ANALYSIS OF ESTRONE SULPHATE, TESTOSTERONE, AND CORTISOL

CONCENTRATIONS AROUND TIME OF EJACULATION AND POTENTIAL

CORRELATION TO SEXUAL BEHAVIOR AND SPERM CHARACTERISTICS IN

STALLIONS

A Thesis

by

JENNIFER LEIGH SEALE

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

MASTER OF SCIENCE

May 2009

Major Subject: Animal Science

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Approved by:

Chair of Committee,   Clay Cavinder
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ABSTRACT

Analysis of Estrone Sulphate, Testosterone, and Cortisol Concentrations Around Time of Ejaculation and Potential Correlation to Sexual Behavior and Sperm Characteristics in Stallions.

(May 2009)

Jennifer Leigh Seale, B.S. Agricultural Development, Texas A&M University

Chair of Advisory Committee: Dr. Clay Cavinder

In the stallion, inconsistent sexual behavior and variable semen quality are common. This reproductive variability has been attributed to differences in circulating hormone concentrations. In order to further examine this relationship, 7 miniature stallions were observed for sexual behavior and semen characteristics. Blood was also drawn from each stallion 15 min before mating (time -15), immediately following ejaculation (time 0) and at times following ejaculation (times +15, +30, and +60). Plasma was later analyzed for concentrations of testosterone (T), estrone sulphate (ES) and cortisol. Semen was evaluated for volume, sperm concentration and progressive motility. Sexual behavior was quantified by assigning a libido score to each stallion, recording reaction time and the number of jumps required for ejaculation.

Upon statistical analysis, data revealed both ES and cortisol increased at the time of semen collection ($P < 0.05$), while T did not. Regression analysis revealed that ES and the ratio of ES to T at times -15, +30, and +60 were negatively correlated to libido

scores. Additionally, a positive relationship was found between ES at times -15 and +60 and reaction time, as well as between cortisol at times -15, 0, and +15 and libido scores. No relationship was observed between T and sexual behavior. However, T at time -15 was positively correlated to progressive motility, and the ratio of ES/T at time -15 was negatively correlated to progressive motility. No other association was detected between ejaculate parameters and hormone concentrations. These results not only serve to enhance understanding of stallion hormone profiles, but also provide further insight into the hormonal control of sexual behavior and sperm production. This knowledge can be used to generate improved management techniques for stallions that are inconsistent in sexual behavior and sperm output.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

The neuroendocrine system is responsible for control of the reproductive organs through the secretion of hormones. Consequently, in many species reproductive variability among males has been attributed to differences in circulating hormone concentrations. In the stallion, traits such as inconsistent sexual behavior and variable semen quality are common[1-3] and can lead to financial loss if these factors prevent the stallion from breeding or being collected on a regular schedule. Furthermore, time losses, and thus labor inefficiencies, are experienced when working with subfertile stallions. While it is possible that the neuroendocrine system is responsible for some of these inconsistencies, the function and relationships between reproductive hormones in the stallion is not completely understood.

Regulation of hormone secretion in the stallion is achieved by a series of positive and negative feedback loops. Neuroendocrine function begins in the hypothalamus which lies in the diencephalon of the brain and regulates, among other things, the release of tropic hormones which correspondingly affect sexual behavior.[4] Neural or hormonal stimulation cause the hypothalamus to synthesize and secrete gonadotropin releasing hormone (GnRH). In response to GnRH, the anterior pituitary releases luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood stream. Leuteinizing hormone elicits a response from Leydig cells of the testes to synthesize

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and secrete testosterone. Testosterone in turn travels down a concentration gradient into the circulatory system where it stimulates reproductive organs and supports spermatogenesis. Elevated blood testosterone concentration also inhibits the hypothalamus, causing a decrease in the pulses of GnRH, and, ultimately, a decline in the synthesis and secretion of testosterone; therefore, being referred to as a negative feedback loop.[4] Estrogen synthesis follows the same pathway as testosterone, but there are also several cells in the stallion, including sertoli cells, that can convert testosterone to estrogen.

The stallion produces larger quantities of estrogens than males of other species.[5] Of the 3 types of estrogens, the sulfated form of estrone is the most abundant plasma estrogen in the stallion.[6] While estrone sulphate is present in the stallion at concentrations almost 100 times that of testosterone, its function in the stallion is not clear.[6,7] Thus, it can be speculated that, due to uniquely high concentration, estrone sulphate may play a central role in stallion reproductive behavior and physiology.

Cortisol is a glucocorticoid that serves several functions and may affect reproductive endocrinology through its interaction with testosterone. Increasing blood cortisol concentration in the stallion through either sexual stimulation or treatment with exogenous cortisol has produced varying results, causing both an increase[8,9] and a decrease in testosterone concentration.[10,11] Still, in other species cortisol and testosterone do not interact.[12-14] Inconsistent results leave the association between cortisol and testosterone unclear.

Research regarding the relationship between the neuroendocrine system and stallion reproductive traits has been documented; however, the results are varied and sometimes contradictory. Furthermore, data correlating stallion hormone concentrations to the intensity of copulation are limited. This research will give insight into the possible relationship between blood hormone concentrations and stallion reproductive physiology and behavior.

The objectives of this study are to:

1. identify circulating blood concentrations of testosterone, cortisol and estrone sulphate around ejaculation, and

2. determine potential correlations between blood hormone concentrations and sexual behavior and sperm characteristics.

The results of this study will provide further understanding of stallion reproductive physiology and behavior. This additional knowledge could eventually lead to the development of more efficient stallion management practices and ultimately decrease the financial burden of reproductively inconsistent stallions.

CHAPTER II

LITERARY REVIEW

Testosterone

Testosterone serves several functions in the stallion including the initiation and maintenance of spermatogenesis. Once a colt reaches puberty, daily sperm output begins to increase synonymous with a rise in circulating testosterone concentration indicating that testosterone plays an important role in sperm production.[3] Consequently, the presence or absence of testosterone can affect sperm production and quality.[15-17] Upon in vitro examination of stallion testes at different ages, a positive correlation was reported between the concentration of testosterone in the testes and the number of germ cells that each sertoli cell can support.[15] Correspondingly, another positive correlation was found between total testicular testosterone and the number of elongated spermatids.[15] Both of these associations demonstrate that testosterone has an effect on the daily sperm production and output in the stallion. Similarly, when testicular testosterone was suppressed in the stallion, a decrease in spermatozoa concentration, progressive motility and percentage of normal spermatozoa was reported.[17] Additionally, when compared with normal fertile stallions, azoospermic stallions had lower concentrations of testosterone.[18]

In other species testosterone affects sperm morphology. An investigation of male rats showed that manual suppression of testicular testosterone resulted in premature detachment of round spermatids.[16] If germ cells are prematurely released as spermatids,

they will not have developed the flagellum or acrosome, both of which are necessary for fertilization of an oocyte. Therefore, it is safe to say that testosterone has an impact on sperm production and quality. This leads to the belief that differences in testosterone concentrations among stallions may account for some of the variability in concentration, progressive motility and volume of the ejaculate.

