PLASMA CONCENTRATIONS OF TESTOSTERONE, LUTEINIZING HORMONE, AND ESTRONE SULFATE IN STALLIONS FOLLOWING HEMICASTRATION

A Thesis

by

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ABSTRACT

Hemicastration is a veterinary surgical procedure in stallions and may be needed for removal of a diseased testicle. The effects of hemicastration on the neuroendocrine system and the hormonal response of the remaining testicle are unclear. In this study, blood plasma concentrations of testosterone, luteinizing hormone, and estrone sulfate were assessed following hemicastration. Miniature stallions (n=8) were used in this study and blood was drawn 7 d prior to hemicastration, and 12 h, 48 h, 14 d, 30 d, and 90 d post hemicastration. Blood samples from all stallions were drawn every 15 min (0, 15, 30, 45, 60 min) for 1 h each sampling period. Plasma was analyzed by RIA for concentrations of testosterone, luteinizing hormone, and estrone sulfate. Compared to pre-surgerical concentrations, plasma luteinizing hormone at 12 h, 48 h, 14 d and 60 d were greater ($P < 0.05$). Compared to 12 h, plasma testosterone values at 48 h, 14 d, and 60 d were higher ($P < 0.05$). Compared to pre-hemicastration values, plasma concentrations of estrone sulfate were lower ($P < 0.05$) at all time periods, but tended to increase up to 60 d. After 30 d, stallions were housed together rather than individually creating a harem group. Luteinizing hormone and testosterone values increased dramatically compared to previous time periods following the housing modification. These results provide insight to better understand the hormonal profiles and compensatory response of the remaining testicle following hemicastration.

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CHAPTER I

INTRODUCTION

The most desirable and financially attractive stallions are those that are reproductively capable of handling a large book of mares per breeding season (Blanchard et al., 2012). Prior to the breeding season, mare owners and managers seek out stallions with impressive pedigrees, notable performance records, and outstanding conformation (Varner et al., 2008) that may successfully cross with a mare and produce sought after offspring. It is imperative to optimally manage and maintain breeding stallions' capabilities to be reproductively efficient and produce quality spermatozoa. Subfertility in stallions leads to lower conception rates and contributes to a reduction in economic sustainability (Roser, 2001). Subfertility is associated with testicular degeneration that may be linked to genetics, aging, effects of long-term medications, or environmental effects, such as scrotal trauma, heat, or fever (Varner et al., 2008).

In order to minimize any potential negative effects of a damaged testicle and reduce the onset of infertility due to a diseased testicle, hemicastration may be an elected procedure (Zhang et al., 1990; Papa et al., 1990). Testicular torsion and inguinal/scrotal hernias may be causes for emergency surgery and removal of a testicle (Horney and Milne, 1964; Pascoe et al., 1981; Threlfall et al., 1990; McKinnon and Voss, 1993).

The neuroendocrine system is important in controlling the function and regulation of spermatogenesis in the stallion (Matsumoto, 1989; Amann, 1993; Weinabuer and Nieschlag, 1993; Roser, 1997). The hypothalamic-pituitary-testicular

(HPT) axis function commences with the production and secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus (Schanbaher, 1982), which in turn stimulates the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Levine, et al., 1982). Luteinizing hormone acts upon the Leydig cells of the testes and causes testosterone to be synthesized and secreted. The major functions of the testes include steroidogenesis, the process of secreting testosterone, estrogens, and other hormones, and spermatogenesis, the production of spermatozoa (Amann and Schanbacher, 1983). The production of spermatozoa and subsequent fertility of a stallion is undoubtedly linked to efficient neuroendocrine coordination.

The effect of hemicastration on the HPT axis is unclear. Though stallion fertility and return to successful breeding following hemicastration are of upmost concern to veterinarians, stallion owners, and stallion managers, research investigating the hormonal response of the remaining testicle will provide insight to researchers and provide sound, comprehensive understanding to stallion reproductive efficiency.

The objective of the current study is to:

measure blood plasma concentrations of testosterone, LH, and estrone sulfate in order to assess the response of the remaining testicle following hemicastration.

CHAPTER II

LITERATURE REVIEW

Hemicastration

Hemicastration is a common procedure for stallion owners, managers, and veterinarians to consider to remove a traumatized testicle and prevent possible thermal damage to the sound testis (Brinsko, 1998; Schumacher, 2012). This procedure is performed with intent to remove any potential negative impact of the damaged testicle. The development of immune-related infertility and degeneration of the damaged testicle as well as impaired function of the other testicle may be associated with negligence to hemicastrate a stallion following unilateral testicular trauma (McKinnon and Voss, 1993). Other reasons for hemicastration include removal of a retained testicle (cryptorchid) or previous surgery that failed to correctly identify the testes and accidently left all or a portion of the testis in the animal (i.e. surgical error) (Cox, 1987; Adams, 1990; Pascoe, 1990; Maxwell, 2005).

Hemicastration serves as a viable approach to surgically manage inguinal and scrotal hernias and epididymitis (Held et al., 1989; Held et al., 1990; McKinnon and Voss, 1993). A reluctance to ejaculate and lameness related to sperm granuloma and inflammation may necessitate hemicastration in the stallion (Blue and McEntee, 1985; Held et al., 1989). The removal of a diseased testicle in the stallion will also require hemicastration. Certain conditions including, testicular neoplasms and testicular tumors have been reported in the horse (Willis and Rudduck, 1943; Baker and Leyland, 1975;

Sundberg et al., 1977; Morse and Whitmore, 1986). Discovery of testicular neoplasms, more specifically benign teratomas, typically occur during routine cryptorchid castration (Stick, 1980). Stallions affected by testicular neoplasms do not exhibit pain; however, an enlargement of the affected testis is standard (Caron et al., 1985). In stallions, severe testicular torsions of more than 270° is associated with colic and will obstruct circulation to the testis, and thusly the testis must be removed. The formation of hematoceles which are linked to testicular torsions or other forms of trauma, are also causes of testicular degeneration (Edwards, 2008). A previous study, however, supports the recovery of spermatogenesis and return of normal fertility due to the removal of a testicle following a trauma to the testicle and subsequent hematocele formation (Gygax et al., 1973).

