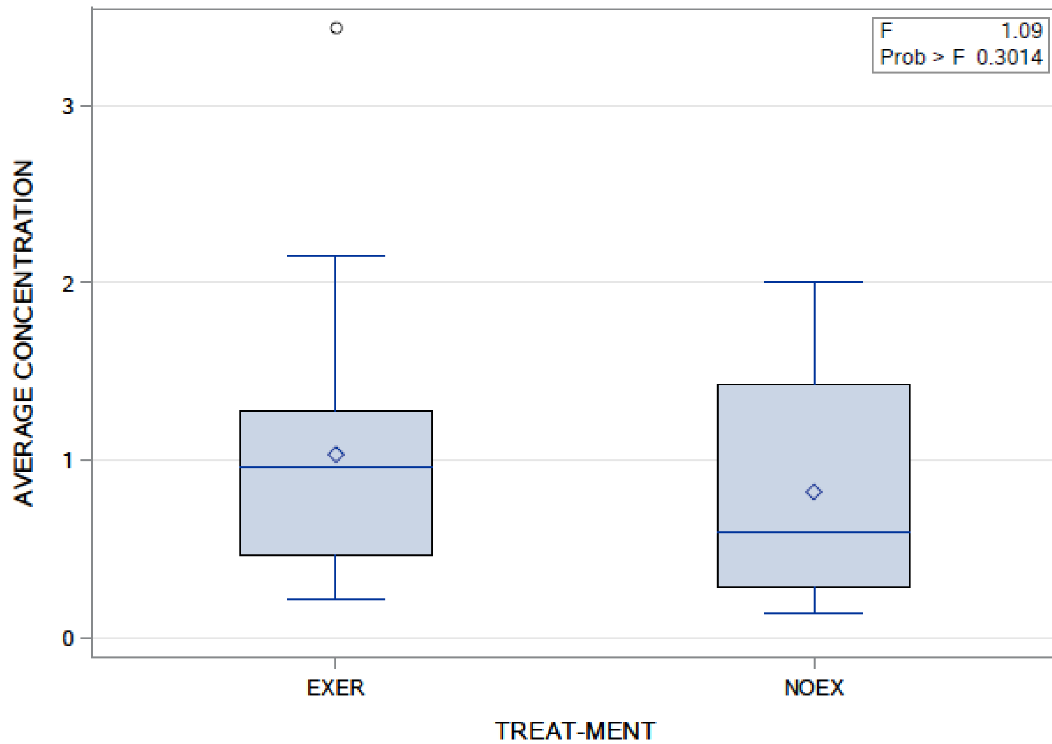

Figure 14. Comparison of LH concentrations between EXER and NOEX mares 5 d prior to confirmed ovulation. Figure from SAS output, 2016.

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Comparing the two groups on d -4 relative to ovulation, the mean for the EXER group was 1.39 ± 0.77 ng/ml (n=28) and the mean for the NOEX group was 0.95 ± 0.74 ng/ml (n=20). The concentration of LH in the EXER group was found to be higher 4 d prior to ovulation when compared to the NOEX group (*P*=0.05) (See Figure 15).

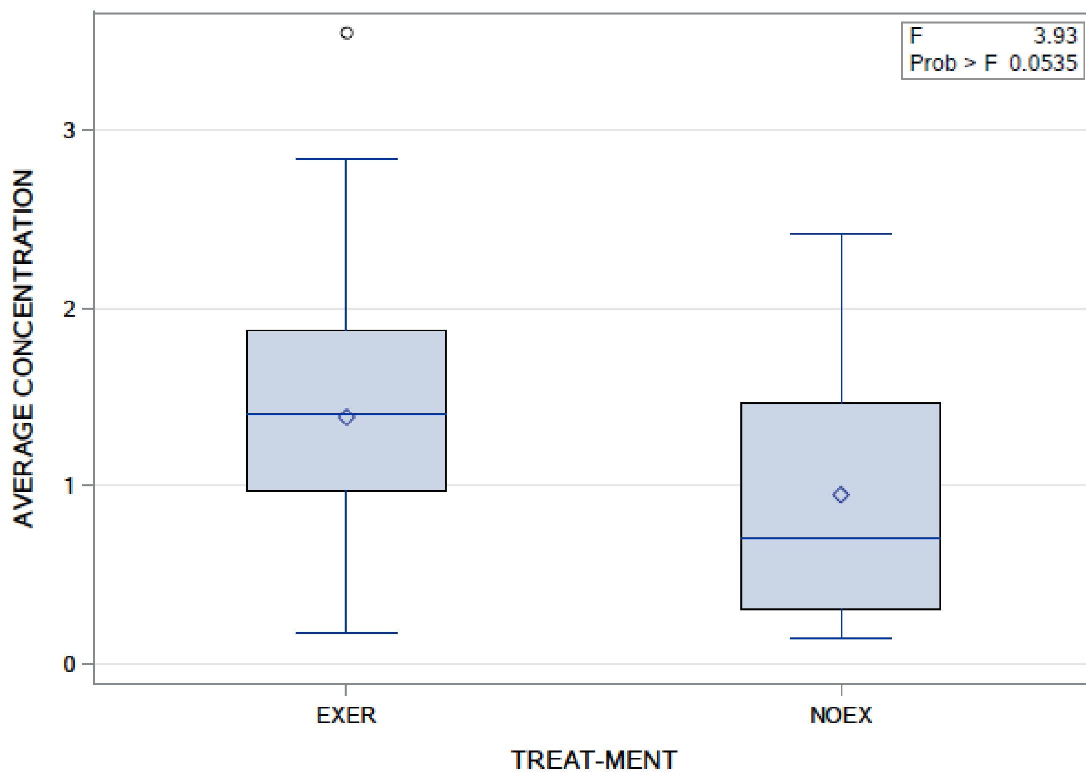


Figure 15. Comparison of LH concentrations between EXER and NOEX mares 4 d prior to confirmed ovulation. Figure from SAS output, 2016.

On d -3 relative to ovulation, the mean concentration of LH in the EXER mares was 1.69 ± 0.67 ng/ml (n=26) and the LH concentration of the NOEX group was 1.13 ± 0.77 ng/ml (n=19). On this day prior to ovulation, the concentration of LH was found to be higher in the EXER group (*P*=0.01) when compared to the NOEX mares (See Figure 16).

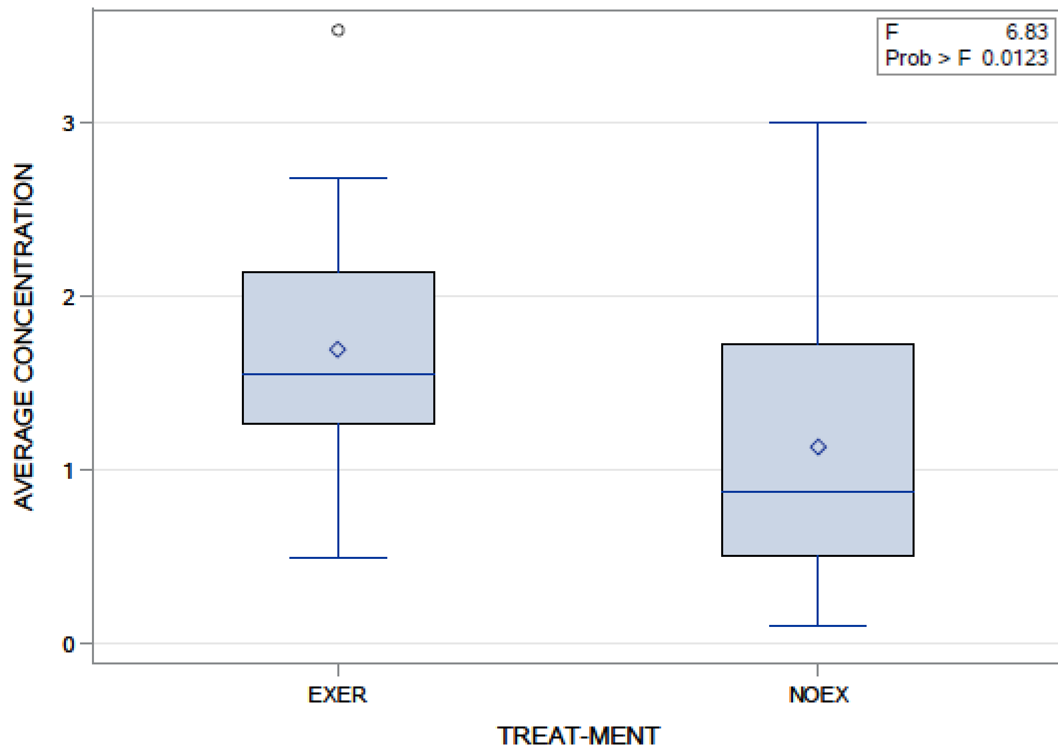


Figure 16. Comparison of LH concentrations between EXER and NOEX mares 3 d prior to confirmed ovulation. Figure from SAS output, 2016.

On d -2 relative to ovulation, the mean concentration of LH in the EXER group was 1.89 ± 0.86 ng/ml (n=28) and was 1.45 ± 1.10 ng/ml (n=23) in the NOEX group. These differences were not found to be statistically significant (*P*=0.11) (See Figure 17).

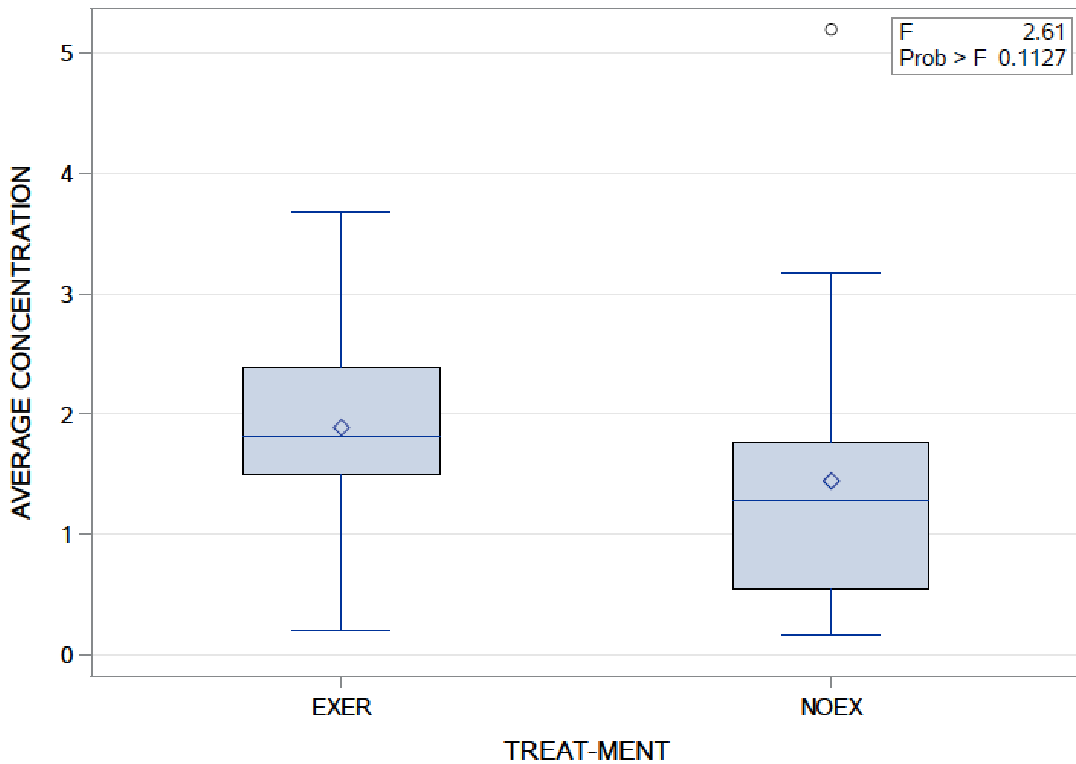


Figure 17. Comparison of LH concentrations between EXER and NOEX mares 2 d prior to confirmed ovulation. Figure from SAS output, 2016.

At -1 d relative to ovulation, the mean concentration of LH in the EXER group was 2.23 ± 1.07 ng/ml (n=30) and the mean concentration of LH in the NOEX group was 1.70 ± 1.15 ng/ml (n=24). At this point of the estrous cycle, the EXER group tended (P=0.09) to have a higher LH concentration when compared to their non-exercising counterparts (See Figure 18).