Testosterone has also been implicated in the maintenance of sexual behavior in the stallion. Although the horse maintains some level of reproductive capability year round, the stallion is still a seasonal breeder. During the spring and summer, when days are long, there is a decrease in melatonin production and consequently an increase in GnRH secretion. This translates to an increase in LH and therefore a rise in circulating testosterone concentrations.[6,19] At the same time, during the natural breeding season the stallion becomes more sexually active, which translates into the stallion responding more quickly to an estrus mare and a decrease in the number of mounts required to ejaculate is observed.[20,21] The simultaneous rise in testosterone and sexual activity leads to the hypothesis that testosterone has a stimulatory effect on stallion libido. However, no significant changes in testosterone concentrations were reported throughout the year in stallions showing normal seasonal changes in libido, suggesting that changes in sexual behavior may not be mediated solely by testosterone.[21] Furthermore, upon endocrine assessment of stallions with poor libido, normal levels of testosterone have been reported.[22] Similarly, differences in circulating blood concentrations of T could not be linked to differences in libido in bulls or rams when exposed to an estrus female for short periods.[23,24]

Administration of exogenous testosterone has also been used in several studies to assess the male sexual response to testosterone. Injections or implants dispensing testosterone propionate restored sexual performance in castrated male rats and rabbits.[25-27] The same increase in libido was reported when geldings were administered testosterone propionate.[28] While it has been consistently illustrated that exogenous testosterone can restore sexual behavior in castrated males, it is unclear whether testosterone alone is responsible for changes in intact male libido. Testosterone interactions with cortisol or aromatization of testosterone to estrogen may impact the importance of testosterone in maintenance of sexual behavior.[8,9,28]

Changes in testosterone concentration in response to copulation have been discussed in several research articles with varying results. Reports have indicated that an increase in circulating testosterone concentration occurs within 10 minutes[9], 2 to 4 hours[7], and is non-existent[29] after stallion exposure to a mare in estrus. However, investigators found a mid-day rise in testosterone that is consistent among stallions.[10] This diurnal variation in testosterone may be partially responsible for the variability in results. Whatever the reason for the inconsistency, it remains unclear if a relationship exists between sexual stimulation and testosterone.

Cortisol

Sensory input from the environment, such as stress or excitement, causes the central nervous system to act on the hypothalamus. The hypothalamus then releases corticotrophin releasing factor (CRF) which triggers the release of adrenocorticotropic

hormone (ACTH) from the anterior pituitary. Adreonocorticotropic hormone acts on the adrenal cortex to release cortisol which acts on muscle, liver and adipose tissue to supply the fuel necessary to withstand stress.[30] Once cortisol concentration in the blood reaches a certain level it begins to negatively feed-back on the hypothalamus to decrease the release of CRF; thus, causing a decrease in cortisol synthesis and secretion.

Corticotropin releasing factor is a neurotransmitter that causes behavioral responses to stress and may also have an inhibitory effect on sexual behavior. Administration of CRF in female rats completely abolished lordosis behavior and increased reaction time in male rats.[31,32] However, in another study CRF had no effect on male reproductive activity.[33] The possibility that CRF may have an effect on sexual behavior leads to the theory that cortisol concentration in the blood may potentially be correlated to male libido.

Circulating cortisol concentrations increase upon sexual stimulation in the stallion, ram, boar, and bull.[9,11-14,34-36] The cause of this increase has been suggested to be both physical and psychic. In one study, stallions were exposed to several different stimuli including sexual stimulation and physical activity. An increase in cortisol was similar for both sexually aroused and physically exercised stallions.[34] However, film induced sexual arousal in men in the absence of physical activity is not found to cause a change in cortisol profiles.[37] These results indicate that the physical activity required for copulation, and not sexual stimulation alone, may be responsible for the increase in cortisol.

Further research exposed stallions to treatments including sexual stimulation (SS), sexual stimulation plus ejaculation (SE) and control.[11] Each stallion was exposed to 1 treatment per day with 2 days of rest between each treatment. A rise in cortisol was reported for both SS and SE treated stallions but not in control stallions that had not yet been exposed to SS or SE treatments. However, upon hearing the vocalization of other stallions being sexually stimulated cortisol concentrations increased in control stallions that had been previously exposed to SS or SE treatments. Physical activity causes an increase in cortisol concentrations, but the above mentioned increase in cortisol was noted with no change in activity of the stallion. These results suggest that the psychic stimulus of sexual arousal may be responsible for the increase in cortisol.

Cortisol may have an effect on male reproductive endocrinology through its relationship with testosterone; yet, research focusing on this association has yielded inconsistent results. Stallions treated with a single injection of 0.5 mg of synacthen[®], a form of ACTH, or an injection of 200 mg of cortisol exhibited an increase in both cortisol and testosterone concentrations.[8] Also, ACTH administration has resulted in an increase in blood testosterone concentration in the boar.[38] Similarly, a sexually stimulated rise in cortisol was accompanied by an increase in peripheral testosterone in stallions.[9] A positive correlation between testosterone and cortisol has also been reported in the boar, ram, and bison.[13,36,39,40] Conversely, in the stallion, administration of synacthen[®] depot twice daily for 5 days caused a prolonged increase in cortisol along with a decrease in testosterone.[8] Administration of ACTH to stallions resulted in an increase in circulating cortisol concentrations and a corresponding suppression of the

midday rise in testosterone that was seen in the control group.[10] Researchers reported that this difference in testosterone concentration was achieved without a change in LH or FSH concentration. The production and secretion of testosterone is controlled by LH, so the suppression of testosterone in the absence of a change in LH suggests that cortisol may have a direct effect on the testis to alter testosterone secretion.[10] This theory is in agreement with a study which reported that cortisol interacts with testosterone secretion by acting directly on the testis of boars through a "cortisol-dependent, hypothalamic pituitary axis-independent mechanism."[41] This same suppression of diurnal variations in testosterone in the presence of cortisol was reported when stallions were sexually stimulated.[11] Still other studies suggest that testosterone and cortisol do not interact, such that increases in cortisol had no effect on testosterone concentration in the boar and bull.[12-14]

Continuously elevated levels of cortisol are associated with Cushing Syndrome or hypercortisolism. The symptoms of hypercortisolism include decreased sexual interest in both males and females. In humans, elevated cortisol and low libido are coupled with depression[42] and it has been reported that circulating cortisol concentration decreases as sexual arousal increases in human males.[43]

Variability in results leaves the relationship between cortisol, sexual behavior and testosterone unclear. Regardless of whether cortisol has a negative or positive correlation with testosterone, any association would indicate that cortisol plays a potential role in stallion reproductive behavior and fertility.

Estrone Sulphate

As previously stated, GnRH secretion from the hypothalamus causes the anterior pituitary to release LH and FSH. Leuteinizing hormone then acts on the leydig cells of the testis to produce androstenedione which can be converted to testosterone or estrone within the cell.[44] Follicle stimulating hormone also regulates the production and release of estrogens from sertoli cells.[4] Both testosterone and estrone can be further converted by the aromatase enzyme into estradiol. Estrogen synthesis can follow several metabolic pathways to produce 3 different types of estrogen: estradiol, estrone, or estriol.[4] The sulphated form of estrone is the most abundant estrogen in the stallion.[45]

The seasonal changes in testosterone and estrogens were examined in a previous study utilizing 15 stallions over a 14 month period.[6] Estrogen concentration, even more so than testosterone, was found to increase during the breeding season. This simultaneous increase during the time when the stallion is most fertile suggests that both testosterone and estrogen work together to influence stallion fertility.