There is speculation concerning post-surgical thermal damage to the remaining testicle following hemicastration which may have a negative impact on semen quality. Consequently, a period of sexual rest to allow adequate functional recovery due to the thermal insult by the testis is generally accepted. In a recent study, semen quality and size of the remaining testicle was assessed following hemicastration (McCormick et al., 2012). Total sperm motility was not significantly different between pre-surgical and post-surgical values (Table A.1). A significant increase in % viable sperm was evident 30 d and 60 d post hemicastration when compared to pre-hemicastration or 14 d post hemicastration values (Table A.1). Furthermore, an increase in distal and cytoplasmic droplets in the ejaculate caused a significant decrease in % morphologically normal sperm 60 d post hemicastration when compared to all other measured time points (Table A.1). Total sperm numbers were observed to be significantly lower 14 and 30 d post

hemicastration than pre-surgical and 60 d post surgery values (Table A.1). A significant increase ($P < 0.05$) in mean testis volume of the remaining testis was estimated by ultrasonography 30 d and 73 d post hemicastration when compared to pre-hemicastration measurements (Table B.1). Upon completion of the project, the remaining testis was removed from each hemicastrated stallion. The weight of the second testis removed was recorded and compared to the weight of the first testis removed at the beginning of the project for each stallion. Weight values of the second testis were averaged and the mean testis weight was significantly greater than the mean testis weight of the first testis removed (Table C.1) (43.8 ± 33.4 g to 64.8 ± 14.9 g; $P < 0.05$).

Testosterone

Leydig cells are responsible for the production and secretion of testosterone and other testicular steroids (Ewing and Brown, 1977; Zirkin et al., 1980). Testosterone is a contributor to spermatogenesis and is an important component of the hypothalamic-pituitary-testicular axis (Roser, 1997). The pulsatile release of LH from the pituitary acts upon the Leydig cells of the testis causing testosterone to be synthesized in an episodic manner. The amount of testosterone produced by Leydig cells is correlated to the volume of smooth endoplasmic reticulum as researched in the rat, rabbit, guinea pig, dog, and hamster (Zirkin et al., 1980). An increase in endogenous testosterone influences the hypothalamus to suppress GnRH and LH concentrations. Consequently, the production of testosterone by the Leydig cells drops, illustrating a negative feedback loop that returns testosterone to basal concentrations (McKinnon and Voss, 1993).

Interestingly, the number of Sertoli cells and germ cells per Sertoli cell can influence the testosterone concentrations measured in stallions during the breeding season (Johnson and Thompson, 1983). The diffusion of testosterone from the Leydig cells into the seminiferous tubules, along with the secretion of FSH from the anterior pituitary, is important in initiating and supporting the development of germ cells (Roser, 2008).

Testosterone is necessary for the production and maturation of spermatozoa (Amann and Schanbacher, 1983). The initiation and maintenance of spermatogenesis as well as normal testicular function is dependent upon the availability of testosterone in adult testes (Weinbauer and Nieschlag, 1993). Johnson and Thompson (1983) found that increases in the concentration of testosterone in peripheral blood coincided with increases in the quantity of spermatozoa produced, suggesting that testosterone is important in regulating spermatogenesis. Other literature explains that immature (< 15 months of age) and castrated stallions had significantly lower concentrations of testosterone than mature, fertile stallions. An increased production of sperm is typical for stallions after the pubertal phase; furthermore, a similar increase in testosterone concentrations was reported in the same stallions (Amann et al., 1979; Johnson and Neaves, 1981; Johnson and Thompson, 1983; Jones and Berndtson, 1986). Analagous to pre-pubertal stallions, infertile, or azoospermic stallions have lower concentrations of testosterone when compared to normal, fertile stallions (Inoue et al., 1993). Suppressed testosterone in the stallion, as evidenced by androgen withdrawal, negatively influenced concentration of spermatozoa, progressive motility, and % normal spermatozoa (Squires

et al., 1981). As a result, testosterone and its relation to the production of spermatozoa must be granted (Squires et al., 1981; Berndtson and Jones, 1989).

The relationship between sexual behavior in the stallion and concentration of testosterone are not completely understood. During the breeding season, an increase in stallion testosterone concentration was measured and associated with increases in the presentation of sexual behavior (Thompson et al., 1977). In another study, harem stallions, which guarded the harem fence line area that allowed visual access to a group of mares, exhibited aggression towards non-dominant, bachelor stallions. Harem stallions exhibited significantly higher testosterone concentrations than those of bachelor stallions (McDonnell and Murray, 1995). Moreover, geldings administered with 175 μg of testosterone propionate obtained the ability to ejaculate and illustrated stallion libido characteristics (Thompson et al., 1980). Other studies, however, indicate no connection between the displays of sexual behavior in stallions or bulls with the concentrations of testosterone measured (Burns and Douglas, 1985; Price et al., 1986). Recently, testosterone levels in stallions were evaluated for premating, immediately after ejaculation, and post-ejaculation periods and differences in concentrations were not found to be significant. This suggests that the concentration of testosterone alone may not be pivotal to the expression of sexual behavior in the stallion (Cavinder et al., 2010). However, testosterone may influence the expression of stallion-like behaviors in mares. Upon examination of mares that exhibited stallion-like behavior, testosterone concentrations were 38.4% higher when compared to mares that this behavior was directed toward (Gastal et al., 2007).

The concentration of testosterone in stallions is influenced by seasonality (Berndtson et al., 1974b). Increases in daylight length do not directly stimulate spermatogenesis. However, the increased length of daylight does indirectly influence the secretion of androgens, including testosterone, in the stallion (Skinner and Bowen, 1968). During the winter months, stallions have been subjected to increased periods of artificial photoperiod, which led to increases in mean testosterone concentrations. Stallions not exposed to an increase in photoperiod during the same time period did not show the same increase (Thompson et al., 1977).

The endocrine response of the remaining testicle following hemicastration shows that testosterone concentrations in one, two, and three-year-old stallions were similar between intact and hemicastrated stallions (Hoagland et al., 1986). Compensatory hypertrophy, however, was exhibited following hemicastration in these stallions and may be associated with an anticipated and presumed increase of intratesticular testosterone production per unit of tissue (Hoagland et al., 1986). Testicular testosterone may be a link to promotion of testicular growth and thus compensatory hypertrophy; however, a definite correlation between testosterone and testicular size is not available (Berndtson and Jones, 1989).

Luteinizing Hormone

Peak testicular function and sperm output and production occurs in the stallion during spring and summer months when the production of melatonin from the pineal gland is low and the production of GnRH high (Clay et al., 1987; Roser, 2008).