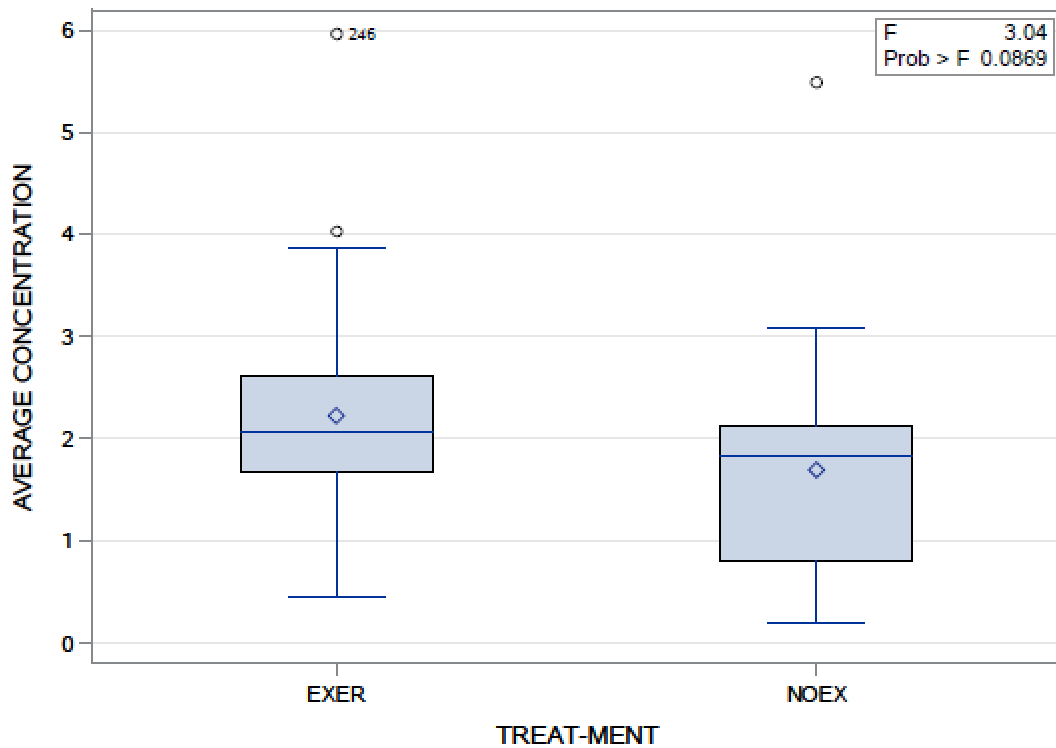


Figure 18. Comparison of LH concentrations between EXER and NOEX mares 1 d prior to confirmed ovulation. Figure from SAS output, 2016.

On the day of confirmed ovulation (d 0), the average concentration of LH in the EXER mares was 3.25 ± 1.39 ng/ml (n=30) and the concentration in the NOEX group was 2.11 ± 1.44 ng/ml (n=24). On this day of confirmed ovulation (d 0), the concentration of LH in EXER mares was determined to be higher than that of the NOEX mares (*P*=0.005) (See Figure 19).

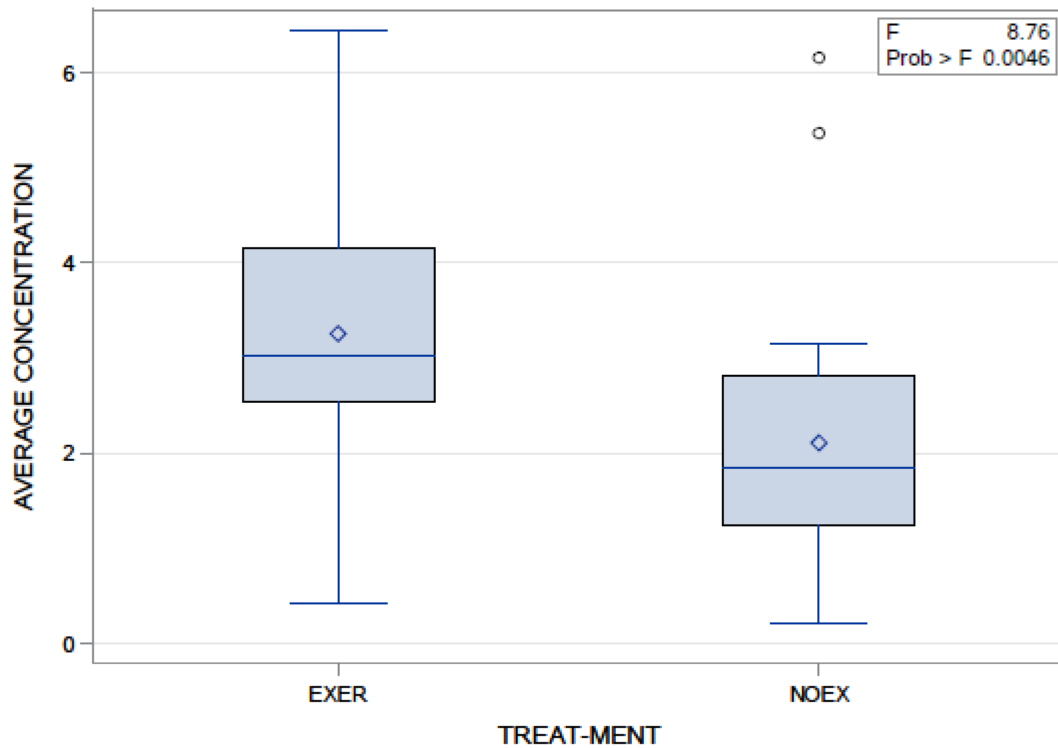


Figure 19. Comparison of LH concentrations between EXER and NOEX mares on the day of confirmed ovulation. Figure from SAS output, 2016.

When evaluating the concentrations of LH between the EXER and NOEX mares on the day following confirmed ovulation (d 1), the mean concentration of LH was 3.94 ± 1.90 ng/ml (n=30) in the EXER group and was 2.49 ± 1.76 ng/ml (n=25) in the control group. This difference was found to be statistically significant (*P*=0.005) (See Figure 20).

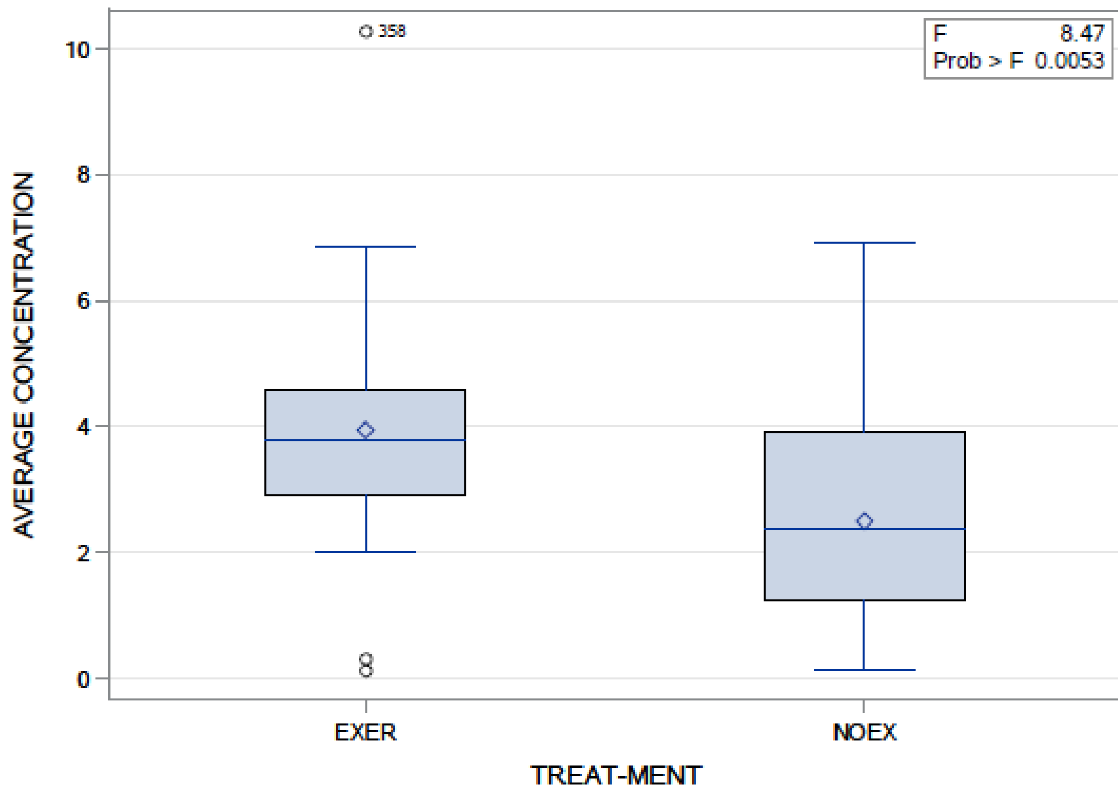


Figure 20. Comparison of LH concentrations between EXER and NOEX mares 1 d post-ovulation. Figure from SAS output, 2016.

On d 2 post-ovulation, the mean concentration of LH in the EXER mares was 5.41 ± 2.63 ng/ml (n=27) and was 3.19 ± 1.92 ng/ml (n=23) in the NOEX group. At this day of the estrous cycle, the EXER group had a significantly higher concentration of LH when compared to the NOEX group (P=0.002). Numerically, this was also the peak day of reported LH concentration in both groups (See Figure 21).

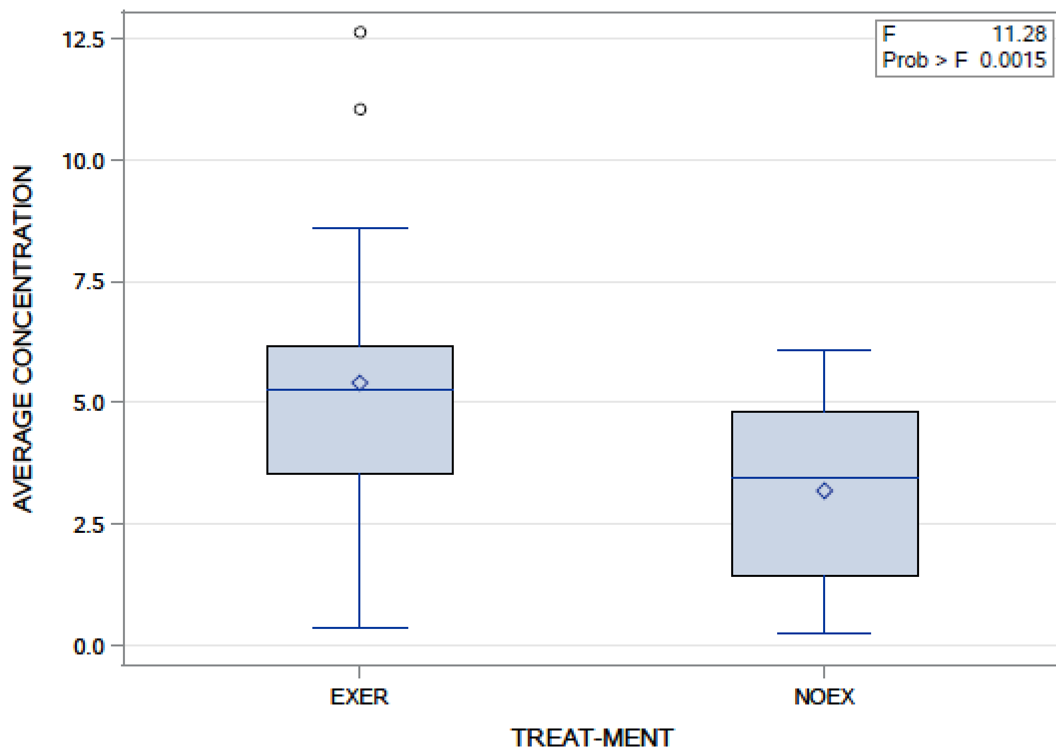


Figure 21. Comparison of LH concentrations between EXER and NOEX mares 2 d post-ovulation. Figure from SAS output, 2016.

Conversely, an equine study published by Kelley et al. (2011) evaluating similarly exercised mares reported opposite findings— this group of researchers reported exercised mares to have a lower peak LH concentration (17.3 ± 16.4 ng/ml) compared to their mean value in rested mares (41.1 ± 5.5 ng/ml).