The concentration of circulating testosterone in infertile stallions has been proven to be highly variable. Case studies of low fertility stallions have reported normal testosterone concentrations but low LH concentrations, or low testosterone concentrations and normal LH.[22] This inconsistency in testosterone concentrations among infertile stallions again leads to the theory that androgen concentrations alone may not be an indicator of reproductive efficiency. Instead, total concentrations of testosterone and estrogen together could be a more accurate marker for stallion fertility.[6,18] This theory is supported by previous research which reported that subfertile

stallions with low blood estrogen conjugate levels (67 ± 5 ng/mL) also had low sperm concentrations ($5.8 \pm 2.2 \times 10^6$ /mL).[46] This correlation implies that a certain level of estrogen concentration may be necessary for normal sperm output. Additionally, estrogens were found to have a stimulatory effect on accessory sex gland secretions resulting in greater volume of ejaculate in the boar and greater weight of accessory sex glands in the rat and in the gelding when administered along with testosterone.[28,47-49] However, when colts were immunized against estrogen, a rise in testosterone was observed.[50] Along with an increase in testosterone, immunized colts exhibited greater daily sperm production, indicating that estrogen may actually suppress the stimulating effects of testosterone on sperm production. Disparity in results leaves the relationship between estrogens and ejaculate parameters unclear.

Estrogens have also been hypothesized to have an effect on reproductive behavior. In one study, 16 young stallions were collected every other day for 12 days.[28] Following each collection the concentration of spermatozoa in the ejaculate was determined and a libido score was given to quantify sexual behavior. Blood samples were also taken before and after each collection to determine the circulating blood concentrations of hormones. The stallions were then gelded and split into 4 treatment groups, each receiving either testosterone propionate (TP), 17β-estradiol-3-benzoate (EB), a combination of the 2 (TE), or control. Treatments were administered at levels to imitate intact stallion hormone concentrations. An increase in sexual behavior scores was seen in TP, EB, and TE treated groups. Upon blood sample analysis, all treated geldings were found to have circulating concentrations of estradiol. The TP group was

not administered estradiol, but testosterone was aromatized causing estradiol to be present when it was not a part of the treatment. The EB group, however, showed no levels of testosterone in circulation and still experienced an increased libido. While EB treated geldings improved in their desire to mount and were able to achieve intromission, they did not ejaculate, which is consistent with reports in hamsters and boars.[48,51] These results again suggest that estrogens may be vital to sexual behavior in the male but they are needed in conjunction with testosterone to achieve copulation. This hypothesis has been supported in several other species. Aromatization inhibiting steroid was administered in the brain of castrated male rats to block testosterone aromatization to estrogens. Treatment with testosterone along with the inhibitor did not restore mounting behavior while testosterone alone did.[52] Estrogen was also required for normal sexual behavior in the castrated ram, rat, and rabbit.[25,53,54]

While exogenous hormone administration in gonadectomized males has revealed that both testosterone and estrogen are related to sexual behavior, study of endogenous hormone concentrations has yielded inconsistent results. No significant differences in testosterone were seen between selected normal and infertile stallions; however, concentrations of estradiol-17β were low in infertile stallions.[55] In the bull, low libido was correlated to a significantly higher estrogen to testosterone ratio as compared to high libido bulls.[14] Conversely, in another study, stallions with normal testosterone and lower than normal estradiol-17β serum levels had normal libido.[56] Due to the variability in the results of different studies, further research into the function of estrogens in the stallion would be beneficial.

The response of estrogen to sexual stimulation has been addressed in several articles with similar results. Research has consistently indicated that upon sexual stimulation circulating estrogen concentrations increase in intact males. This increase occurs very rapidly, within 10 min, and then returns to basal levels within approximately 30 minutes.[7,9] This further supports the theory that estrogens may play an important role in stallion libido. Research that correlates estrogen concentration around the time of sexual stimulation to individual stallion libido may provide valuable insight into the differences in libido among stallions.

Stallion Size

Testicular size is highly correlated to parenchyma weight, which in turn is linked to daily sperm production.[57] Also, the accessory sex glands are responsible for producing the majority of ejaculate volume.[44] There is a great difference in testicular and accessory sex gland size between full-size and miniature stallions. Consequently, miniature stallions produce fewer spermatozoa and have a smaller ejaculate volume. However, values for motility and concentration are not affected by stallion size.[44,58]

CHAPTER III

MATERIALS AND METHODS

Animals

Miniature stallions (n=7) between the ages of 3 and 13 y were used. They were

housed in individual stalls at the Texas A&M Horse Center and had no visual contact

with mares. Each stallion was fed 1.5% of BW in alfalfa hay/d and 0.5% of BW in

concentrate/d with *ad libitum* access to fresh water. Stallions were turned loose in

outside pens every other day for exercise. Horses used in this study were maintained

under the approval of the Texas A&M University Institutional Agricultural Animal Care

and Use Committee using guidelines set forth by the Federation of Animal Science

Societies (1999).

Collection and Evaluation of Semen Samples

Semen was collected from each stallion once a day for 3 consecutive days in

order to provide a more uniform ejaculate on the third collection, followed by 11 days of

sexual rest. The first 2 collections were discarded and ejaculate gel-free volume,

concentration and motility of the third collection were recorded. A total of 5 ejaculates,

collected over 55 days from August to October, were evaluated from each stallion.

Collection was achieved using an artificial vagina (AV, Missouri model). Upon

collection the gel portion of the ejaculate was filtered off in order to measure the volume

of the sperm rich gel-free portion. Spermatozoa concentration in the ejaculate was

measured using a densimeter (Animal Reproduction Systems, Chino, CA). The sample

was then extended using a commercial extender (INRA-96® Breeder's Choice, Aubrey,

TX) to achieve a concentration of 25 to 50 x 10^6 spermatozoa/mL, which is consistent

with accurate analysis of progressive motility using computer-assisted semen analysis

(CASA, Ceros Motility Analyzer, Hamilton-Thorne, Beverly, MA).[59] The samples were

maintained at 37°C until analysis.

Evaluation of Sexual Behavior

      Libido scores (Table 1) were assigned by 2 individual appraisers to each stallion

when presented with an estrus mare in order to quantify the intensity of sexual arousal.

Table 1. Libido score description

Score	Description
0	no interest in an estrus mare
1	slight vocalization and interest initially but quickly fades
2	moderate vocalization and interest in mare however interest dissipates
3	moderately interested with consistent contact with mare
4	highly interested with vocalization and squealing, consistent contact or attempt to mount

Similar scores have been used to quantify sexual behavior in the bull and stallion.[28,60,61]

Additionally, the number of mounts required before ejaculation and the reaction time for each stallion was recorded. Timing began when the stallion entered the breeding facility and a time was recorded upon ejaculation.

Plasma Sampling

In order to measure the changes in circulating hormone concentrations around copulation, blood was collected via jugular venipuncture using vacutainers. Blood sampling took place on each third consecutive semen collection 15 minutes prior to entering the breeding facility, immediately following ejaculation, and 15, 30 and 60 min following ejaculation. Blood plasma was centrifuged at 2500 rpm for 20 min, in a refrigerated centrifuge. Blood plasma was then collected and stored in micro centrifuge tubes at $-20^{\circ}C$ until assayed for T, cortisol and ES by radioimmunoassay.

Radioimmunoassay (RIA) Procedures

Plasma T was measured by an RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX) previously validated for use in horses.[62] A sample size of 50 µL was used with a sensitivity of 0.08 ng/mL. Standards were run in triplicate while controls and samples were run in duplicate. The inter-assay coefficient of variation between the controls was between 11 and 20%, respectively (n=7 assays).