Gonadotropin releasing hormone, secreted from the hypothalamus, signals the release of FSH and LH from the anterior pituitary which act on the testis (Roser, 2008). The apparent cause-and-effect relationship between GnRH and LH is recognized as distinct pulses of GnRH that lead to immediate, pulsatile discharges of LH (Lincoln, 1979). Gonadotropic hormones released from the anterior pituitary, including LH and FSH, control the testes, responsible for the production of spermatozoa and biosynthesis of androgens. Testicular regression was initiated with the removal of the pituitary gland in both rats and sheep, supporting the importance that the anterior pituitary and gonadotropins from this gland have on testicular function (Smith, 1927; Courot, 1967). Spermatogenesis, however, was restored and testicular size increased following the administration of LH and FSH in young lambs after hypophysectomy (Courot, 1967; Schanbacher, 1980).

Testosterone and estrogens are produced and released from the Leydig cells in the stallion following LH stimulation (Eisenhauer et al., 1994; Eisenhauer and Roser, 1995; Roser, 1997). Research in rams shows that LH is released in pulses, between 1 and 12 times in a 24 h period, and these pulses are generally followed by a pulse in testosterone concentrations (Sanford et al., 1974; Lincoln, 1976; Schanbacher, 1982). Specific receptors on the plasma membrane of Leydig cells activate adenyl cyclase. This enzyme serves as the catalyst to the cascade of events that converts cholesterol to testosterone. Adenyl cyclase initiates the conversion of ATP to cAMP, which then, as a second messenger, stimulates a cytoplasmic protein kinase to transform cholesterol to testosterone (Neaves, 1975; Schanbacher, 1982). As previously described, testosterone

is fundamental for the production and maturation of spermatozoa (Amann and Schanbacher, 1983); thus, it is established that availability of LH can be associated with the maintenance of spermatogenesis through its relationship with the androgen testosterone. The regulation of pituitary LH and FSH, as well as hypothalamic GnRH, is linked to a negative feedback system involving testosterone and other testicular steroids. Testosterone does not directly decrease the secretory pattern of LH; instead, the inhibition of GnRH by testosterone has an effect on the pituitary, causing lower concentrations of LH to be secreted (Schanbacher, 1980; Roser, 2008). Castrated rams and bulls have decreased concentrations of testosterone and increased concentrations of LH and FSH, illustrating the negative feedback loop that testosterone has on gonadotropins (Schanbacher and Ford, 1977; Amann and Schanbacher, 1983; Amann and Walker, 1983). Similarly, infertile stallions, such as those diagnosed with azoospermia, have elevated concentrations of LH and FSH when compared to normal, fertile stallions (Blue et al., 1991). There are, however, conflicting results describing impotent stallions with lower levels of LH and estradiol than normal stallions (Wallach et al., 1983).

Increased concentrations of LH and testosterone due to copulation and sexual stimulation have been reported in the rat, bull, ram, and stallion (Taleisnik et al., 1966; Katongole et al., 1971; Purvis and Haynes, 1974; Schanbacher et al., 1987; Rabb et al., 1989; McDonnell and Murray, 1995). The effect that sexual stimulation and ejaculation have on concentrations of LH, though, is variable in the stallion. Within 1 h after ejaculation, Irvine et al. (1985) found a 34% increase in plasma concentrations of LH in

stallions. On the contrary, Bono et al. (1982) and Rabb et al. (1989) did not see an increase in plasma LH concentrations in stallions immediately following copulation or after sexual stimulation. As a result and in contrast to findings in other species, an increase in LH from the anterior pituitary does not result from sexual stimulation or ejaculation in the stallion (Rabb et al., 1989).

Pulses of LH are critical for testicular growth in the bull (Schanbacher, 1981; Schanbacher, 1982; Thompson et al., 1985). In unilaterally castrated stallions, serum LH concentrations were evaluated in order to assess any physiological effects. A significant increase in serum LH concentrations, as well as in serum FSH concentrations, was associated with unilateral castration in the group of stallions. Hoagland et al. (1986) suggests that the compensatory hypertrophy in the remaining testis of the unilaterally castrated stallions may be justified by the increased serum concentrations of LH and FSH. Those results are conflicting, however, to a study conducted with bulls. Even though the remaining testis of hemicastrated bulls significantly increased in weight due to an increase in tubule length, concentrations of LH were not affected and were similar to pre-surgical values (Leidl et al., 1980). As a result, it is not known whether LH can be considered a direct contributor to compensatory hypertrophy.

Estrone Sulfate

Mature breeding stallions possess large quantities of estrogens in peripheral blood plasma and in semen (Claus et al., 1992). The production of estrogens originates at the testicular level in the stallion, with high quantities of this steroid typical in the

species (Nyman et al., 1959; Bedrak and Samuels, 1969; Raeside and Christie, 1997). High concentrations of estrogens have been identified in stallion testes (Raeside and Christie, 1997). When compared to quantities measured in nonpregnant mares by examining urine excretion, daily estrogen output in the stallion was greater by a factor of 100 to 200 (Pigon et al., 1961; Raeside and Christie, 1997). Although testosterone is considered to be the "steroid of greatest physiologic importance" (McKinnon and Voss, 1993), Leydig cells within the testis produce larger quantities of estrogens than testosterone.

The synthesis of estrogens can follow different metabolic pathways to produce three common estrogens, including estradiol, estrone, and estriol. Androstenedione, a product derived from Leydig cells in response to pulses of LH and FSH, is converted into testosterone, estrone, or estradiol (McKinnon and Voss, 1993). The conversion of androstenedione to estrone or estradiol requires the enzyme aromatase (Santen, 1975). In addition, estradiol and free estrone have been measured in testes. The most abundant estrogen in the stallion testis is estrone conjuated with a sulfate (Raeside et al., 1969; McKinnon and Voss, 1993). Concentrations of estrone sulfate are reported as being 100 times greater than testosterone concentrations in peripheral blood (Raeside, 1978/1979). Thus, the analysis of estrone sulfate may provide insight to the endocrinologic activity of stallion testes.

The effects of copulation on blood plasma estrone sulfate concentrations have been previously studied and have provided inconsistent and variable results (Bono et al., 1982; Claus et al., 1992). Plasma estrone sulfate concentrations increased within 15 min

after copulation in stallions (Bono et al., 1982) and significantly increased immediately after ejaculation, with concentrations returning to basal values within 30 min after ejaculation in miniature stallions (Cavinder et al., 2010). This suggests that estrone sulfate may be a pivotal contributor in stallion sexual behavior and is in agreement with previous research that found an increase in libido in geldings administered estradiol (Thompson et al., 1980).