On d 3 post-ovulation, the average concentration LH in the EXER and NOEX mares was 4.12 ± 2.47 ng/ml (n=28) and 2.47 ± 1.98 ng/ml (n=26), respectively. On this day relative to ovulation, the mean concentration of LH was found to be significantly lower (*P*=0.009) in the NOEX group compared to the EXER group. It is worth noting

that the outliers were not removed for statistical analysis, and that this day of the cycle exhibited the greatest number of statistical outliners when compared to the other days evaluated (See Figure 22).

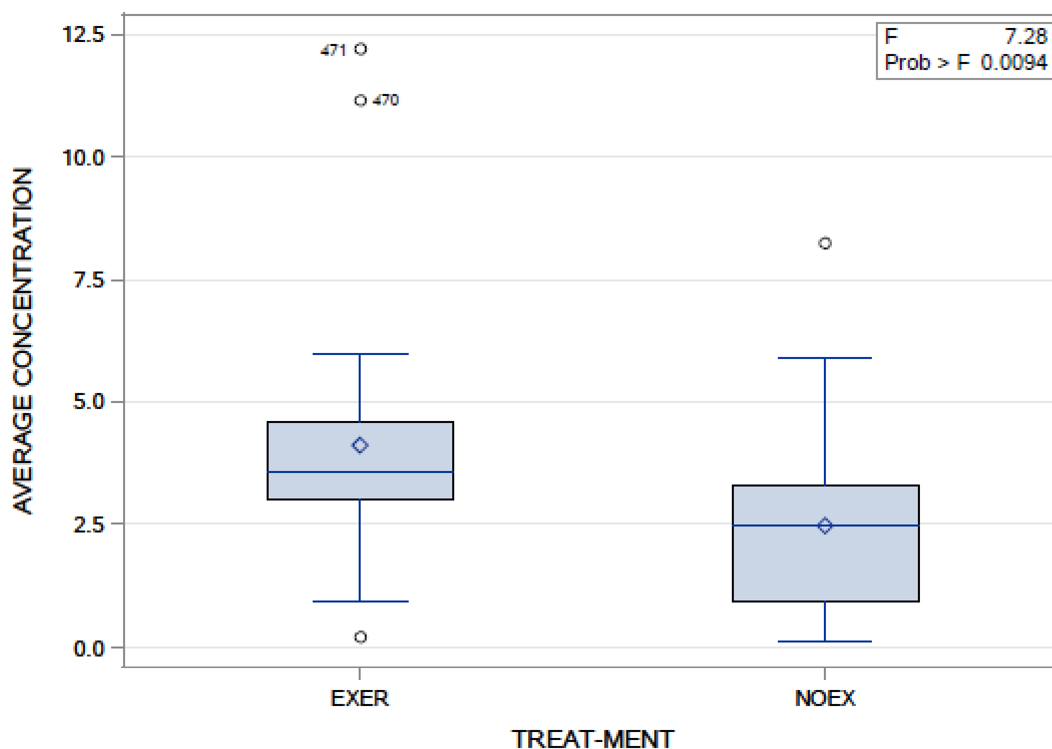


Figure 22. Comparison of LH concentrations between EXER and NOEX mares 3 d post-ovulation. Figure from SAS output, 2016.

On the fourth day post-ovulation, the mean concentration of LH was 3.12 ± 2.52 ng/ml (n=30) in the EXER group and was 1.68 ± 1.23 ng/ml (n=25) in the NOEX group. These values were found to be significantly different (*P*=0.05), with the EXER group

continuing its higher concentration of LH compared to the non-exercised group (See Figure 23).

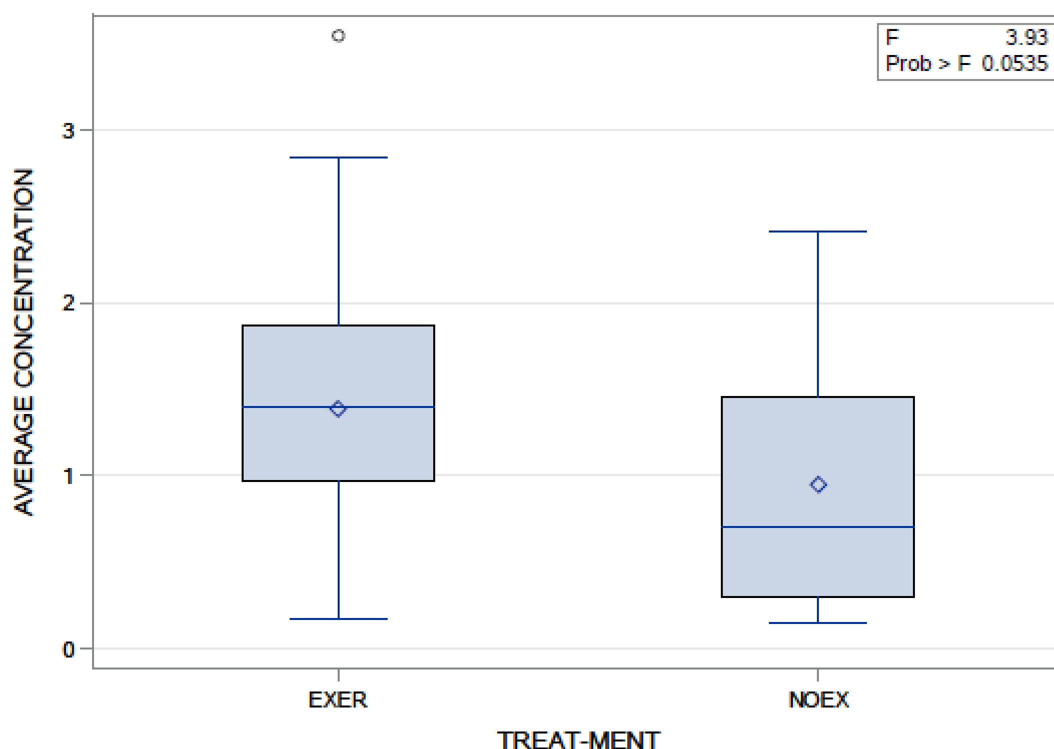
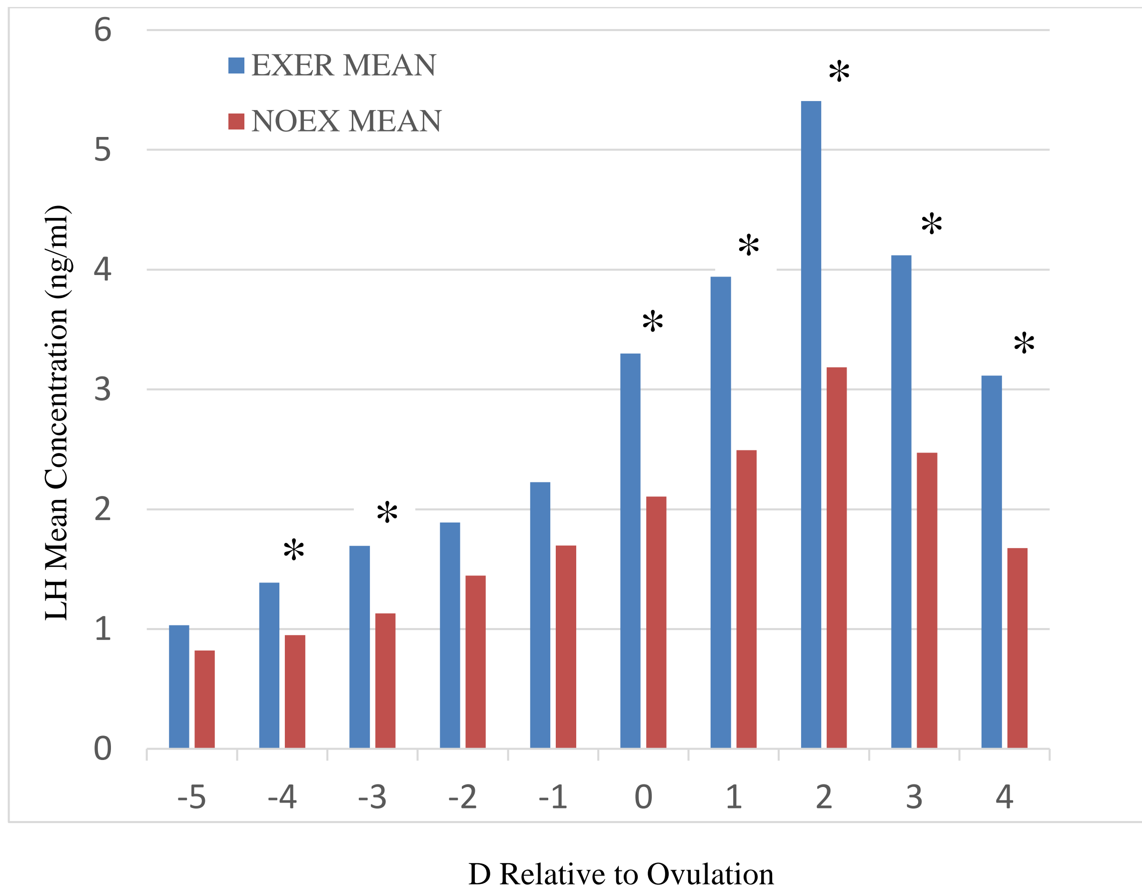


Figure 23. Comparison of LH concentrations between EXER and NOEX mares 4 d post-ovulation. Figure from SAS output, 2016.

Evaluating these LH data on a daily basis is tedious and unlikely to be applicable to the general horse-industry participant, therefore, it is more interesting to consider this data as a whole. Throughout the evaluated period of 5 d prior to ovulation through 4 d post-ovulation, it is interesting that the EXER mares demonstrated a higher LH concentration, although the average concentrations were only numerically different on d

-5 and -2, and only tended to be different on d -4, -1, and 4, but were significantly different on d -3, 0, 1, 2, and 3 relative to ovulation (See Figure 24).



D Relative to Ovulation

*Figure 24. Overall comparison of LH concentrations between EXER and NOEX mares from d -5 to d 4 relative to ovulation. Column sets indicated with * represent a significant difference of P ≤ .05 between the EXER and NOEX groups. Figure created by author.*

To evaluate the LH data in a different way and increase the sample size, the data were pooled into groups of d -4 and -3, d -2 and -1, d 0 (day of confirmed ovulation), d

1 and 2, and d 3 and 4 relative to ovulation. When evaluated together, there was a significant difference between the EXER and NOEX mares at all groupings (See Table 6 and Table 7).

Table 6. Mean summary of EXER LH concentrations for pooled groups of d -4 and -3, d -2 and -1, d 0, d 1 and 2, and d 3 and 4 relative to ovulation. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS_OVULATION_GROUP	N Obs	N	N Miss	Mean	Std Dev
A. 5 DAYS PRE-OVULATION	27	26	1	1.0314642	0.6808805
B. 3 AND 4 DAYS PRE-OVULATION	56	54	2	1.5343589	0.7337371
C. 1 AND 2 DAYS PRE-OVULATION	59	58	1	2.0642281	0.9823731
D. OVULATION	30	30	0	3.2497510	1.3907612
E. 1 AND 2 DAYS POST-OVULATION	60	57	3	4.6362072	2.3728991
F. 3 AND 4 DAYS POST-OVULATION	60	58	2	3.6007419	2.5229290

Table 7. Mean summary of NOEX LH concentrations for pooled groups of d -4 and -3, d -2 and -1, d 0, d 1 and 2, and d 3 and 4 relative to ovulation. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS_OVULATION_GROUP	**N Obs**	**N**	**N Miss**	**Mean**	**Std Dev**
A. 5 DAYS PRE-OVULATION	20	19	1	0.8199795	0.6543719
B. 3 AND 4 DAYS PRE-OVULATION	42	39	3	1.0372859	0.7466474
C. 1 AND 2 DAYS PRE-OVULATION	50	47	3	1.5743134	1.1229204
D. OVULATION	26	24	2	2.1045958	1.4395001
E. 1 AND 2 DAYS POST-OVULATION	51	48	3	2.8251885	1.8504881
F. 3 AND 4 DAYS POST-OVULATION	53	51	2	2.0815514	1.6875013

The mean LH concentration at d -4 and -3 for the EXER mares was 1.53 ± 0.73 ng/ml (n=54) and was 1.04 ± 0.75 ng/ml (n=39) for the NOEX mares. For this grouping, the EXER concentration was higher (*P*=0.002) (See Figure 25).