Plasma cortisol was analyzed using an RIA kit (Coat-A-Count[®], Siemens Medical Solutions Diagnostics, Los Angeles, CA) as previously described for horse samples.[63] A sample size of 25 μL was used with a sensitivity of 0.2 μg/dL. Standards were run in triplicate and unknowns were run in duplicate. The inter-assay coefficient of variation between the controls was 5% (n=6 assays).

Plasma ES was measured using an RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX) previously validated for horse samples.[9] The kit recommended a 100 μL sample size; however, due to high levels of estrone sulphate in plasma, the sample size was reduced to 25 μL in order to fit the standard curve. Kit sensitivity was 0.01 ng/mL. Standards were run in triplicate while controls and samples were run in duplicate. The inter-assay coefficient of variation between the controls was between 4 and 5%, respectively (n=5 assays).

All samples were counted using Perkin-Elmer Packard Cobra II Auto-Gamma counter (Packard Systems, Meridan, CT).

Statistical Analysis

Data were subjected to one-way ANOVA to determine differences between stallions in all parameters measured (T, ES, C, ES:T, concentration, volume, progressive motility, number of jumps, reaction time and libido score). A two-way ANOVA was used to determine the differences in hormone concentrations over time. In this analysis 'time' was used as the fixed factor and 'stallion' as the random factor. Correlation coefficients were calculated using regression analysis to determine

relationships between sexual behavior, semen parameters and hormone concentrations. Hormone concentrations in the stallion vary throughout the year and because data were collected over a period of 3 months, ANOVA was used to determine if date had an effect on hormone concentrations. All statistical analyses were performed with SPSS computer software (SPSS Inc., 16.0 graduate student version, 2007).

CHAPTER IV

RESULTS

Mean Ejaculate Parameters and Sexual Behavior Measurements

There were some variations in concentration, progressive motility and volume between the stallions (Table 2). All stallions were similar in number of jumps; however, some variation in reaction time was found (Table 3). Mean libido score for stallion 7 was significantly different from all other stallions (Table 3).

Table 2. Mean (± SD) concentration (Conc.), progressive motility (PM), volume (Vol.), and total sperm per ejaculate (Total) for each stallion

Stallion	Conc. (x 10^6)	PM (%)	Vol. (ml)	Total
1	257.80 ± 99.33^a	65.20 ± 3.03^{abc}	12.82 ± 5.89^a	2958.68 ± 576.23^{ac}
2	467.25 ± 156.10^b	74.75 ± 6.75^{ab}	3.80 ± 1.78^{bcd}	1590.23 ± 509.3^{bcdf}
3	373.40 ± 163.67^{ab}	57.20 ± 5.12^{abc}	$6.28 \pm 3.34b^c$	$2124.96 \pm 912.74^{abcd}$
4	362.80 ± 126.64^{ab}	58.60 ± 9.48^{abc}	3.78 ± 2.64^{bcd}	$1201.38 \pm 911.28^{bcdef}$
5	266.20 ± 35.27^a	53.00 ± 4.47^{acd}	5.90 ± 2.78^{bc}	1578.6 ± 844.19^{bcdf}
6	207.00 ± 129.04^a	46.60 ± 26.75^{acd}	1.60 ± 1.65^{bd}	390.3 ± 373.27^{def}
7	486.40 ± 195.27^b	36.80 ± 26.92^{cd}	2.15 ± 1.80^{bcd}	883.61 ± 656.99^{bdef}
Overall	342.26 ± 159.30	55.47 ± 17.98	5.23 ± 4.62	1530.84 ± 1030.16

[abcdef] Means not sharing the same superscript within a column are different (P < 0.05)

Table 3. Mean (± SD) reaction time, number of jumps, and libido score for each stallion

Stallion	Reaction Time (min)	Jumps	Libido
1	5.24 ± 1.99^{abcd}	1.60 ± 0.55^a	3.20 ± 0.27^a
2	3.67 ± 1.33^{abc}	1.25 ± 0.50^a	3.63 ± 0.48^a
3	2.85 ± 0.75^{ab}	1.60 ± 0.89^a	3.80 ± 0.27^a
4	3.19 ± 1.08^{abc}	1.20 ± 0.45^a	3.90 ± 0.22^a
5	4.23 ± 1.41^{abc}	1.60 ± 0.55^a	3.40 ± 1.08^a
6	6.06 ± 2.66^{acd}	1.40 ± 0.55^a	3.00 ± 1.00^a
7	7.24 ± 4.38^{cd}	1.60 ± 0.55^a	1.80 ± 1.04^b
Overall	4.67 ± 2.6	1.47 ± 0.563	3.15 ± 0.87

[abcd] Means not sharing the same superscript within a column are different ($P < 0.05$)

Mean Hormone Concentrations

There were some differences in pre-mating values of T among the stallions (Table 4). However, at all post-mating measurement times each stallion had similar blood plasma T concentrations. Stallions varied greatly in ES concentration at all measurement times (Table 5). Some variations in cortisol were found between stallions at each time measurement (Table 6). As previously mentioned, stallion 7 was significantly lower in libido score; however, possessed higher ES/T at times -15, +30 and +60 than the rest of the stallions (Table 7). In order to better define any relationships that may exist between hormone concentrations and sexual behavior or semen parameters, regression analysis was used to analyze the data.

Table 4. Mean (± SD) plasma testosterone (ng/ml) concentrations for each stallion

Stallion	Time -15	Time 0	Time +15	Time +30	Time +60
1	1.23 ± 0.46^{abc}	1.19 ± 0.52^{a}	0.99 ± 0.43^{a}	0.96 ± 0.34^{a}	0.95 ± 0.60^{a}
2	1.30 ± 0.32^{ab}	1.47 ± 0.69^{a}	1.58 ± 0.99^{a}	1.18 ± 0.79^{a}	0.98 ± 0.62^{a}
3	0.79 ± 0.46^{acd}	1.02 ± 0.34^{a}	1.08 ± 0.44^{a}	0.98 ± 0.45^{a}	1.15 ± 0.60^{a}
4	0.85 ± 0.27^{abcd}	0.93 ± 0.27^{a}	1.1 ± 0.46^{a}	1.44 ± 1.05^{a}	0.96 ± 0.66^{a}
5	0.60 ± 0.28^{cd}	0.62 ± 0.42^{a}	0.86 ± 0.44^{a}	0.98 ± 0.40^{a}	1.12 ± 0.19^{a}
6	0.79 ± 0.43^{acd}	0.78 ± 0.51^{a}	$o.91 \pm 0.63^{a}$	0.93 ± 0.64^{a}	0.84 ± 0.76^{a}
7	0.52 ± 0.19^{cd}	0.69 ± 0.25^{a}	0.87 ± 0.63^{a}	0.56 ± 0.23^{a}	0.51 ± 0.18^{a}
Overall	0.86 ± 0.42^{e}	0.94 ± 0.48^{e}	1.04 ± 0.55^{e}	0.99 ± 0.61^{e}	0.93 ± 0.54^{e}

[abcd] Means not sharing the same superscript within a column are different ($P < 0.05$)
[e] Means not sharing the same superscript within a row are different ($P < 0.05$)

Table 5. Mean (± SD) plasma estrone sulphate (ng/ml) concentrations for each stallion