The role of estrone sulfate and estrogens in stallions is not completely understood. Thompson and Honey (1984) reported that sperm concentration was suppressed by estrogens. Cavinder et al. (2010) describes the absence of a correlation between ejaculate volume, progressive motility, and spermatozoa concentration with concentrations of plasma estrone sulfate. Roser and Hughes (1992), on the other hand, reported a connection between concentration of estrogens and sperm concentration. This study observed that low concentrations of estrogens coincided with very low sperm concentrations in a group of azoospermic stallions, and suggests that varying concentrations of estrogens may be associated with varying semen quality. Further investigation is necessary to gain knowledge about the physiological and behavior effects mediated by estrone sulfate and other estrogens.

CHAPTER III

MATERIALS AND METHODS

Stallions, Housing, and Diets

Eight Miniature Horse stallions between the ages of 4 and 15 y were used in this study (mean = 7.9 y). Stallions were housed at the Texas A&M University Horse Center in individual stalls (1.8 m x 1.8 m) and were allowed free exercise every other day in individual turnout pens (7.3 m x 1.8 m). Stallions were housed individually prior and up until 60 d post hemicastration. This portion of the study occurred during the winter months, from November to January. Upon completion of the semen collection 60 d post hemicastration, stallions were housed in a harem-bachelor group for the remainder of the project. Stallions were housed in this harem-bachelor group from January to February of and had visual access to mares located in an adjacent pasture through mutual fence line. Each stallion was fed 0.5% of BW in concentrate/d and 1.5% of BW in coastal Bermudagrass hay/d. *Ad libitum* access to water was provided. The Texas A&M University Insitutional Agricultural Animal Care and Use Committee provided project approval using guidelines set forth by the Federation of Animal Science Societies (2010).

Blood Sampling

Blood samples were collected for evaluation of plasma concentrations of T, LH, and ES prior to and post hemicastration. Blood samples (10 mL) were drawn via jugular

venipuncture using vacutainers and placed into blood collection tubes every 15 min (T0, T15, T30, T45, T60) for 1 h, 7 d prior to unilateral orchiectomy. The samples were kept on ice until they were centrifuged. Additionally, blood samples (10 mL) were collected 12 h, 48 h, 14 d, 30 d, and 90 d post hemicastration with the use of jugular catheterization with a 7.62 cm 20-gauge I.V. catheter at intervals of 15 min (T0, T15, T30, T45, T60) for 1 h into blood collection tubes. Upon completion of each blood draw, the catheters were flushed with heparinized saline and the blood collection tubes were placed on ice until they were centrifuged.

Plasma Processing

Following each sampling period, blood plasma was refrigerated until time of processing (within 2 h of collection). Blood samples were centrifuged for 20 min at 2500 rpm in a refrigerated centrifuge. Blood plasma was then aspirated with disposable pipettes, placed in micro centrifuge tubes (1.8 mL), and stored at -20°C until the plasma was assayed for T, LH, and ES concentrations.

Radioimmunoassay (RIA) Procedures

Plasma T was evaluated with a Testosterone RIA Kit (Diagnostic Systems Laboratories, Inc., Webster, TX) previously validated for horse samples (Johnson et al., 2003). A 50 μL sample of plasma was measured for T concentration in duplicate. Controls were run in duplicate, while standards were run in triplicate. The measurement range of the RIA was 0.18-100.0 pg/mL and kit sensitivity was 0.18 pg/mL. The

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

Plasma ES was analyzed using a RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX) previously validated for use with horses (Villani et al., 2006). A 100 µL sample of plasma was recommended by the kit to measure ES concentration; however, due to high concentrations of ES in the plasma, 25 µL of plasma were measured in duplicate in order to fit the standard curve. Controls were also run in duplicate, while standards were run in triplicate. The measurement range of the RIA was 2.0-320.0 ng/mL with a sensitivity of 0.01 ng/mL. The samples were counted using Perkin-Elmer Packard Cobra II Auto-Gamma counter (Packard Systems, Meridan, CT).

Plasma LH concentrations were measured by RIA as described and validated for the horse (Williams et al., 1982). On day 1 of the assay, 500 µL of 1% phosphate buffered saline (PBS) with egg white (PBS-EW) was added to the non-specific binding (NSB) and 0 standard tubes. Two hundred mL of standard and 300 µL of 1% PBS-EW were added to the standard tubes. Three hundred mL of 1% PBS-EW and 200 µL of the unknown plasma sample were then added to the unknown tubes. Three hundred mL of 1% PBS-EW and 200 µL of reference preparations were added to the reference preparation tubes. Next, anti-eLH primary antibody was diluted with PBS-EDTA and a normal rabbit serum (NRS) in a 1:400 ratio. Two hundred mL of the PBS-EDTA and NRS without the primary antibody were added to the NSB tubes. Two hundred mL of anti-eLH (diluted with PBS-EDTA and 1:400 NRS) was pipetted into all tubes except the NSB and total count tubes. One hundred mL of 125I-eLH tracer (20,000

counts/minute/100 µL diluted in 0.1% PBS-EW) were added to all tubes. Following the addition of the tracer, all tubes were vortexed briefly and incubated for 24 h at 4°C. On the second day of the assay, 200 µL of sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS were added to all the tubes except the total count tubes. The tubes were again vortexed briefly and incubated in a refrigerator for 48 h at 4°C. On the final day of the assay, 3.0 mL of ice cold PBS (0.01 M; pH 7.0) were added to all the tubes except the total counts. All tubes were then centrifuged for 1 h at 4°C at 4200 RPM in the Beckman J-6 M/E (JS-4.2 Rotor). The supernatant was then decanted into a waste receptacle and the tubes were counted using the Packard Autogamma 5780 Counter.