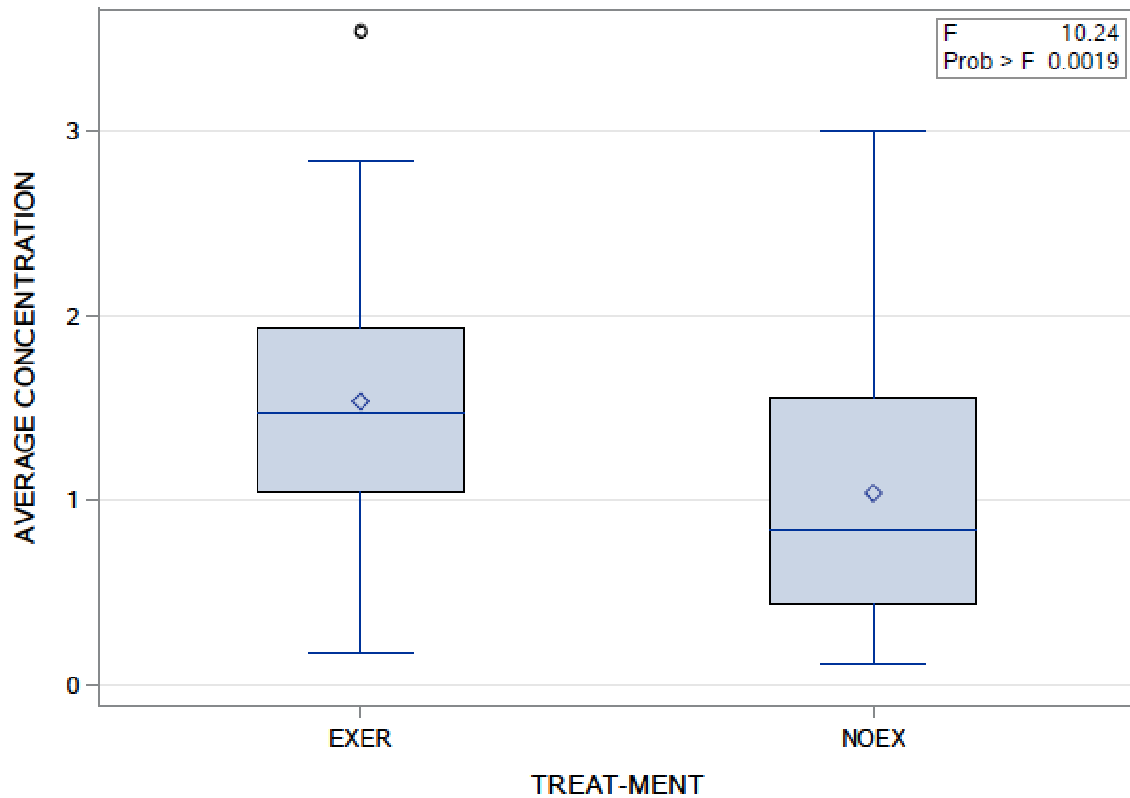


Figure 25. Comparison of pooled LH concentrations between EXER and NOEX mares at d -4 and -3 d relative to ovulation. Figure from SAS output, 2016.

For the d -2 and -1 data group, the average LH concentration was 2.06 ± 0.98 ng/ml (n=58) for the EXER group and was 1.57 ± 1.12 ng/ml (n=47) for the NOEX group. This concentration was significant at P=0.02 (See Figure 26).

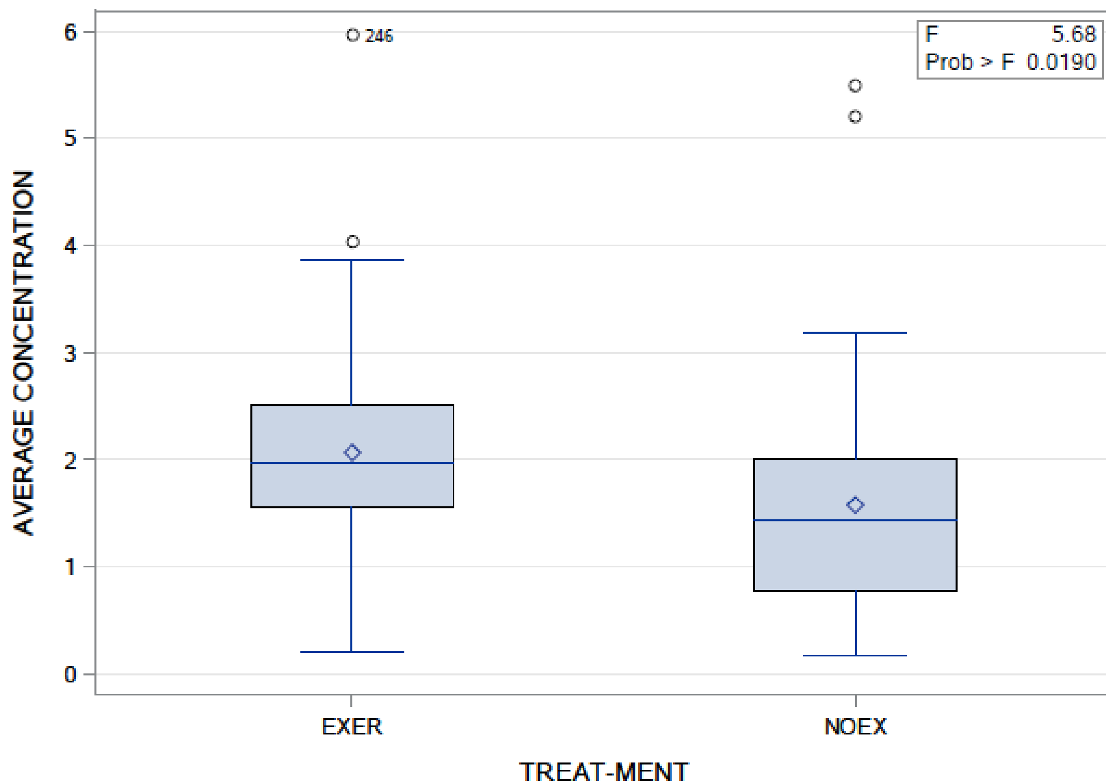


Figure 26. Comparison of pooled LH concentrations between EXER and NOEX mares at d -2 and -1 d relative to ovulation. Figure from SAS output, 2016.

As previously reported on d 0, the EXER LH concentration was higher than the NOEX (*P*=0.005). In the d 1 and 2 post-ovulation grouping, the EXER LH mean was 4.64 ± 2.37 ng/ml (n=57) was higher (*P* <0.0001) than the NOEX mean of 2.83 ± 1.85 ng/ml (n=48) (See Figure 27).

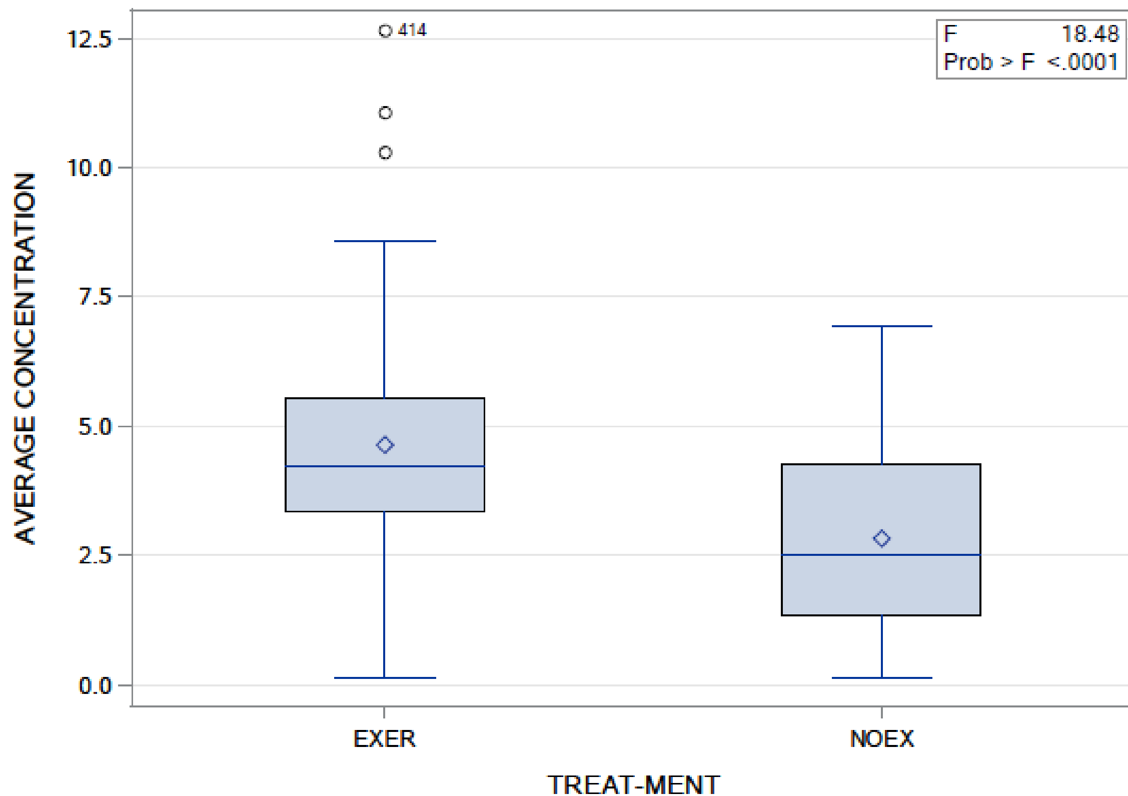


Figure 27. Comparison of pooled LH concentrations between EXER and NOEX mares at d 1 and 2 post ovulation. Figure from SAS output, 2016.

At d 3 and 4 post-ovulation, the EXER average concentration of LH was 3.60 ± 2.52 ng/ml (n=58) and was found to be significantly higher (*P*=0.0004) than the NOEX mean of 2.08 ± 1.69 ng/ml (n=51) (See Figure 28).

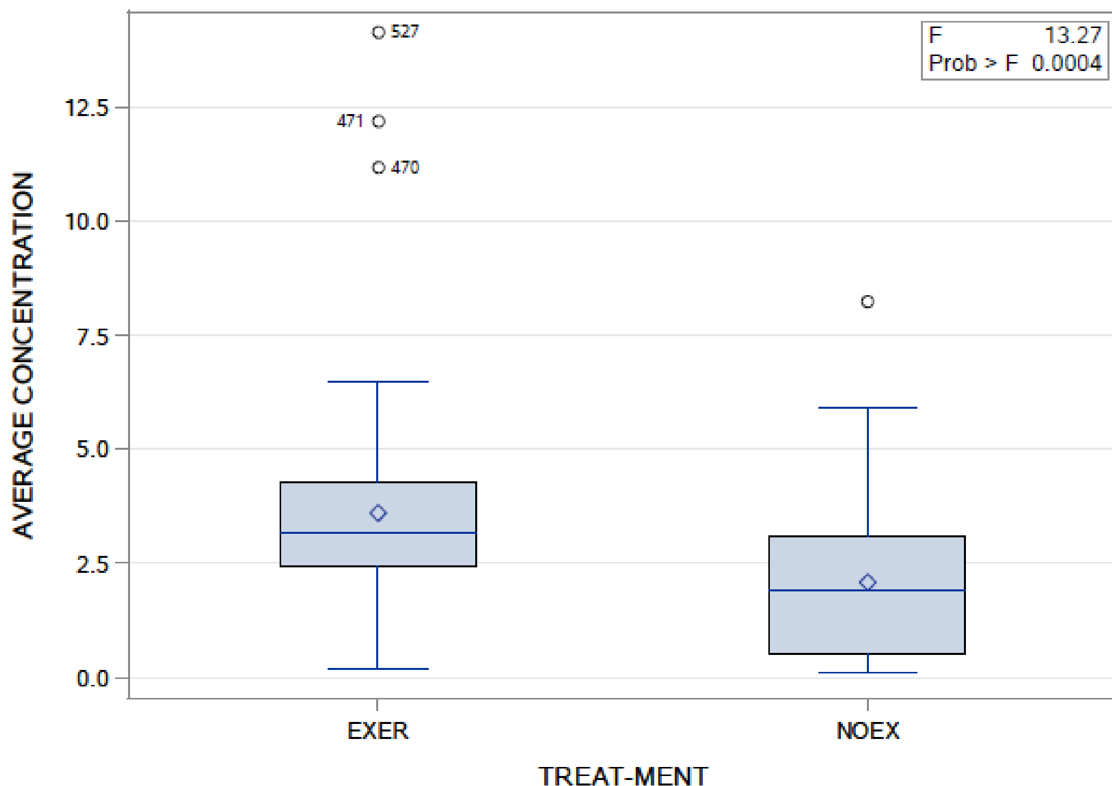


Figure 28. Comparison of pooled LH concentrations between EXER and NOEX mares at d 3 and 4 post ovulation. Figure from SAS output, 2016.