Stallion	Time -15	Time 0	Time +15	Time +30	Time +60
1	121.8 ± 24.2^{abcdf}	232.4 ± 19.1^{abc}	161.7 ± 13.3^{abc}	138.6 ± 31.8^{abcdf}	160.6 ± 24.5^{ab}
2	150.7 ± 67.1^{abcf}	187.1 ± 89.7^{abd}	157.1 ± 85.8^{abcd}	142.5 ± 64.1^{abcdf}	150.2 ± 57.4^{abc}
3	112.2 ± 32.1^{abcde}	261.2 ± 28.4^{abc}	195.5 ± 44.2^{ab}	131.9 ± 24.0^{abcde}	94.8 ± 4.2^{bcd}
4	108.8 ± 31.4^{abcde}	274.1 ± 52.2^{ac}	192.1 ± 66.7^{ab}	168.7 ± 62.6^{abcf}	148.7 ± 59.7^{abc}
5	85.3 ± 12.5^{acde}	126.8 ± 42.2^{bd}	106.1 ± 33.7^{acd}	88.2 ± 4.8^{abdef}	83.6 ± 8.7^{cd}
6	68.1 ± 12.9^{cde}	121.7 ± 68.3^{bd}	88.2 ± 20.2^{cd}	74.5 ± 12.4^{bde}	74.8 ± 8.5^{cd}
7	172.9 ± 76.5^{abf}	261.0 ± 92.2^{abc}	223.9 ± 79.7^{ab}	199.2 ± 79.9^{ac}	187.8 ± 82.8^{ab}
Overall	116.2 ± 51.2^{g}	209.9 ± 82.5^{h}	160.8 ± 67.9^{i}	134.6 ± 59.4^{g}	128.0 ± 58.1^{g}

[abcdef] Means not sharing the same superscript within a column are different ($P < 0.05$)
[ghi] Means not sharing the same superscript within a row are different ($P < 0.05$)

Table 6. Mean (± SD) plasma cortisol (µg/dL) concentrations for each stallion

Stallion	Time -15	Time 0	Time +15	Time +30	Time +60
1	4.35 ± 6.4^{ab}	5.38 ± 5.7^{ade}	5.43 ± 4.2^{ac}	5.11 ± 9.5^{ace}	4.71 ± 8.2^{ac}
2	5.15 ± 4.4^{abc}	7.30 ± 4.6^{b}	7.28 ± 3.6^{b}	7.09 ± 8.1^{b}	7.56 ± 19.0^{b}
3	4.21 ± 6.7^{ab}	4.33 ± 9.8^{ce}	4.90 ± 4.1^{acd}	4.33 ± 2.8^{acd}	3.87 ± 4.8^{acd}
4	4.29 ± 14.1^{ab}	4.37 ± 7.1^{ce}	4.49 ± 8.7^{cd}	4.05 ± 2.8^{cd}	3.44 ± 3.4^{cde}
5	5.76 ± 13.8^{bc}	5.64 ± 5.9^{ad}	6.27 ± 5.1^{e}	5.6 ± 8.9^{ae}	4.50 ± 11.5^{acd}
6	4.13 ± 10.7^{ab}	4.72 ± 8.3^{ace}	4.61 ± 4.8^{cd}	3.99 ± 7.6^{cd}	3.46 ± 7.7^{cde}
7	2.43 ± 4.7^{d}	2.25 ± 4.0^{f}	1.89 ± 2.5^{f}	1.78 ± 1.0^{f}	2.28 ± 6.4^{de}
Overall	4.31 ± 1.3^{eh}	4.79 ± 1.5^{fg}	4.91 ± 1.6^{fg}	4.5 ± 1.6^{efh}	4.17 ± 1.7^{eh}

[abcd] Means not sharing the same superscript within a column are different ($P < 0.05$)
[efgh] Means not sharing the same superscript within a row are different ($P < 0.05$)

Table 7. Mean (± SD) ratio of estrone sulphate (ng/ml) to testosterone (ng/ml) concentration for all stallions

Stallion	Time -15	Time 0	Time +15	Time +30	Time +60
1	109.3 ± 35.9^{a}	219.0 ± 74.1^{a}	193.7 ± 96.7^{a}	154.3 ± 54.6^{a}	238.7 ± 163.0^{abd}
2	111.4 ± 31.2^{a}	141.6 ± 89.2^{a}	121.4 ± 87.9^{a}	163.0 ± 104.8^{a}	175.8 ± 56.4^{abcde}
3	173.1 ± 78.8^{a}	290.8 ± 134.8^{a}	202.7 ± 88.4^{a}	147.0 ± 35.7^{a}	104.1 ± 54.3^{bcde}
4	144.8 ± 72.2^{a}	320.0 ± 129.7^{a}	212.3 ± 123.4^{a}	161.8 ± 87.6^{a}	220.1 ± 118.1^{abcd}
5	180.3 ± 123.2^{a}	357.2 ± 288.9^{a}	155.7 ± 84.1^{a}	110.1 ± 64.8^{a}	77.2 ± 21.8^{bce}
6	98.2 ± 33.4^{a}	164.2 ± 35.8^{a}	121.6 ± 49.0^{a}	97.1 ± 33.0^{a}	141.8 ± 92.9^{abcde}
7	353.8 ± 147.5^{b}	449.2 ± 260.2^{a}	274.3 ± 114.5^{a}	435.1 ± 274.6^{b}	371.5 ± 117.0^{f}
Overall	168.9 ± 115.0^{g}	281.5 ± 185.8^{h}	184.9 ± 99.9^{g}	181.8 ± 155.6^{g}	190.4 ± 131.3^{g}

[abcdef] Means not sharing the same superscript within a column are different ($P < 0.05$)
[gh] Means not sharing the same superscript within a row are different ($P < 0.05$)

Hormones Over Time

Testosterone

     Mean blood testosterone concentration (± SD) was not significantly different at any measured point from pre-mating to 60 min after ejaculation (Figure 1).

Figure 1. Mean (± SD) testosterone concentration for all stallions for pre-mating (time -15), immediately following ejaculation (time 0), and post-ejaculation periods (times +15, +30, +60).

Estrone Sulphate

Mean estrone sulphate concentration (± SD) showed a significant increase immediately following ejaculation (from 116.18 ± 51.16 ng/ml to 209.88 ± 82.49 ng/ml; $P < 0.001$). Concentrations then returned to basal values within 30 min following ejaculation (Figure 2).

Figure 2. Mean (± SD) estrone sulphate concentration for all stallions for pre-mating (time -15), immediately following ejaculation (time 0), and post-ejaculation periods (times +15, +30, +60).
* Signifies significant increase from time -15 (P < 0.001)