Semen Collection and Analysis

Semen was collected from Miniature Horse stallions in order to assess the capability for stallions to return to successful breeding after hemicastration. Semen quality was evaluated during the following collection periods: 7 d before hemicastration, 14 d, 30 d, and 60 d post hemicastration (McCormick et al., 2012). Ejaculates were collected for 5 consecutive d, with semen from the final 2 d per collection period serving as a representation of the actual seminal parameters being studied. Semen collections took place at the Texas A&M University Horse Center using a 33 cm Missouri-model AV (Nasco, Ft. Atkinson, WI). Stallions were exposed to Miniature Horse mares exhibiting behavioral estrus to induce sexual stimulation. A commercial semen extender (INRA-96, Breeder's Choice, Aubrey, TX) was used to dilute raw samples at a ratio of

1:4 (raw semen:extender). Semen was transported in coolers (Koolatron FI3, Koolatron™ , Brantford, Ontario) at 16°C from the Texas A&M University Horse Center to the Theriogenology Laboratory at the Texas A&M School of Veterinary Medicine, located 2 km from the Texas A&M Horse Center, for analyses. An automated cell counter (NucleoCounter SP-100; ChemoMetec, Allerød, Denmark) was used to define both sperm concentration as well as % viable sperm. Extended semen samples were analyzed for total sperm motility using computer-assisted spermatozoa analysis (CASMA, IVOS version 12.2 L, Hamilton-Thorne Biosciences, Beverly, MA). Raw, gel-free semen samples, buffered in 10% formol saline, were analyzed to determine % morphologically normal sperm using differential interference contrast (DIC) microscopy (Olympus BX60, Olympus America, Inc., Melville, NY, 1250X magnification) as previously validated and described (Edmond et al., 2012).

Statistical Analysis

The SAS mixed repeated measures analysis of variance was used to analyze data (SAS v 9.2; SAS Inst. Inc., Cary, NC). Hormone type (T, LH, ES) was considered a fixed effect and stallion was random and time the repeated measure. In order to determine significant differences between hormone concentrations and time periods, least squares means and differences of least squares means differences were evaluated. Data were considered to be significantly different at $P \leq 0.05$.

CHAPTER IV

RESULTS

Mean Hormone Concentrations

Mean blood plasma for each stallion at each collection time was used to calculate results. A single blood draw was used to calculate results 7 d prior to hemicastration, 12 h, 30 and 90 d post hemicastration for stallion 3 and 14 d post hemicastration for stallion 6. A decrease in plasma testosterone concentrations ($P < 0.05$) was demonstrated 12 h, 14 d, and 30 d post hemicastration when compared to pre-surgical values (Table 1). Plasma testosterone concentrations 90 d post hemicastration, however, dramatically increased ($P < 0.0001$) from values 30 d post hemicastration. Plasma LH concentration were greater ($P < 0.05$) 12 h, 48 h, and 14 d post hemicastration when compared to pre-surgical values (Table 2). Similarly to the increase in testosterone concentration found 90 d post hemicastration, the plasma concentration of LH also significantly increased ($P < 0.0001$) at this time period as compared to values 30 d post hemicastration. In contrast, blood plasma estrone sulfate concentrations were lower at every measured post-castration time period ($P < 0.05$) and never recovered to pre-surgical values (Table 3). Though plasma estrone sulfate concentrations were lower at every time period following hemicastration than pre-surgical concentrations, estrone sulfate tended ($P < 0.05$) to increase up to 90 d. There were some differences in plasma testosterone, LH, and estrone sulfate when analyzing concentrations before and after hemicastration (Table 4).

Table 1. Mean (± SE) plasma testosterone (ng/mL) concentrations for individual stallions before (-7 d) and after (12 h, 48 h, 14 d, 30 d, 90 d) hemicastration.

	Time Period					
Stallion	-7 d	12 h	48 h	14 d	30 d	90 d
1	3.86 ± 1.33	1.16 ± 0.30	0.42 ± 0.02	0.82 ± 0.17	1.07 ± 0.28	4.48 ± 0.47
2	2.63 ± 0.18	0.59 ± 0.03	0.30 ± 0.05	1.72 ± 0.69	2.36 ± 0.38	3.26 ± 0.62
3	2.06 ± 0.32	0.25 ± 0.00	1.26 ± 0.12	0.90 ± 0.13	1.04 ± 0.10	1.30 ± 0.00
4	0.95 ± 0.09	0.48 ± 0.10	1.76 ± 0.43	1.29 ± 0.40	0.55 ± 0.21	4.40 ± 1.02
5	0.75 ± 0.29	0.63 ± 0.05	0.62 ± 0.12	1.06 ± 0.21	0.31 ± 0.13	2.26 ± 0.62
6	0.95 ± 0.33	0.53 ± 0.09	1.26 ± 0.11	0.90 ± 0.05	0.59 ± 0.06	4.73 ± 0.47
7	1.24 ± 0.18	0.52 ± 0.13	2.53 ± 0.52	1.47 ± 0.67	0.28 ± 0.05	1.67 ± 0.24
8	1.09 ± 0.19	1.18 ± 0.22	3.32 ± 0.38	2.03 ± 0.21	1.80 ± 0.61	7.50 ± 0.59
Mean	1.69 ± 0.18^{d}	0.67 ± 0.05^{a}	1.43 ± 0.16^{cd}	1.27 ± 0.09^{bc}	1.00 ± 0.12^{ab}	3.92 ± 0.41^{e}

[abcdefg] Differing superscripts indicate significant difference from time points in a row ($P < 0.05$)

Table 2. Mean (± SE) plasma LH (ng/mL) concentrations for individual stallions before (-7 d) and after (12 h, 48 h, 14 d, 30 d, 90 d) hemicastration.

	Time Period					
Stallion	-7 d	12 h	48 h	14 d	30 d	90 d
1	0.42 ± 0.09	1.16 ± 0.18	1.31 ± 0.15	0.43 ± 0.07	0.35 ± 0.05	1.49 ± 0.22
2	0.55 ± 0.09	1.26 ± 0.29	0.88 ± 0.86	1.33 ± 0.11	0.84 ± 0.07	3.87 ± 0.22
3	0.27 ± 0.08	0.36 ± 0.22	0.20 ± 0.08	0.28 ± 0.06	0.34 ± 0.03	0.62 ± 0.00
4	0.15 ± 0.05	0.42 ± 0.03	0.79 ± 0.07	0.15 ± 0.01	0.16 ± 0.05	1.11 ± 0.13
5	0.54 ± 0.17	0.76 ± 0.14	1.17 ± 0.07	0.97 ± 0.12	0.55 ± 0.40	1.01 ± 0.23
6	0.10 ± 0.01	0.16 ± 0.06	0.24 ± 0.30	0.10 ± 0.00	0.11 ±0.03	1.59 ± 0.03
7	0.18 ± 0.05	0.18 ± 0.06	0.14 ± 0.04	0.35 ± 0.05	0.11 ± 0.02	0.84 ± 0.24
8	0.18 ± 0.08	0.57 ± 0.13	0.49 ± 0.10	0.44 ± 0.08	0.35 ± 0.11	1.70 ± 0.21
Mean	0.30 ± 0.03^{a}	0.61 ± 0.07^{b}	0.65 ± 0.07^{b}	0.51 ± 0.06^{b}	0.35 ± 0.04^{a}	1.61 ± 0.21^{c}

[abc] Differing superscripts indicate significant difference from time points in a row ($P < 0.05$)

Table 3. Mean (± SE) plasma estrone sulfate (ng/mL) concentrations for individual stallions before (-7 d) and after (12 h, 48 h, 14 d, 30 d, 90 d) hemicastration.