The actual numerical normal or expected concentration of LH during the estrous cycle has been difficult for researchers to determine, as different studies have reported wide variations. In the well-accepted and respected textbook *Reproductive Biology of the Mare: Basic and Applied Aspects* by Ginther (1992) the concentrations of LH reported in the cycling mare seem to more closely resemble those found and reported in the current study compared to the numerical values reported by Kelley et al. (2011). In Ginther's text (p. 234) a graph represents the lowest mean LH concentration to be less than 2 ng/ml and be found 12 to 15 d post-ovulation. This text also reports the peak of

the LH curve to occur approximately 2 d post-ovulation and the mean concentration to be approximately 8 ng/ml. However, another very popular and valued text by McKinnon and Voss (1993) entitled *Equine Reproduction*, reports equine LH RIA values ranging from approximately < 5 ng/ml at the nadir d 12 post-ovulation to a mean peak of over 80 ng/ml approximately 2 d post ovulation. Ginther (1992) suggests that these numerical differences (seen to be as much as 191-fold in various published works) may be due to widely divergent assay procedures among equine researchers and particularly related to the purity of LH assay standards that may or may not be available.

However, the overall LH profile can be visually evaluated. Both the EXER and NOEX groups' peak concentrations of LH were reported in the samples collected 2 d after ovulation was confirmed, which is similar to what has been widely published and accepted. Although the differences in absolute values show variation when compared to other studies, the overall LH profile curves for both EXER and NOEX mare from the current study are similar to what has been historically reported by other researchers. (Ginther, 1992; McKinnon and Voss, 1993).

The consistently higher concentration (although of varying degrees of significance) of LH seen in the EXER mare compared to the NOEX mares is interesting. These findings conflict with the findings of other published equine research, such as Kelley and associates (2011), which reported the peak, mean, and nadir concentration of LH to be significantly lower in exercised mares when compared to their rested counterparts. Research in other species subjected to stress has also been conflicting. Similar to the current study, Roman-Ponce, et al. (1981) reported an increase in LH in

heat-stressed dairy cattle and Ozawa et al., (2005) reported no change in peak LH concentration in heat-stressed goats. However, the bulk of published research has reported a decline in LH concentration (Madan et al., 1973; Wise et al., 1988; Gilad et al, 1993; Dobson and Smith, 2000), pulse amplitude (Gilad et al., 1993), or pulse frequency (Wise et al., 1988, Tilbrook et al., 1999) like what was reported in the equine by Kelley et al. (2011).

Progesterone Concentration in Exercising and Non-Exercising Mares

Although blood samples were collected and processed daily, researchers determined to only analyze progesterone concentrations at days 9, 15, and 21 post-ovulation in the exercised treatment (EXER) group and control group (NOEX). These days were selected under the assumptions that they would address the key parts of the estrous cycle, with d 9 giving an indication of the progesterone production when the CL should be fully productive, d 15 representing the peak of the progesterone curve, and d 21 illuminating a period where the progesterone should be on the decline in preparation for the next estrous cycle to begin, or be immediately following the next ovulation in the case of some individual shorter estrous cycles (before the CL is hormonally functional) (See Table 8 and Table 9).

Table 8. Summary of progesterone concentrations of EXER mares at d 9, 15, and 21 post-ovulation. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS POST-OVULATION	N Obs	N	N Miss	Mean	Std Dev
9	22	22	0	11.9679727	5.2118287
15	22	22	0	6.7827841	3.9366702
21	22	8	14	4.1754538	4.5056244

Table 9. Summary of progesterone concentrations of NOEX mares at d 9, 15, and 21 post-ovulation. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS POST-OVULATION	N Obs	N	N Miss	Mean	Std Dev
9	21	21	0	14.1512286	5.4199379
15	20	19	1	9.4586368	3.9959604
21	19	15	4	7.1486640	4.4218041

At d 9 post ovulation, the average concentration of progesterone in the EXER group was determined to be 11.97 ± 5.21 ng/ml (n=22) compared to 14.15 ± 5.42 ng/ml (n=21) the NOEX control group. This difference was not found to be statistically significant (*P*= 0.19) (See Figure 29).

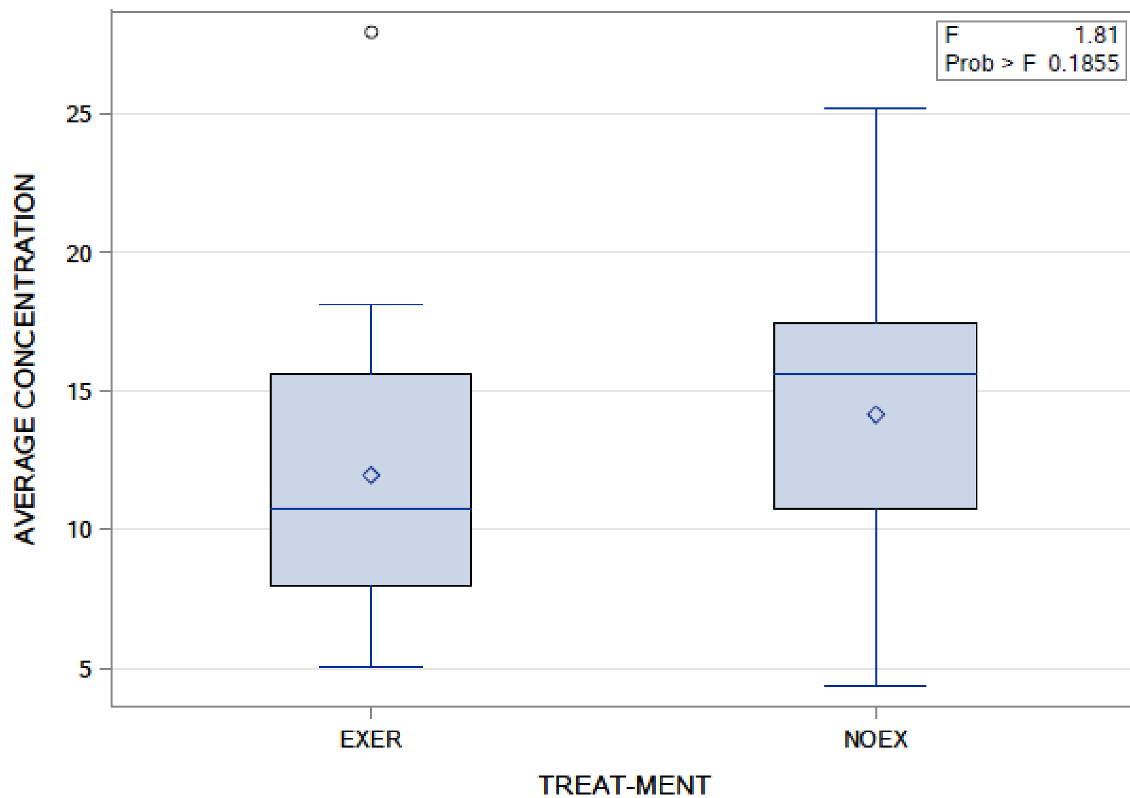


Figure 29. Comparison of progesterone concentration between EXER and NOEX mares at d 9 post-ovulation. Figure from SAS output, 2016.

However, at d 15 post-ovulation, mean progesterone concentrations was found (*P*=0.04) to be lower in the EXER group (6.78 ± 3.94 ng/ml, n=22) compared to the NOEX group (9.46 ± 4.00 ng/ml, n=19) (See Figure 30).

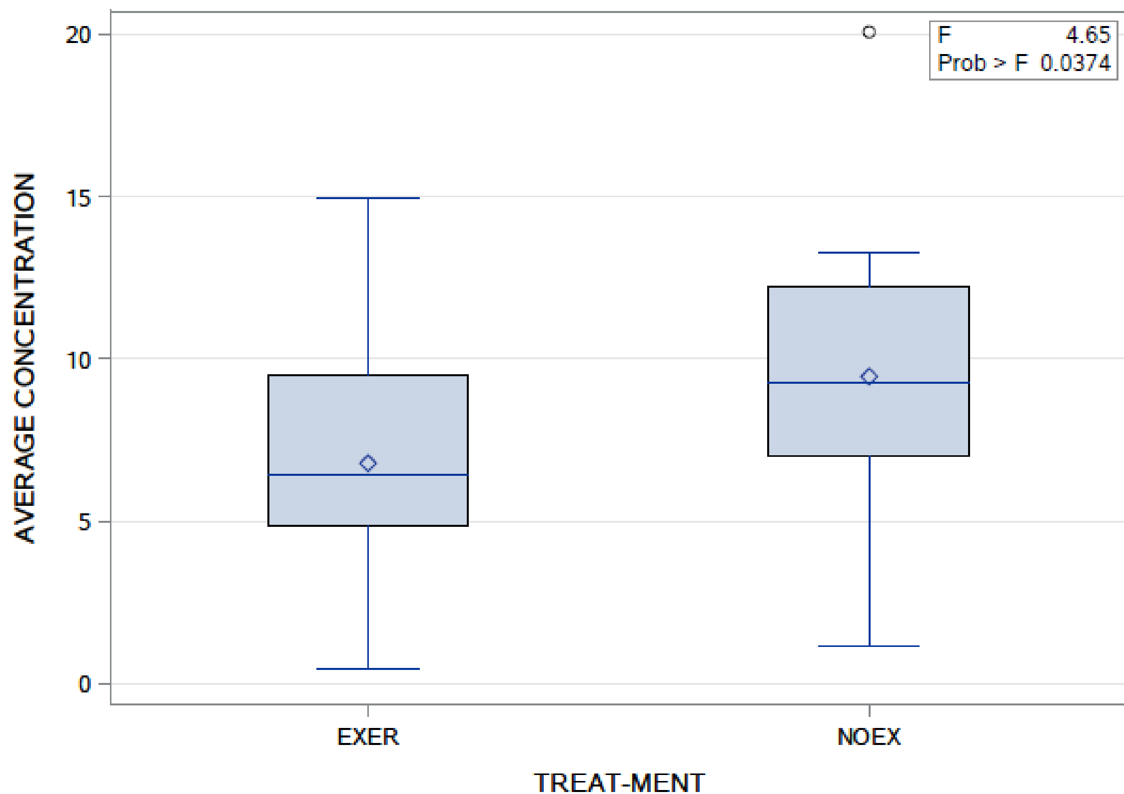


Figure 30. Comparison of progesterone concentration between EXER and NOEX mares at d 15 post-ovulation. Figure from SAS output, 2016.

At d 21 post-ovulation, initial analysis of the available data found the mean progesterone concentration to be 4.18 ± 4.51 ng/ml for the EXER group (n=8) and 7.15 ± 4.42 ng/ml for the NOEX group (n=15). Analysis of these data demonstrated no difference in the concentration of progesterone between the EXER and NOEX groups at d 21 post ovulation (*P*=0.14) (See Figure 31).