Cortisol

Mean cortisol concentrations rose significantly from pre-mating values until 15 min following ejaculation (from 4.31 ± 1.31 µg/dL to 4.92 ± 1.63 µg/dL; $P < 0.05$). Concentrations then returned to pre-mating values within 30 min following ejaculation (Figure 3).
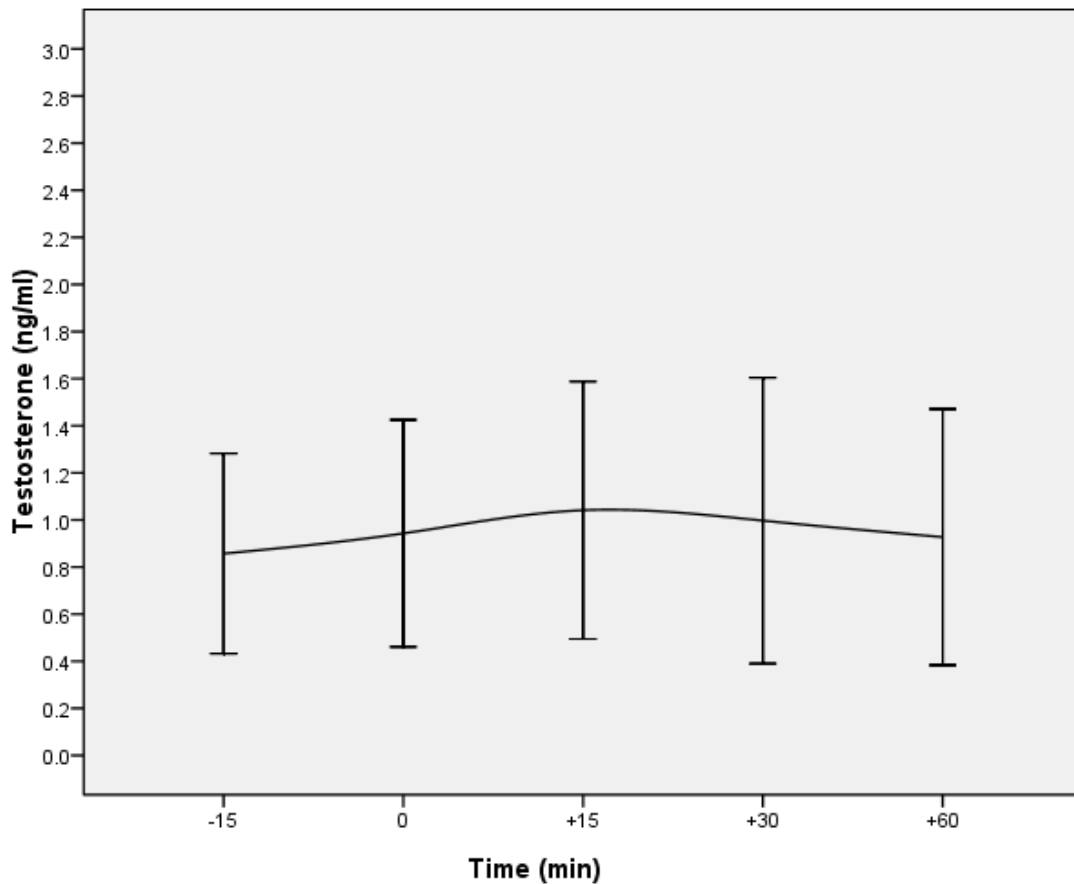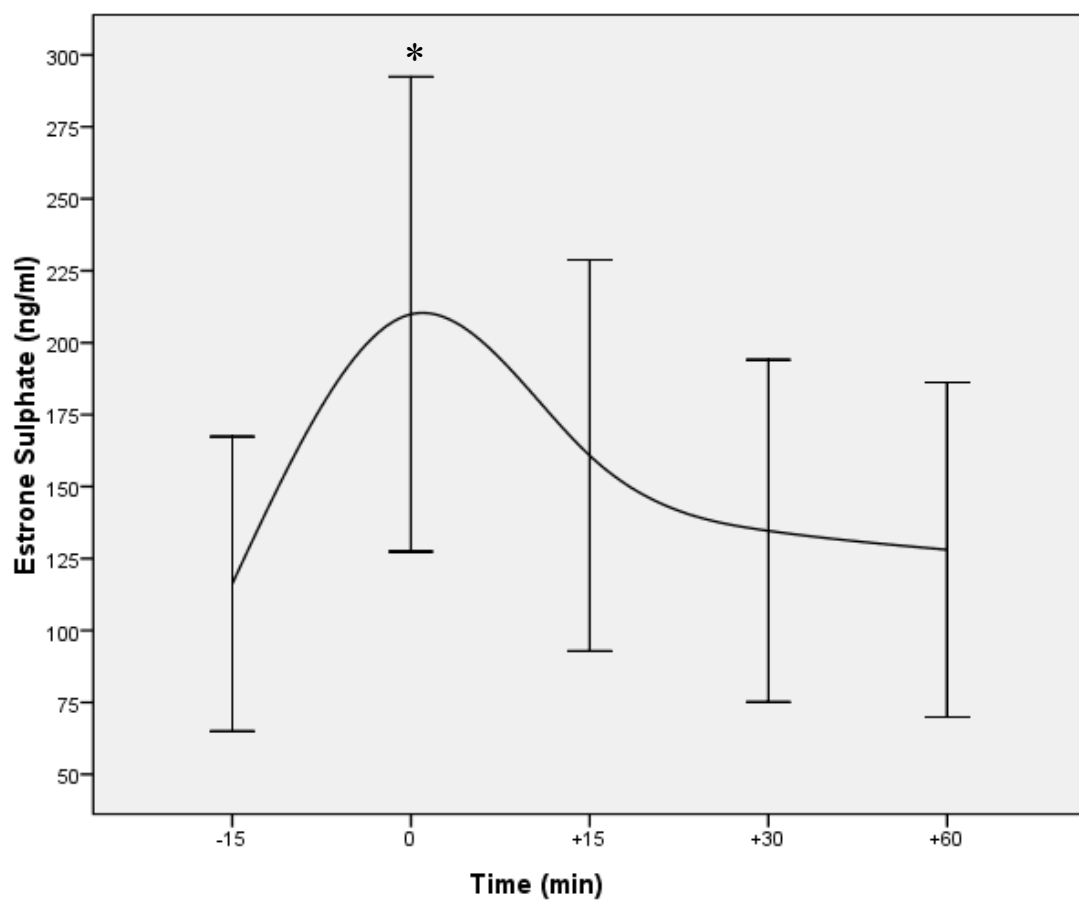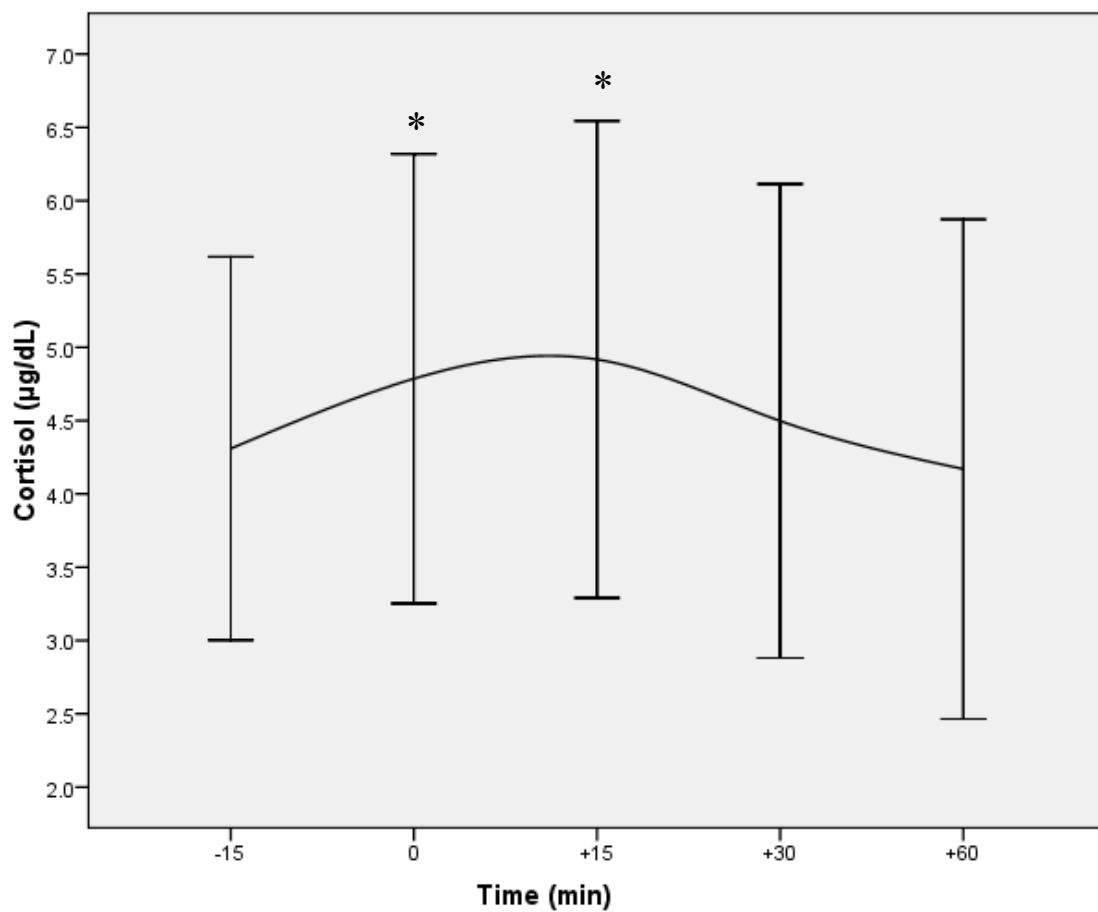