	Time Period					
Stallion	-7 d	12 h	48 h	14 d	30 d	90 d
1	29.35 ± 5.81	24.65 ± 5.05	19.76 ± 3.50	18.63 ± 3.26	18.70 ± 2.44	16.21 ± 0.30
2	44.93 ± 2.91	30.47 ± 4.07	23.39 ± 1.61	32.20 ± 4.05	36.80 ± 3.10	22.77 ± 3.24
3	80.00 ± 0.00	52.33 ± 1.63	36.22 ± 1.44	77.25 ± 3.95	80.00 ± 0.00	73.74 ±1.47
4	79.03 ± 2.16	65.99 ± 9.61	57.11 ± 7.56	56.53 ±11.50	62.83 ± 14.13	71.76 ± 8.52
5	44.24 ± 14.21	35.38 ± 8.27	30.95 ± 7.90	32.41 ± 4.72	33.02 ± 7.57	34.75 ± 7.78
6	19.89 ± 5.43	10.61 ± 1.86	15.38 ± 2.00	16.92 ± 1.08	30.09 ± 5.63	58.28 ± 8.03
7	62.72 ± 7.11	38.93 ± 5.39	37.06 ± 6.26	41.33 ± 2.35	40.60 ± 6.66	44.47 ± 7.04
8	70.75 ± 13.13	44.65 ± 5.28	37.77 ± 5.72	40.46 ± 3.84	42.02 ± 3.92	53.36 ± 7.55
Mean	53.87 ± 3.58^{a}	37.88 ± 2.68^{b}	32.21 ± 2.10^{c}	39.47 ± 3.07^{bd}	43.01 ± 3.08^{de}	45.75 ± 4.32^{e}

[abcdef] Differing superscripts indicate significant difference from time points in a row ($P < 0.05$)

Table 4. Mean (± SE) plasma testosterone, LH, and estrone sulfate (ng/mL) concentrations before (-7 d) and after (12 h, 48 h, 14 d, 30 d, 90 d) hemicastration.

	Time Period					
	-7 d	12 h	48 h	14 d	30 d	90 d
T	1.69 ± 0.18^{d}	0.67 ± 0.05^{a}	1.43 ± 0.16^{cd}	1.27 ± 0.09^{bc}	1.00 ± 0.12^{ab}	3.92 ± 0.41^{e}
LH	0.30 ± 0.03^{a}	0.61 ± 0.07^{b}	0.65 ± 0.07^{b}	0.51 ± 0.06^{b}	0.35 ± 0.04^{a}	1.61 ± 0.21^{c}
ES	53.87 ± 3.58^{a}	37.88 ± 2.68^{b}	32.21 ± 2.10^{c}	39.47 ± 3.07^{bd}	43.01 ± 3.08^{de}	45.75 ± 4.32^{e}

[abcde] Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).

Hormonal Response to Hemicastration

Testosterone

Mean plasma testosterone concentrations (± SE) were significantly lower 12 h post hemicastration when compared to pre-surgical values (0.67 ± 0.05 ng/mL to 1.69 ± 0.18 ng/mL; $P < 0.05$) (Figure 1). Concentrations returned to values similar to pre-hemicastration values within 48 h, however, a steady decline in plasma testosterone concentrations occurred by 14 d and 30 d. Plasma testosterone concentrations significantly increased between 30 d and 90 d, and also showed a difference in testosterone concentrations 90 d post hemicastration when compared to pre-surgical values (3.92 ± 0.41 ng/mL to 1.69 ± 0.18 ng/mL; $P < 0.05$) (Figure 1). Testosterone increased significantly after stallions were housed in a harem-like herd as evidenced by the difference in values 30 d and 90 d post hemicastration (1.00 ± 0.12 ng/mL to 3.92 ± 0.41 ng/mL). This is in agreement with a previous study that describes harem stallions having higher concentrations of testosterone (McDonnell and Murray, 1995).