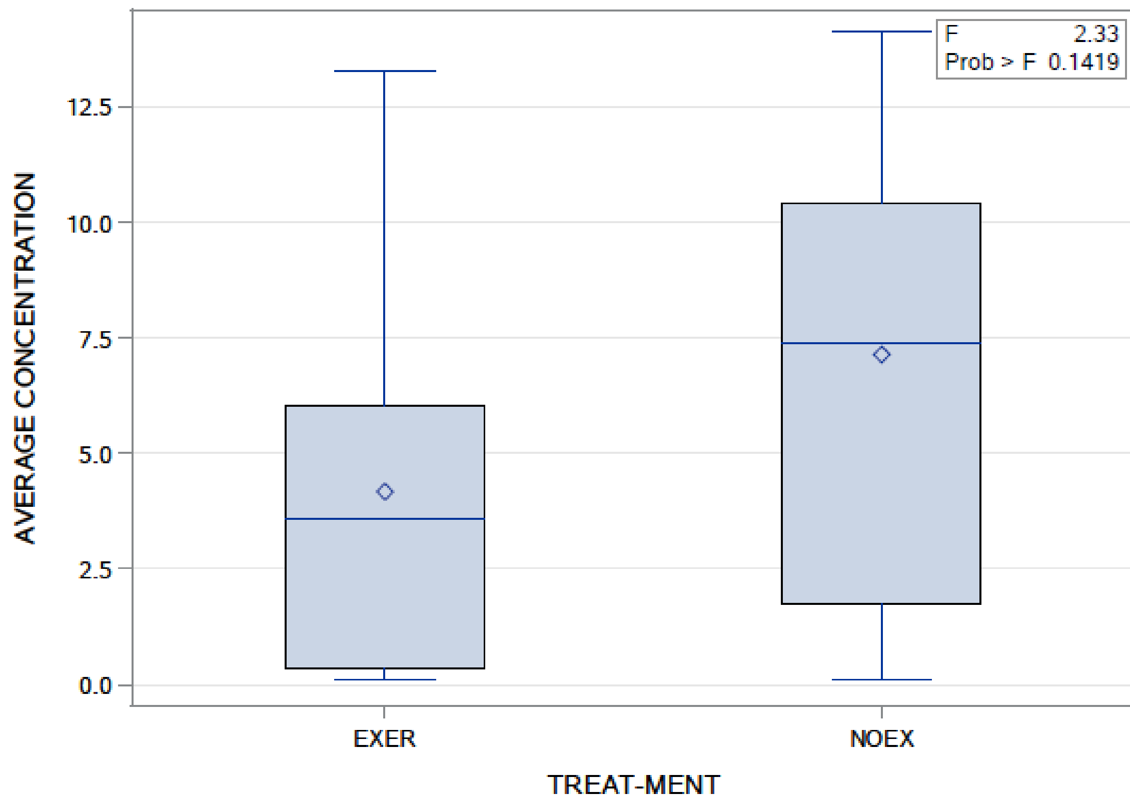


Figure 31. Comparison of progesterone concentration between EXER and NOEX mares at d 21 post-ovulation. Figure from SAS output, 2016.

The sensitivity of the RIA used to evaluate these concentrations is known to be sensitive from 0.10 ng/ml to 40.00 ng/ml. At this late point of the estrous cycle, it is expected for progesterone levels to be very low as the mare prepares to return to estrus. Therefore, researchers speculate that it may be a possibility for the actual levels progesterone to be below the sensitivity of the assay, resulting in the missing data points. When evaluating the raw data there were readings collected for the EXER group as low as 0.106 and 0.196 ng/ml, and readings as low as 0.114 ng/ml and 0.164 ng/ml in the NOEX group, which supported that this event could be a possibility. To attempt to

combat this possibility, the missing data points for both d 21 groups were arbitrarily completed with 0.1 ng/ml values and analyses were recompleted. Once reanalyzed in this manner, progesterone concentration was found to be significantly lower (P= 0.0029) in the revised EXER group mean of 1.58 ± 3.29 ng/ml (n=22) compared to the original NOEX mean of 5.67 ± 4.89 ng/ml (n=19). Again, these data are only valuable if it could be determined that the missing values of progesterone concentration at d 21 were due to the actual values being below the sensitivity of the RIA (See Table 10, Table 11, and Figure 32).

Table 10. Revised summary of progesterone concentrations at d 9, 15, and 21 of EXER mares with data supplemented with 0.1 ng/ml values at missing data points. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS POST-OVULATION	N Obs	N	N Miss	Mean	Std Dev
9	22	22	0	11.9679727	5.2118287
15	22	22	0	6.7827841	3.9366702
21	22	22	0	1.5819832	3.2853289

Table 11. Revised summary of progesterone concentrations at d 9, 15, and 21 of NOEX mares with data supplemented with 0.1 ng/ml values at missing data points. Table from SAS output, 2016.

The MEANS Procedure

Analysis Variable : AVERAGE_CONCENTRATION AVERAGE CONCENTRATION					
DAYS POST-OVULATION	N Obs	N	N Miss	Mean	Std Dev
9	21	21	0	14.1512286	5.4199379
15	20	19	1	9.4586368	3.9959604
21	19	19	0	5.6647347	4.8911979

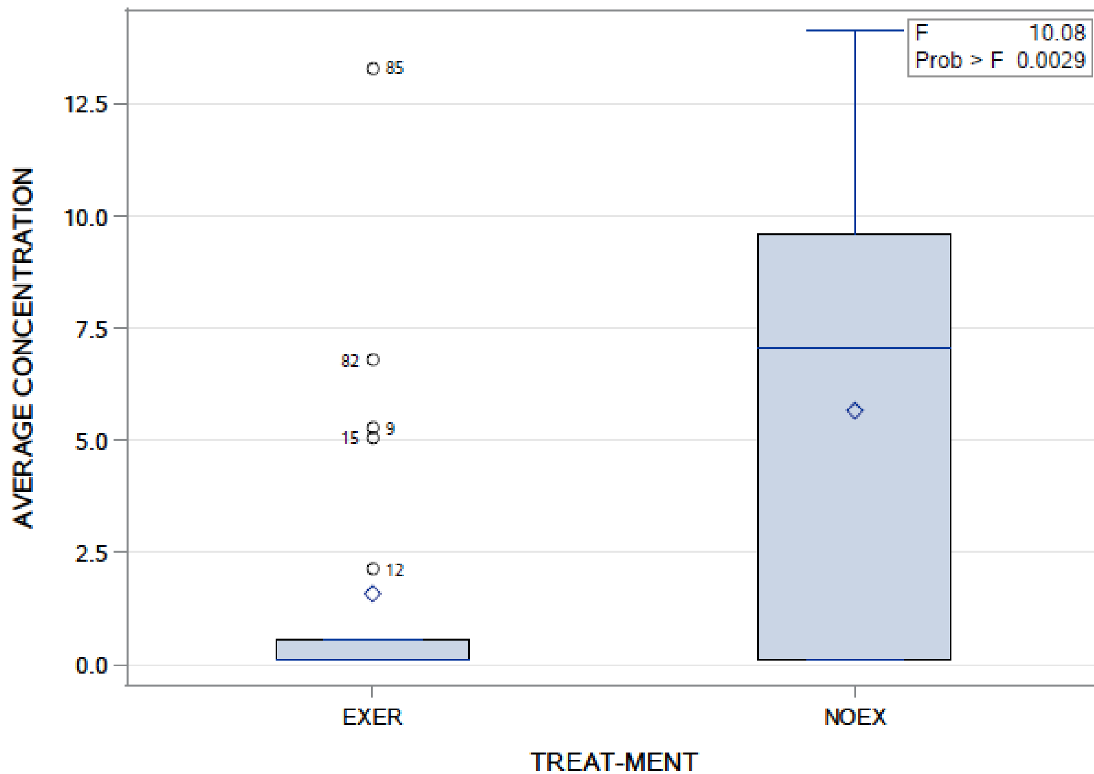


Figure 32. Revised mean comparison of progesterone concentration of EXER and NOEX mares at d 21 post-ovulation (with supplemented values of 0.1 ng/ml at missing data points due to suspected assay limitations). Figure from SAS output, 2016.

The current study may pose more questions than it does to provide answers regarding the presence of any influence of exercise-induced stress on progesterone concentrations in the equine, especially considering concentrations at d 21 post-ovulation. Published research in dairy cattle is also very conflicting, with some researchers reporting an increase in progesterone in heat-stressed cattle (Roman-Ponce, et al., 1981; and review by Rensis and Scaramuzzi, 2003) or exercise-stressed humans (Bonen et al., 1979) with others reporting a decline (Rosenberg et al., 1977; Younas et

al., 1993; Jonsson et al., 1997, Ronchi et al., 2001) in animal exposed to stressful situations. Rensis and Scaramuzzi (2003) suggested that extraneous factors beyond the stressor of consideration may be influencing the production and concentration of progesterone during the luteal phase of the estrous cycle. This is very likely, and further research to evaluate the potential change in progesterone production in the equine female athlete is warranted.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study was designed to evaluate the potential effect of exercise-induced stress upon the ovarian activity and the concentrations of cortisol, progesterone, and LH in the mare. To accomplish this, a study was conducted over two physiologic breeding seasons. Six mares were subjected to an exercise protocol similar to that which could be experienced by a horse engaged in a training program within the equine industry, and their ovarian dynamics and concentrations of cortisol, LH, and progesterone were evaluated and compared to six non-exercising counterparts. The experimental treatment group was exercised five d each wk, and blood samples and ovarian activity were collected or evaluated daily for later analysis. Cortisol and progesterone concentrations were determined with commercially-available RIA kits and LH concentrations were determined with a double-antibody RIA procedure. Follicular dynamics were evaluated by transrectal ultrasonography.

Although the interovulatory period and ultimate size of the dominate follicle were not found to differ, some differences in ovarian activity and follicular dynamics were found between the exercising and rested mares. At d -5 relative to ovulation, the rested mares had more small follicles <10 mm present on the ovaries, but the exercised mares had more medium and large follicles present on the ovaries, 10-15 mm and 15.1-25 mm, respectively. When evaluating the total number of follicles present, the exercised mares were found to have significantly (P=.001) more total antral follicles

present on the ovaries, however, the actual means were 17.81 ± 1.12 for the exercised mares and 17.43 ± 1.78 for the rested mares, suggesting a complication in statistical analyses and the unlikelihood of any applicable difference. This is likely due to the small sample size available and a wide degree of individual variation. Although the influence of exercise on the total number of follicles present at d -5 relative to ovulation is debatable, the increased number of 10-25 mm follicles in exercised mares, particularly at this time of follicular deviation, may indicate a reduced dominance of the ovulatory follicle. This is supported by the findings and conclusions of other researchers that have evaluated heat- and exercise- induced stress in the equine and other species.

Immediately prior to confirmed ovulation, there tended to be more total follicles present on the ovaries of the exercised group in the 10-15 mm subgrouping. There were significantly more follicles in the 15.1-25 mm and the >25 mm subgroups in the rested mares immediately prior to ovulation. This shift in follicle size population at ovulation is interesting, and conflicts with the idea of reduced dominance of the ovulatory follicle at this time of the estrous cycle. However, the differences found in ovarian dynamics between the two groups, coupled with the existing body of published works in the related fields, lead the current researchers to believe that exercise-induced stress does exert an influence on the ovarian dynamics of the exercising female.

In regard to the lack of difference in basal cortisol levels between the exercising and rested mares, similar results have been reported by others equine and non-equine species. The acute increase in cortisol concentration following a stressful event is well documented and understood in many species, but it is interesting to find that the basal

cortisol concentration does not appear to increase when an individual is subjected to a long-term exercise program. Therefore, the current study suggests that the changes seen in the ovarian and reproductive hormone differences are not due the influence of an increase in basal cortisol concentration.

The general profiles of LH concentration and expression in the exercised and rested mares were similar to that which is historically reported in the equine—with a dramatic increase in LH on the days preceding ovulation, a peak in concentration approximately 2 d post-ovulation, and a sharp decline over the following days. However, the elevated LH in the exercising mares at d -4, -3, 0, 1, 2, 3, and 4 (or as grouped at d -4 and -3, -2 and -2, 0, 1 and 2, and 3 and 4) relative to ovulation was interesting and unexpected. These findings contradict those reported by other animal scientists, many of which have consistently reported a decline in LH concentration in response to a stressor. This increase in LH seen in the exercising mares could potentially be explained by a decline in progesterone production and its associated feedback on the HPG axis, however, our progesterone results were inconclusive. Therefore, it was interesting to find the LH profile of the exercising mare to elevated compared to their resting counterparts, but the cause is not clearly defined. However, the current group of researchers suggest that a moderate exercise program, similar to what was employed in the present study, does not appear to result in reduction in LH concentration like that which may be witnessed in more severe stressors.