Figure 3. Mean ($\pm$ SD) cortisol concentration for all stallions for pre-mating (time -15), immediately following ejaculation (time 0), and post-ejaculation periods (times +15, +30, +60).
* Signifies significant increase from time -15 ($P < 0.05$)

Correlation of Sexual Behavior and Hormone Concentrations

Estrone sulphate was negatively correlated ($P < 0.05$) to stallion libido score at times -15, +30 and +60, when ES concentration was again approaching pre-mating values (Table 8). A similar negative correlation ($P < 0.05$) also exists between libido score and ES/T at times -15, +30 and +60 (Table 8). Testosterone, however, was not related ($P > 0.05$) to libido score at any measurement time (Table 8). Estrone Sulphate concentration was also positively correlated ($P > 0.05$) to reaction time at times -15 and +60 (Table 10). Cortisol concentration was positively correlated to libido score at times -15, 0, and +15 (Table 8). No significant correlations were found between number of jumps and hormone concentrations at any time measured (Table 9).

Table 8. Correlation coefficients across all data for libido scores and Testosterone (T), Estrone Sulphate (ES), Cortisol (C), and ratio of Estrone Sulphate to Testosterone (ES/T)

Time	T	ES	C	ES/T
-15	0.149	-0.418 *	0.377*	-0.402 *
0	0.212	-0.079	0.355*	-0.318
+15	0.093	-0.224	0.448 *	-0.211
+30	0.246	-0.339*	0.323	-0.406 *
+60	0.178	-0.415 *	0.246	-0.386 *

* signifies significant correlation at $P < 0.05$

Table 9. Correlation coefficients across all data for number of jumps and Testosterone (T), Estrone sulphate (ES), Cortisol (C) and ratio of Estrone Sulphate to Testosterone (ES/T)

Time	T	ES	C	ES/T
-15	0.171	0.221	-0.206	-0.018
0	0.216	0.097	-0.155	-0.027
+15	0.178	0.123	-0.071	-0.053
+30	0.049	0.114	-0.016	0.068
+60	0.002	0.110	-0.083	0.125

* signifies significant correlation at $P < 0.05$

Table 10. Correlation coefficients across all data for reaction time and Testosterone (T), Estrone Sulphate (ES), Cortisol (C) and ratio of Estrone Sulphate to Testosterone (ES/T)

Time	T	ES	C	ES/T
-15	0.021	0.407 *	-0.325	0.246
0	-0.064	0.029	-0.199	0.117
+15	-0.002	0.085	-0.321	0.048
+30	-0.073	0.201	-0.270	0.284
+60	-0.065	0.341*	-0.262	0.329

* signifies significant correlation at $P < 0.05$

Correlation of Semen Parameters and Basal Hormone Concentrations

Testosterone at time -15 is positively correlated to progressive motility, and ES/T is negatively correlated to progressive motility (Table 11). No significant relationships were observed between hormone concentrations and sperm concentration or volume of the ejaculate (Table 11).

Table 11. Correlation coefficients across all data for semen parameters and Testosterone (T), Estrone Sulphate (ES), Cortisol (C), and ratio of Estrone Sulphate to Testosterone (ES/T) at time -15

Hormone	Conc. (x 10^6)	Vol. (ml)	PM (%)
T	-0.086	0.203	0.400*
ES	0.148	0.034	0.086
C	-0.283	0.195	0.200
ES/T	0.182	-0.166	-0.347*

* signifies significant correlation at $P < 0.05$

Correlation of T and Cortisol

In the present study there was no significant correlation between cortisol and T at any time measurement (Table 12).

Table 12. Correlation coefficients and associated p-values across all data for Cortisol (C) (ng/ml) and Testosterone (T) concentrations

Time	T (ng/ml)
-15	0.003 ($P < 0.988$)
0	0.254 ($P < 0.147$)
+15	0.194 ($P < 0.271$)
+30	0.144 ($P < 0.418$)
+60	0.110 ($P < 0.537$)

CHAPTER V

DISCUSSION

Male fertility and sexual behavior are controlled in part by the production and secretion of hormones such as testosterone, estrone sulphate and cortisol. Therefore, the differences in hormone concentrations surrounding the time of mating may contribute to the differences in sexual behavior and ejaculate characteristics among stallions.

Testosterone

In earlier investigations T concentration has been attributed to greater libido as well as higher sperm concentration and progressive motility.[15,17,20,21] The concentrations of T found in the present study are within the normal range previously reported for stallions.[6,29,46] Testosterone was measured both before and after ejaculation to determine its importance in the change of stallion behavior when presented with an estrus mare. No significant differences were observed between time periods (-15, 0, +15, +30 and +60). This is in agreement with previous research which found no change of T when stallions were presented with an estrus mare or upon ejaculation.[29] However, when measured in stallions at rest over a 24 hr period, T showed significant diurnal variations.[10,29] This diurnal variation may be the cause for T differences surrounding the time of ejaculation found by other researchers.[7,9] The lack of significant variation in T at any time surrounding mating leads to the possibility that T may not play a major role in stallion sexual behavior.

To further investigate this theory, regression analysis was used to analyze the data to determine if any significant correlations existed between T and sexual behavior. There were no significant ($P > 0.05$) relationships between T and libido scores, reaction times, or number of jumps; which supports the theory that T does not play a substantial role in stallion sexual behavior at or near time of mating. This theory is in agreement with several other studies which stated that T concentration could not be linked to libido.[21-24] However, previous reports state that sexual performance could be restored when castrated males were administered exogenous T.[25-27] This contradiction in results may be due to the fact that T can be converted to estrogens.[64] When geldings were administered T alone, an increase in libido was observed, and upon subsequent blood collections both T and estrogens were observed;[28] thus demonstrating that T can be aromatized to estrogen in the stallion. Further, when castrated male rats were administered T only, an increase in the desire to mount was observed; however, when T was given in conjunction with an aromatization inhibiting steroid, the increase in libido was not observed.[52] These results indicate that the aromatization of T to estrogens, and not T alone, may be responsible for the changes in stallion sexual behavior.