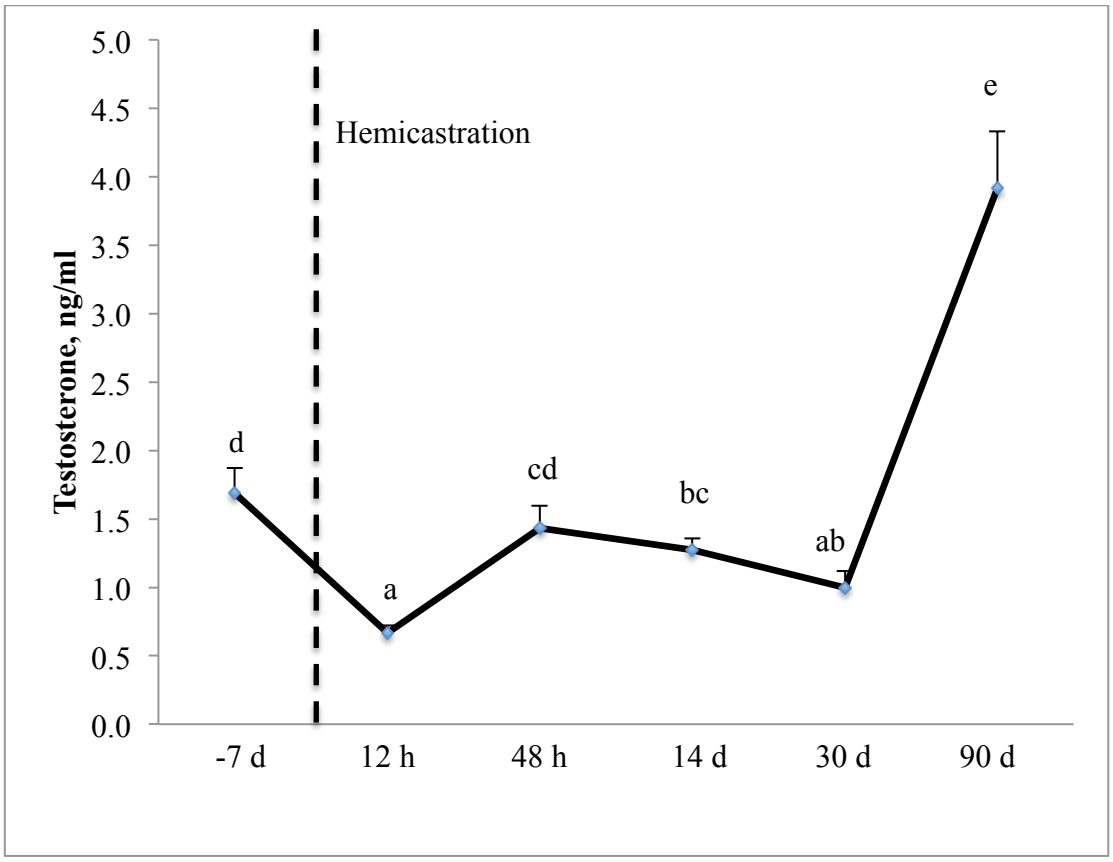

Figure 1. Mean (± SE) plasma testosterone concentrations (ng/mL) 7 d before and 12 h, 48 h, 14 d, 30 d, 90 d post hemicastration. Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).

Luteinizing Hormone

Mean plasma LH concentrations were significantly higher 12 h, 14 d, and 30 d following hemicastration when compared to values pre-surgical values. However, concentrations 30 d after hemicastration were similar to pre-surgical values ($P = 0.51$). When comparing pre-surgical concentrations to those measured 90 d post hemicastration, a significant and substantial increase was recorded (0.30 ± 0.03 ng/mL to 1.61 ± 0.21 ng/mL; $P < 0.05$) (Figure 2).

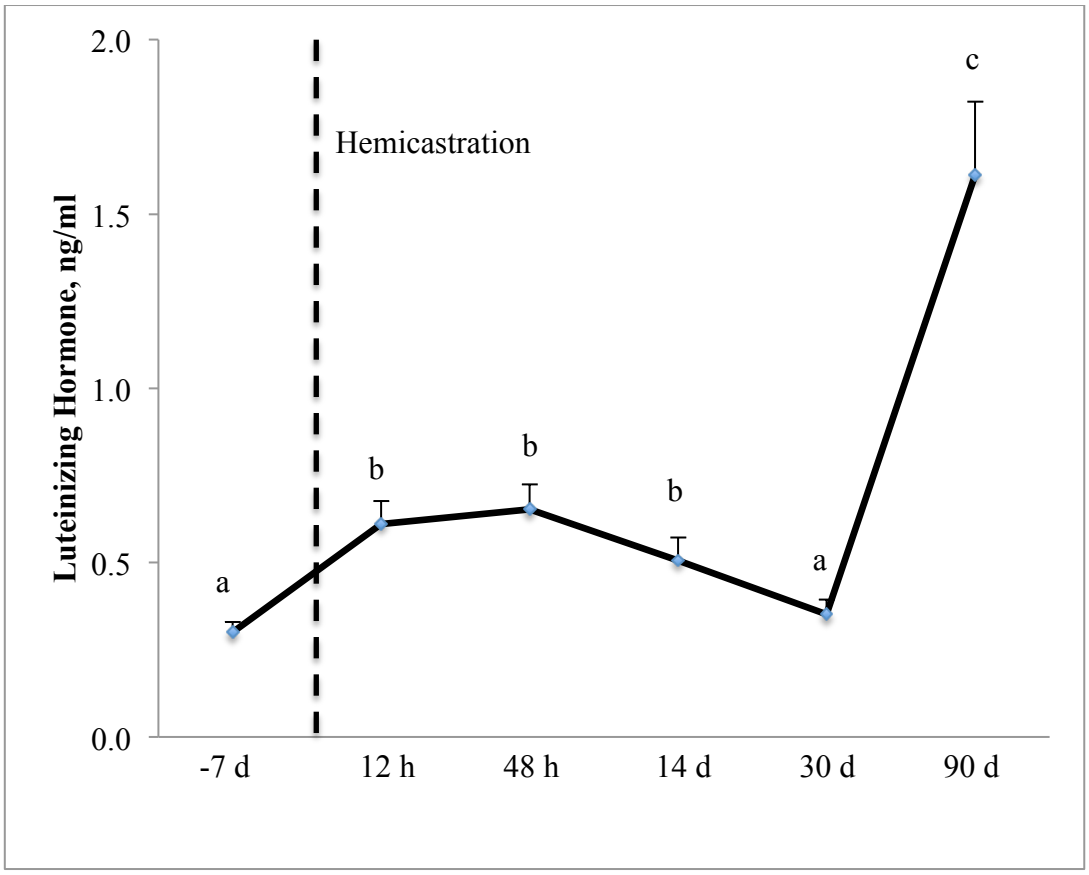

Figure 2. Mean (± SE) plasma LH concentrations (ng/mL) 7 d before and 12 h, 48 h, 14 d, 30 d, 90 d post hemicastration. Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).

Estrone Sulfate

Mean plasma estrone sulfate concentrations significantly decreased ($P < 0.0001$) 12 h and 48 h post hemicastration. Plasma concentrations remained lower than pre-surgical values 14 d, 30 d, and 90 d post hemicastration; however, concentrations steadily rose up to 90 d after hemicastration (Figure 3).