In regard to progesterone, the limitations of the assay employed were unexpected and unfortunate. At d 9 post-ovulation, when progesterone production and

concentration should be on the rise, it was interesting to find that there was no difference in progesterone levels between the exercising and rested groups of mares. Interestingly, at d 15 post-ovulation (when the corpus luteum should be at its most productive), the exercised mares had a significantly lower mean concentration of progesterone compared to the non-exercised mares. However, the lower mean progesterone concentration seen in the exercised mares was still found to be considered to be clinically acceptable for that stage of the estrous cycle and may not ultimately effect the success of a pregnancy.

Due to a limitation in sensitivity for the assay utilized, the difference in circulating progesterone between the exercising and non-exercising mares at d 21 post-ovulation is suspected to be lower in exercising mares, but that could not be positively concluded. Many samples from the exercising mares at this time period were unreadable by this commercially-available assay kit. Researchers believe that this was due to these samples' actual values falling below the lowest extreme of the assay's capabilities of 0.1 ng/ml. When the missing data points were excluded from analysis, greatly reducing the sample size, there was no difference in progesterone concentration found between the two treatment groups. However, when the missing data points were substituted with the lowest limit of the assay (0.1 ng/ml), which may be higher than the majority of the actual values, the exercised mares were found to have a lower concentration of progesterone at d 21 post-ovulation. Although it may offer little practical application, it would be interesting to continue studying the progesterone profile exercising mares to solidify the presence or absence of an influence of exercise on progesterone concentration.

It is important to consider how this new information could impact the human medical field. Fitness continues to become more important in our society, and many doctors allow or recommend exercise to continue when women are attempting to conceive or maintain a pregnancy. The existing body of literature, coupled with the findings of the current study, support the existence of an influence of exercise-induced stress on the follicular dynamics and the concentrations of progesterone and LH in the female. Additionally, other research has strongly suggested that these and other changes seen in stressed females may be a detriment to fertility and reproductive success. Therefore, livestock producers and human medical professionals should all consider the need to reduce stress, exercise-induced or otherwise, in reproductively active females—not only to maximize profit margins in livestock operations, but to also help women conceive and attain motherhood.

LITERATURE CITED

Abilay, T.A., H.D. Johnson, and M. Madan. 1975. Influence of environmental heat on peripheral plasma progesterone and cortisol during the bovine estrous cycle. J. Dairy Sci. 58 (12): 1836-1840.

Allen, W.R. and R.M. Moor. 1972. The origin of the equine endometrial cups. J. Repro. Fertility. 29(2) 313-316.

Allen, W.R. 2001. Luteal deficiency and embryo mortality in the mare. Repro. in Dom. Ani. 36(3-4): 121-131.

Anton, J.E., K.L. Vernon, D.E. Kelley, J.R. Gibbons, G. Birrenkott, and C.J. Mortensen. 2014. Exercising the pregnant mare from day 16 to day 80 of gestation. J. Equine Vet. Sci. 34: 415-420.

Bonan, A., A.N. Belcastro, A.A. Simpson, and W. Ling. 1978. Comparison of LH and FSH concentrations in age-group swimmers, moderately active girls and adult women. Inter. Series on Sport Sci. 6: 70-78.

Bonan, A., W.Y. Ling, K.P. MacIntyre, R. Neil, J.C. McGrail, and A.N. Belcastro. 1979. Effects of exercise on the serum concentrations of FSH, LH, progesterone, and estradiol. Eur. J. Appl. Physiol. 42: 15-23.

Desmecht, D., A. Linden, H. Amory, T. Art, and P. Lekeux. 1996. Relationship of plasma lactate production to cortisol release following completion of different types of sporting events in horses. Vet. Research Communications. 20 (4): 371-379.

Dobson, H. 1987. Effect of stress during shearing on the LH response to GnRH in anestrous ewes. J. Agric. Sci. 110: 673-676.

Dobson, H., and Smith, R.F. 2000. What is stress, and how does it affect reproduction? Ani. Repro. Sci. 60-61: 743-752.

Dworaczyk, M.C., M.M. Vogelsang, B.D. Scott, D.H. Sigler, S.G. Vogelsang, and T.H. Welsh. 2010. The effect of exercise-induced heat stress on ovarian dynamics in the mare. ISER 10 Program. Ani. Repro. Sci. 121 (1): 47-48.

Engler, D., T. Pham, M.J. Fullerton, G. Ooi, J.W. Funder, and I.J. Clarke. 1989. Studies of the secretion of corticotrophin-releasing factor and arginine vasopressin into the hypophysial-portal circulation of the conscious sheep. Neuroendocrinology. 49: 367-381.

Gilad, E., R. Meidan, A. Berman, Y. Graber, and D. Wolfenson. 1993. Effect of heat stress on tonic and GnRH-induced gonadotrophin secretion in relation to concentration and estradiol in plasma of cyclic cows. J. Repro. Fert. 99: 315-321.

Ginther, O.J. 1992. Reproductive biology of the mare: Basic and applied aspects. Cross Plains, WI: Equiservices Publishing.

Ginther, O.J., M.A. Beg, D.R. Bergfelt, F.X. Donadeu, and K. Kot. 2001. Follicle selection in monovular species. Bio. Repro. 65: 638-647.

Ginther, O.J., M.A. Beg, M.O. Gastal, and E.L. Gastal. 2004. Follicle dynamics and selection in mares. Anim. Repro. 1(1): 45-63.

Goldstein, D.S. 1987. Stress-induced activation of the sympathetic nervous system. Bailliere's Clin. Endo. Met. 1 (2): 253-278.

Guzeloglu, A., J.D. Ambrose, T. Kassa, T. Diaz, M.J. Thatcher, and W.W. Thatcher. 2001. Long-term follicular dynamics and biochemical characteristics of dominant follicles in dairy cows subjected to acute heat stress. Ani. Repro. Sci. 66 (1-2): 15-34.

Henneke, D.R., G.D. Potter, and J.L. Kreider. 1981. A condition score relationship to body fat content of mares during gestation and lactation. Proc 7th ENPS, Warrenton, VA. 105-110.

Irvine, C.H.G. and S.L. Alexander. 1994. Factors affecting the circadian rhythm in plasma cortisol concentrations in the horse. Dom. Ani. Endo. 11 (2): 227-238.

Jonsson, N.N., M.R. McGowan, K. McGuigan, T.M. Davison, A.M. Hussain, M. Kafi, and A. Matschoss. 1997. Relationships among calving season, heat load, energy balance and postpartum ovulation of dairy cows in a subtropical environment. Ani. Repro. Sci. 47 (4): 315-326.

Jordan, E.R. 2003. Effects of heat stress on reproduction. J. Dairy Sci. 86 (E. Suppl.): E104-E114.

Jurkowski, J.E., N.L. Jones, W.C. Walker, E.V. Younglai, and J.R. Sutton. 1978. Ovarian hormonal response to exercise. J. Appl. Physiol. 44: 109-114.

Kelley, D.E., J.R. Gibbons, S.E. Pratt, R.L. Smith, and C.J. Mortensen. 2008. The effect of exercise on folliculogenesis in mares. Repro. Fertility Dev. 21 (1): 185-186.

Kelley, D.E., J.R. Gibbons, S.E. Pratt, R.L. Smith, and C.J. Mortensen. 2009. J. of Equine Vet. Sci. 29 (5): 337-338.

Kelley, D.E., J.R. Gibbons, R. Smith, K.L. Vernon, S.E. Pratt-Phillip, and C.J. Mortensen. 2011. Exercise effects both ovarian follicular dynamics and hormone concentrations in mares. Theriogenology. 76: 615-622.

Kasimanickam, R., S. Schroeder, M. Assay, V. Kasimanickam, D.A. Moore, J.M. Gay, and W.D. Whittier. 2014. Influence of temperament score and handling facility on stress, reproductive hormone concentrations, and fixed time AI pregnancy rates in beef heifers. Repro. Dom. Anim. 49 (5): 775-782.

Lassourd, V., V. Gayrard, V. Laroute, M. Alvinerie, P. Benard, D. Courtot, and P.L. Toutain. 1996. Cortisol disposition and production rate in horses during rest and exercise. Amer. J. Physiology. 271 (1): R25-R33.

Madan, M.L. and H.D. Johnson. 1973. Environmental heat effects on bovine luteinizing hormone. J. Dairy Sci. 56 (11): 1420-1423.

Marc, M., N. Parvizi, F. Ellendorff, E. Kallweit, and F. Elsaesser. 2000. Plasma cortisol and ACTH concentrations in the warmblood type horse in response to a standardized treadmill exercise test as physiological markets for evaluation of training status. J. Ani. Sci. 78 (7): 1936-1946.

McKinnon, A.O. and J.L. Voss. 1993. Equine reproduction. Philadelphia: Lea and Febiger.

Mesa, A.M., D.Y. Kim, and C.J. Mortensen. 2015. Effect of exercise on ovarian function in cycling gilts. Unpublished manuscript.

Mastorakos, G., M. Pavlatou, E. Diamanti-Kandarakis, G.P. Chrousos. 2005. Exercise and the stress system. Hormones. 4: 73-89.

Mihm, M., Baguisi, A., Boland, M.O., and Roche, J.F. 1994. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. J. Repro. Fert. 102: 123-130.

Mortensen, C.J., Choi, Y.H., Hinrichs, K., Ing, N.H., Kraemer, D.C., Vogelsang, S.G., and Vogelsang, M.M. 2009. Embryo recovery from exercised mares. Ani. Repro. Sci. 110, 237-244.

Nagata, S., F. Takeda, M. Kurosawa, K. Mima, A. Hiraga, and K. Taya. 1999. Equine Vet. J., Suppl. 30: 570-574.

Nambo, Y., M. Oikawa, T. Yoshihara, A. Kuwano, S. Hobo, S. Nagata, G. Watanabe, and K. Taya. 1996. J. Equine. Sci. 7 (1): 1-5.

Nanda, A.S., W.R. Dobson, and W.R. Ward. 1990. Relationship between an increase in plasma cortisol during transport-induced stress and failure of estradiol to induce a luteinizing hormone surge in dairy cows. Anim. Sci. Technol. 65: 25-28.

Olive, D.L. 2010. Exercise and fertility: an update. Current Opinion in Ob. Gyn. 22: 259-263.

Ozawa, M., D. Tabayashi, T.A. Latief, T. Shimizu, I. Oshima, and Y. Kanai. 2005. Alterations in follicular dynamics and steroidogenic abilities induced by heat stress during follicular recruitment in goats. Reproduction. 129: 621-630.

Peyser, M.R., D. Ayalon, A. Harell, R. Toaff, and T. Cordova. 1973. Stress induced delay of ovulation. Ob. Gyn. 42 (5): 667-670.

Plotsky, P.M., E.T. Cunningham, Jr., and E.P. Widmaier. 1989. Catecholaminergic modulation of corticotrophin-releasing factor and adrenocorticotropin secretion. Endocrine Reviews. 10(4): 437-458.

Rensis, F.D. and Scaramuzzi. 2003. Heat stress and seasonal effects on reproduction in the dairy cow— a review. Theriogenology. 60: 1139-1151.

Rivier, C. and S. Rivest. 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Bio. Repro. 45 (4): 523-532.

Roman-Ponce, H., W.W. Thatcher, and C.J. Wilcox. 1981. Hormonal interrelationships and physiological responses of lactating cows to a shade management system in a subtropical environment. Theriogenology. 16 (2): 139-154.

Romero, L.M. and L.K. Butler. 2007. Endocrinology of Stress. International J. Comparative Psychology. 20: 89-95.

Ronchi, B., G. Strandaioli, A. Verini Supplizi, U. Bernabucci, N. Lacetera, P.A. Accorsi, A. Nardone, and E. Seren. 2001. Influence of heat stress of feed restriction on plasma progesterone, estradiol-17β, LH, FSH, prolactin and cortisol in Holstein heifers. Livestock Prod. Sci. 68 (2-3): 231-241.

Rosenberg, M., Z. Herz, and Y. Folman. 1977. Seasonal variations in post-partum plasma progesterone levels and conception in primiparous and multiparous dairy cows. J. Repro. Fert. 51: 363-367.

Roth, Z., R. Meidan, R. Braw-Tal, and D. Wolfenson. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. J. Repro. Fert. 120: 83-90.

Selye, H. 1946. The general adaptation syndrome and the diseases of adaptation. J. Clin. Endo. 6: 117-230.

Senger, P.L. 1997. Pathways to pregnancy and parturition. Current Conceptions, Inc. 1615 NE Eastgate Blvd.