Ejaculate parameters were also compared to T to ascertain if any association exists. Testosterone was not related to volume or sperm concentration. However, a positive correlation was observed between T and progressive motility. This same relationship was observed when testicular testosterone was suppressed in the stallion.[17] However, several articles state that a positive relationship was observed between T and spermatozoa concentration.[15,17] In the present study very little variation was observed

between the stallions in reference to sperm concentration. This is most likely due to the small number of stallions included in the study and may be why the same correlation between T and concentration was not observed.

Estrone Sulphate

The range of blood ES concentration in stallions is highly variable. Values have been reported from a minimum of 2.447 ± 1.996 ng/ml to a maximum of 180.85 ± 120.63 ng/ml.[9,65] The mean range of ES found in the present research ranged from 116.18 ± 51.16 ng/ml to 209.88 ± 82.49 ng/ml, which is within concentrations previously reported. However, a significant variation between the stallions in ES concentration was found.

When measured at times surrounding mating, mean estrone sulphate concentration (± SD) showed a significant increase immediately following ejaculation, time 0 (from 116.18 ± 51.16 ng/ml to 209.88 ± 82.49 ng/ml; $P < 0.001$). Concentrations then returned to basal values within 30 min following ejaculation. These data are consistent with prior results for stallions.[7,9] A rise in estrone sulphate was observed in previous research within 10 min after ejaculation (from 124.77 ± 71.12 ng/ml to 180.85 ± 120.63 ng/ml) and a subsequent return to pre-mating values within 30 min of ejaculation.[9]

The rise in ES following mating leads to the speculation that ES may play a substantial role in stallion sexual behavior. Upon further statistical analysis, ES was found to have a negative correlation with libido scores at times -15, +30, and +60 ($P <$

0.05). Due to the lack of variation between stallions in number of jumps required to ejaculate, no relationship was found between ES and number of jumps. However, ES was significantly positively correlated to reaction time 15 min prior to ejaculation and +60 ($P < 0.05$). These relationships identify ES as a major factor in stallion sexual behavior. Previously, studies have reported both negative and positive relationships between equine libido and the estrogen, estradiol-17β.[28,55,56] However, estrone sulphate is more abundant in stallion plasma than estradiol-17β.[4,45] This led to the theory that ES may play a larger role in stallion libido, which is supported by the results of the current study as ES blood level could be an indication of reaction time and libido prior to mating.

Neither volume, progressive motility nor spermatozoa concentration were correlated to ES in the present data. Earlier studies suggested that estrogen concentration was positively correlated to sperm concentration and volume of the ejaculate.[27,28,46-49] Another study reports that estrogens suppress spermatozoa concentration.[50] However, these studies measured estrone and estradiol-17β in their free form; whereas, in the present study the sulfated form of estrone was measured. The current results indicate that ES has neither a stimulatory nor inhibitory effect on ejaculate volume or concentration.

Ratio of Estrone Sulphate to Testosterone

Previous research indicates that both testosterone and estrogens are required for normal copulation in males of several species.[28,48,51] Administration of estrogens to

castrated males enhanced sexual arousal; however, intromission and ejaculation were not achieved unless testosterone was also administered. Furthermore, in the bull, low libido was correlated to significantly higher estrogen to testosterone ratios.[14] This same relationship was found in the current data. The ratio of ES/T at times -15, +30, and +60 was negatively correlated to libido scores ($P < 0.05$). The ratio of ES to T was also negatively correlated to progressive motility ($P < 0.05$). However, ES/T was not linked to reaction time or number of jumps. Still, the relationships discovered indicate that pre-mating blood concentrations of ES values and the ratio of ES to T are more accurate predictors of stallion libido than T concentrations alone.

Cortisol

The concentration of cortisol previously reported for normal stallions is between 19.88 ± 7.49 ng/ml and 70 ng/ml.[9,35] The mean cortisol range found in the present study is between 4.31 ± 1.31 µg/dL and 4.92 ± 1.63 µg/dL, which is within the values described in the past. However, there was significant variation between the stallions in cortisol concentration.

Mean cortisol concentrations rose significantly from pre-mating values until 15 min following ejaculation (from 4.31 ± 1.31 µg/dL to 4.92 ± 1.63 µg/dL; $P < 0.05$). Concentrations then returned to pre-mating values within 30 min following ejaculation. A similar pattern was observed in another study which reported an increase in cortisol concentration from pre-mating values to a peak concentration 10 min following ejaculation (19.88 ± 7.49 ng/ml to 33.65 ± 9.38 ng/ml).[9] However, this same study also

reported that cortisol remained at peak values through 30 min after ejaculation and still another study reported that cortisol remained at peak levels until 120 min post-ejaculation.[9,35] These sustained high levels were not found in the current study as cortisol returned to basal values within 30 min of mating. Like T, cortisol shows a diurnal variation which is most likely the cause of the variations between studies.[66-68] Regardless of the pattern, the change in cortisol in the time surrounding ejaculation leads to the theory that cortisol may play a significant role in stallion sexual behavior. This theory is supported in the present study as libido scores were found to be significantly positively correlated to cortisol at times -15, 0, and +15 ($P < 0.05$). These findings contradict previous research in human and rat males which stated that a negative relationship exists between cortisol and sexual arousal.[32,43] However, in the stallion it has been consistently shown that cortisol increases when sexual arousal is stimulated either by presenting an estrus mare or upon hearing another male being stimulated.[9,11,34,35] These findings in conjunction with the present results lead to the conclusion that, unlike humans and rats, the stallion exhibits a positive relationship between cortisol and libido. Cortisol concentrations were not related to number of jumps or reaction time in the present study.

Previous researchers have speculated that cortisol may have an effect on male reproductive endocrinology through its relationship with testosterone.[8-11] However, in the present study cortisol was not significantly correlated to T at any time surrounding ejaculation. Research stating a negative association between cortisol and T reported that an increase in cortisol caused a depression in the T diurnal variations seen in

controls.[10,11] Blood collection times in the current study did not extend far enough after

ejaculation to make the same determination. Further, both T and cortisol undergo

natural concentration fluctuations throughout the day, which contributes to variation

between studies.[10,29,66,68] It appears from the present data that cortisol and T do not

interact; however, this association requires further investigation that takes diurnal

variation of these hormones into consideration. Cortisol concentration was not related to

any ejaculate parameters measured.

CHAPTER VI

SUMMARY

The objectives of the present study were to identify the circulating plasma concentrations of T, cortisol and ES around ejaculation; and to determine if relationships exist between blood hormone concentrations and sexual behavior or sperm characteristics. The results indicate that around the time of ejaculation both ES and cortisol increase, while T does not. These fluctuations indicate that ES and cortisol may play an important role in stallion sexual behavior.

Upon analysis, a negative correlation was found between libido and both ES and the ratio of ES to T. Additionally, a positive relationship was observed between ES and reaction time. Cortisol concentrations were positively correlated to libido scores, however, there was no correlation between libido scores and T. These associations indicate that cortisol and ES may be more accurate predictors of stallion sexual behavior than T alone. In application, manipulation of ES and cortisol could alleviate problems in stallions who exhibit poor libido. This in turn would decrease time expenditure and financial losses experienced by stallion managers.

Additional analysis revealed a positive correlation between T and progressive motility, which supports previous research. Similarly, a negative relationship was seen between ES/T and progressive motility. However, no other relationships were detected between ejaculate parameters and hormone concentrations. This could be due to the

small number of stallions observed and only minor variations between the stallions in

sperm concentration.

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