Smart, D., A.J. Forhead, R.F. Smith, and H. Dobson. 1994. Transport stress delays the estradiol-induced LH surge by a non-opioidergic mechanism in the early postpartum ewe. J. Endocrinol. 142: 447-451.

Smith, R.L., K.L. Vernon, D.E. Kelley, J.R. Gibbons, and C.J. Mortensen. 2012. Impact of moderate exercise on ovarian flow and early embryonic outcomes in mares. J. Anim. Sci. 90: 3770-3777.

Stoebel, D.P. and G.P. Moberg. 1982. Repeated acute stress during the follicular phase and luteinizing hormone surge of dairy heifers. J. Dairy Sci. 65: 92-96.

Stott, G.H. 1981. What is animal stress and how is it measured? J. Anim. Sci. 52 (1): 150-153.

Sutton, J.R., M.L. Coleman, J. Casey, L. Lazarus. 1973. Androgen responses during physical exercise. Br. Med. J. 1: 520-523.

Tillbrook, A.J., B.J. Canny, M.D. Serapiglia, and T.J. Amborse. 2000. Suppression of the secretion of luteinizing hormone due to isolation/restraint stress in

gonadectomised rams and ewes is influenced by sex steroids. J. Endocrinology. 160: 469-481.

Trout, J.P., L.R. McDowell, and P.J. Hansen. 1998. Characteristics of the estrous cycles and antioxidant status of lactating Holstein cows exposed to heat stress. J. Dairy Sci. 81 (5): 1244-1250.

Vaught, L.W., D.E. Monty, Jr., and W.C. Foote. 1977. Effect of summer heat stress on serum luteinizing hormone and progesterone values in Holstein-Friesian cows in Arizona. Amer. J. Vet. Research. 38 (7): 1027-1030.

Warren, M.P. and N.E. Perlroth. 2001. The effects of intense exercise on the female reproductive system. J. Endo. 170: 3-11.

Williams, R.J., D.L. Marlin, N. Smith, R.C. Harris, W. Haresign, and M.C. Davies Morel. 2002. Effects of cool and hot humid environmental conditions on neuroendocrine responses of horses to treadmill exercise. Vet. J. 164 (1): 54-63.

Williams, G.L., M. Amstalden, G.P. Blodgett, J.E. Ward, D.A. Unnerstal, and K.S. Quirk. 2007. Continuous administration of low-dose GnRH in mares I. control of persistent anovulation during the ovulatory season. Theriogenology. 68 (1) 67-75.

Wilson, S.J., R.S. Marion, J.N. Spain, D.E. Spiers, D.H. Keisler, and M.C. Lucy. 1998. Effects of controlled heat stress on ovarian function of dairy cattle. 1. Lactating cows. J. Dairy Sci. 81 (8): 2124-2131.

Wise, M.E., R.E. Rodriguez, D.V. Armstrong, J.T. Huber, F. Wiersma, and R. Hunter. 1988. Fertility and hormonal responses to temporary relief of heat stress in lactating dairy cows. Theriogenology. 29 (5): 1027-1035.

Wolfenson, D., W.W. Thatcher, L. Badinga, J.D. Savio, R. Meidan, B.J. Lew, R. Braw-tal, and A. Berman. 1995. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. Bio. Repro. 52: 1106-1113.

Wolfenson, D., B.J. Lew, W.W. Thatcher, Y. Graber, and R. Meidan. 1997. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. Ani. Repro. Sci. 47 (1-2): 9-19.

Younas, M., J.W. Fuquay, A.E. Smith, and A.B. Moore. 1993. Estrous and endocrine responses of lactating Holsteins to forced ventilation during summer. J. Dairy Sci. 76 (2): 430-436.

APPENDIX A

Equine LH RIA Protocol

Additional methodology

Equine LH radioimmunoassay

1. Iodinated Product: Iodination grade eLH (AFP-5130A)

2. Antibody: Anti-equine LH (AFP-240580). Dilution 1:100,000

3. Standards (stds): Iodination grade equine LH (AFP-5130A; 0.1 – 20.0 ng/mL)

4. References (ref): equine LH added to equine serum

5. RIA Procedure:

   Day 1: Begin Assay

   1. NSB – 500 µl of 1% PBS-EW (egg white)
   2. 0 Std – 500 µl of 1% PBS-EW
   3. Stds – 200 µl std + 300 µl of 1% PBS-EW
   4. Ref – 200 µl ref + 300 µl of 1% PBS-EW
   5. Unknown – 200 µl unknown sample + 300 µl of 1% PBS-EW
   6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only
   7. Pipette 200 µl of anti-eLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
   8. Pipette 100 µl 125I-eLH (20,000 cpm/100 µl diluted in 1% PBS-EW) into all tubes
   9. Vortex tubes briefly and incubate for 24 h at 4°C

Equine LH RIA Protocol, Continued.

Day 2: Add Second Antibody

1. Pipette 200 µl of Sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS into all tubes except TC tubes
2. Vortex tubes briefly and incubate for 48-72 h at 4°C

Day 4: Pour Off Assay

1. Add 3 ml ice cold PBS (0.01 M; pH 7.0) to all test tubes except TC tubes
2. Centrifuge tubes for 1 h at 4°C at 3600 rpm
3. Decant supernatant
4. Count radioactivity of each tube using a gamma counter

APPENDIX B

Appendix Table 1. The GLM Procedure Output for basal cortisol concentration overall by treatment.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	40.57940	40.57940	0.20	0.6581
Error	161	33227.73282	206.38343		
Corrected Total	162	33268.31222			

R-Square	Coeff Var	Root MSE	NG_ML Mean
0.001220	33.27801	14.36605	43.16980

Appendix Table 2. The GLM Procedure Output for basal cortisol concentration between treatments for Week 3.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	21.492852	21.492852	0.09	0.7668
Error	10	2314.187955	231.418795		
Corrected Total	11	2335.680806			

R-Square	Coeff Var	Root MSE	NG_ML Mean
0.009202	32.20706	15.21246	47.23329

Appendix Table 3. The GLM Procedure Output for basal cortisol concentration between treatments for Week 6.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	49.721663	49.721663	0.34	0.5731
Error	10	1465.189241	146.518924		
Corrected Total	11	1514.910904			

R-Square	Coeff Var	Root MSE	NG_ML Mean
0.032822	29.57698	12.10450	40.92541

Appendix Table 4. The GLM Procedure Output for basal cortisol concentration between treatments for Week 9.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	145.700023	145.700023	0.85	0.3878
Error	7	1203.020306	171.860044		
Corrected Total	8	1348.720328			

R-Square	Coeff Var	Root MSE	NG_ML Mean
0.108028	32.90662	13.10954	39.83861

Appendix Table 5. The GLM Procedure Output for basal cortisol concentration by week within EXER treatment group

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	158.560112	79.280056	0.40	0.6812
Error	13	2605.857225	200.450556		
Corrected Total	15	2764.417337			

R-Square	Coeff Var	Root MSE	NG_ML Mean
0.057358	33.01895	14.15806	42.87858

Appendix Table 6. The GLM Procedure Output for basal cortisol concentration by week within NOEX control group.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	414.742259	207.371130	1.22	0.3243
Error	14	2376.540276	169.752877		
Corrected Total	16	2791.282535			

R-Square	Coeff Var	Root MSE	NG_ML Mean
0.148585	30.32494	13.02892	42.96439

Appendix Table 7. The GLM Procedure Output for LH concentration at d -5 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.49098992	0.49098992	1.09	0.3014
Error	43	19.29760331	0.44878147		
Corrected Total	44	19.78859323			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.024812	71.10299	0.669912	0.942171

Appendix Table 8. The GLM Procedure Output for LH concentration at d -4 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2.23217249	2.23217249	3.93	0.0535
Error	46	26.15464287	0.56857919		
Corrected Total	47	28.38681536			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.078634	62.65464	0.754042	1.203489

Appendix Table 9. The GLM Procedure Output for LH concentration at d -3 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	3.48560464	3.48560464	6.83	0.0123
Error	43	21.95406606	0.51055968		
Corrected Total	44	25.43967069			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.137015	49.05868	0.714535	1.456490

Appendix Table 10. The GLM Procedure Output for LH concentration at d -2 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2.49005095	2.49005095	2.61	0.1127
Error	49	46.77220030	0.95453470		
Corrected Total	50	49.26225125			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.050547	57.82965	0.977003	1.689450

Appendix Table 11. The GLM Procedure Output for LH concentration at d -1 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	3.73814880	3.73814880	3.04	0.0869
Error	52	63.84548088	1.22779771		
Corrected Total	53	67.58362968			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.055311	55.63171	1.108060	1.991778

Appendix Table 12. The GLM Procedure Output for LH concentration at d 0 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	17.4850714	17.4850714	8.76	0.0046
Error	52	103.7519833	1.9952304		
Corrected Total	53	121.2370547			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.144222	51.53713	1.412526	2.740793

Appendix Table 13. The GLM Procedure Output for LH concentration at d 1post-ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	28.6096475	28.6096475	8.47	0.0053
Error	53	179.0300597	3.3779257		
Corrected Total	54	207.6397071			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.137785	55.97168	1.837913	3.283649

Appendix Table 14. The GLM Procedure Output for LH concentration at d 2 post-ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	61.3138807	61.3138807	11.28	0.0015
Error	48	260.9731489	5.4369406		
Corrected Total	49	322.2870296			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.190246	53.16965	2.331725	4.385444

Appendix Table 15. The GLM Procedure Output for LH concentration at d 3 post-ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	36.6294811	36.6294811	7.28	0.0094
Error	52	261.7026232	5.0327428		
Corrected Total	53	298.3321042			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.122781	67.42769	2.243378	3.327086

Appendix Table 16. The GLM Procedure Output for LH concentration at d 4 post-ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2.23217249	2.23217249	3.93	0.0535
Error	46	26.15464287	0.56857919		
Corrected Total	47	28.38681536			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.078634	62.65464	0.754042	1.203489

Appendix Table 17. The GLM Procedure Output for LH concentration at d -4 and -3 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	5.59520175	5.59520175	10.24	0.0019
Error	91	49.71794968	0.54635110		
Corrected Total	92	55.31315143			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.101155	55.74709	0.739156	1.325909

Appendix Table 18. The GLM Procedure Output for LH concentration at d -2 and -1 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	6.2312832	6.2312832	5.68	0.0190
Error	103	113.0119528	1.0972034		
Corrected Total	104	119.2432360			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.052257	56.77576	1.047475	1.844933

Appendix Table 19. The GLM Procedure Output for LH concentration at d 1 and 2 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	85.4619189	85.4619189	18.48	<.0001
Error	103	476.2587972	4.6238718		
Corrected Total	104	561.7207162			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.152143	56.46382	2.150319	3.808313

Appendix Table 20. The GLM Procedure Output for LH concentration at d 3 and 4 relative to ovulation between treatments.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	62.6319823	62.6319823	13.27	0.0004
Error	107	505.1977744	4.7214745		
Corrected Total	108	567.8297567			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.110301	75.18857	2.172895	2.889928

Appendix Table 21. The GLM Procedure Output for progesterone concentration at d 9 relative to ovulation between treatment groups.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	51.213303	51.213303	1.81	0.1855
Error	41	1157.940862	28.242460		
Corrected Total	42	1209.154164			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.042355	40.77241	5.314364	13.03421

Appendix Table 21. The GLM Procedure Output for progesterone concentration at d 15 relative to ovulation between treatment groups.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	72.9989893	72.9989893	4.65	0.0374
Error	39	612.8634066	15.7144463		
Corrected Total	40	685.8623959			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.106434	49.41091	3.964145	8.022813

Appendix Table 22. The GLM Procedure Output for progesterone concentration at d 21 relative to ovulation between treatment groups.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	46.1216306	46.1216306	2.33	0.1419
Error	21	415.8374754	19.8017845		
Corrected Total	22	461.9591060			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.099839	72.77646	4.449920	6.114504

Appendix Table 23. The GLM Procedure Output for progesterone concentration at d 21 relative to ovulation between treatment groups with 0.1 ng/ml values substituted for missing values in EXER mares.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	169.9410631	169.9410631	10.08	0.0029
Error	39	657.2898119	16.8535849		
Corrected Total	40	827.2308750			

R-Square	Coeff Var	Root MSE	AVERAGE_CONCENTRATION Mean
0.205434	118.1728	4.105312	3.473990