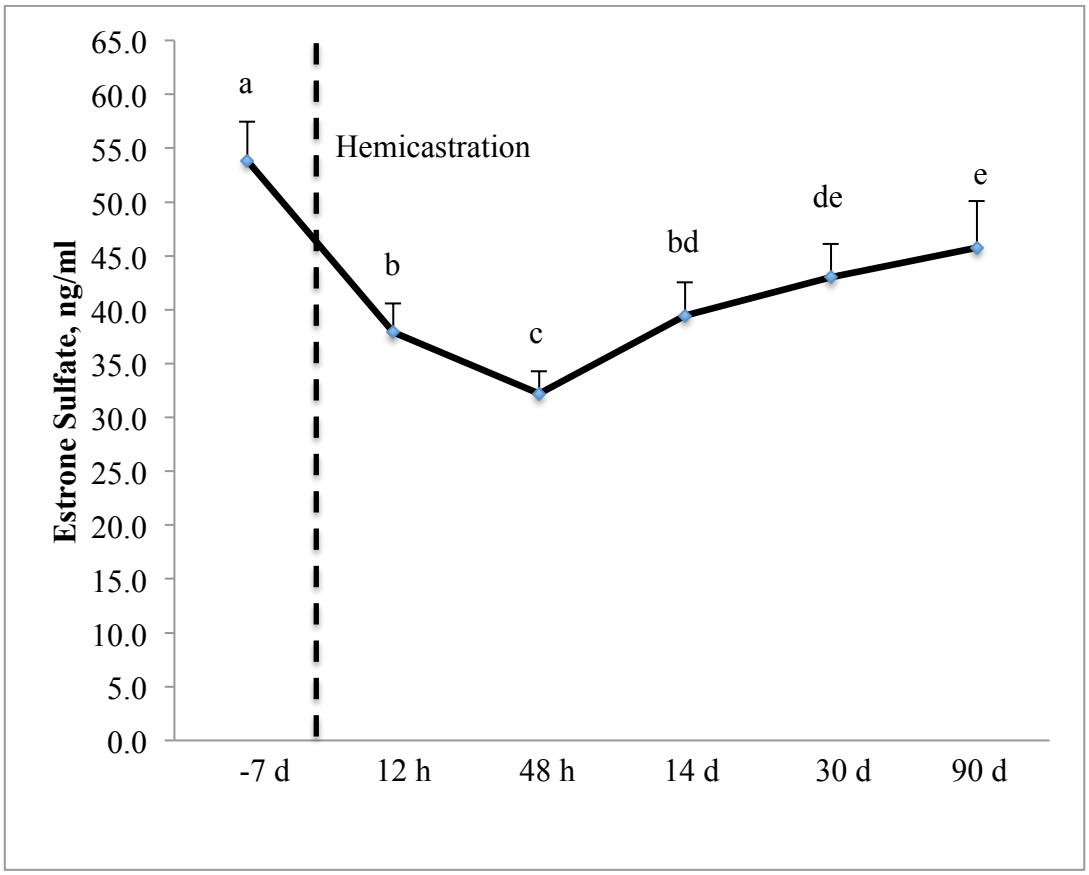

Figure 3. Mean (± SE) plasma estrone sulfate concentrations (ng/mL) 7 d before and 12 h, 48 h, 14 d, 30 d, 90 d post hemicastration. Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).

CHAPTER V

DISCUSSION

Hemicastration is a routine, common surgical technique performed by veterinarians in removing a diseased, injured, or traumatized testicle; however, the potential for impaired stallion fertility by removing one testicle may seem discouraging to stallion owners and managers. The thermal damage associated with healing following hemicastration is anticipated to have a negative impact on the remaining testis and it is assumed the stallion should be sexually rested for at least one full spermatogenic cycle before resuming normal breeding capacity. This interruption of breeding capabilities in stallions following hemicastration may cause a reduction in financial opportunities if hemicastration is performed during the breeding season.

Previous research indicates that stallions with a diseased or impaired testis returned to normal fertility following hemicastration and suggests that hemicastration could provide an opportunity to remove the negative impact of a damaged testis (Gygax et al., 1973). Moreover, Amelar et al. (1971) also described a recovery of fertility following hemicastration in men. The effects of hemicastration on stallion seminal parameters and testicular function of the remaining testicle have been studied (Table A.1) (McCormick et al., 2012) in collaboration with the present study that investigated the concentrations of reproductive hormones in stallions following hemicastration. These results provide insight to the seminal response to hemicastration by the stallion.

Testosterone

Testosterone concentrations were measured 7 d prior to hemicastration, 12 and 48 h, 14, 30, and 90 d post hemicastration to investigate the trends of the production of this androgen in response to hemicastration. No significant differences were detected between pre-surgical and 48 h post-surgical values. A significant decrease in the concentration of testosterone occurred 12 h post hemicastration and concentrations remained lower than pre-surgical values at 14 d and 30 d after surgery. These results following the assessment of testosterone concentrations differ from the findings of Hoagland et al. (1986) where testosterone concentrations were similar between control and unilaterally castrated groups of one-, two-, and three-year-old stallions. The difference in results between the current study and the study presented by Hoagland et al. (1986) may be a consequence of the differing blood sample collection periods. Other studies have also demonstrated similar concentrations of testosterone in unilaterally castrated bulls when compared to bulls that had not received treatment (Johnson, 1978; Leidl et al., 1980). This suggests that one testis can produce an amount of testosterone that is comparable to amounts produced by two functional testes.

Interestingly, a significant increase in plasma testosterone concentrations occurred between 30 d and 90 d post hemicastration (1.00 ± 0.12 ng/mL to 3.92 ± 0.41 ng/mL; $P < 0.05$), which coincides with the modification of stallion housing between these two time periods. Stallions were housed in individual pens up to 30 d post hemicastration and then pastured together in a harem group for the remainder of the study. Additionally, when compared to pre-surgical values, the concentration of

testosterone was significantly greater 90 d post hemicastration (3.92 ± 0.41 ng/mL to 1.69 ± 0.18 ng/mL; $P < 0.05$). The dramatic increase in plasma testosterone concentrations by 90 d post hemicastration suggests that the remaining testis is more than capable at generating and surpassing the concentrations of testosterone when two testes were present. McDonnell and Murray (1995) describe the endocrinologic response by stallions pastured in a harem band. In the McDonnell and Murray (1995) study, stallions were grouped in a pasture and only one stallion, the harem stallion, had control and main access to a small portion of fence line adjacent to a pasture housing mares. Stallions that did not have control of the fence line next to the mares were considered bachelors and had lower concentrations of testosterone when compared to the harem stallion. In the current study, all stallions were housed in a harem group. It was not possible for a single stallion to solely control the fence line and his proximity to the mares due to the vast distance needed to travel to control the entire fence line. All stallions had the ability to visually and tactically sense the mares from the mutual fence line between their pastures. As a result, all stallions could be classified as harem stallions. In accordance with previous literature (McDonnell and Murray, 1995), harem stallions had elevated plasma testosterone concentrations as evidenced by an almost 300% increase in testosterone measured.

The normal range of testosterone concentrations in stallions have been previously reported (Berndtson et al., 1974). Despite the evaluation of testosterone concentrations during the winter months, when day length is shortest, testosterone impressively increased following the housing modification. However, plasma testosterone

concentrations were higher 90 d post hemicastration than the highest mean concentration of testosterone reported by Berndtson et al. (1974) (3.92 ng/mL to 3.2 ng/mL). The current study also demonstrates that following hemicastration the remaining testicle only has the ability to produce large concentrations of testosterone and that spermatogenesis is not significantly hindered following this procedure (McCormick et al., 2012).

Luteinizing Hormone

The stimulatory effects of LH on Leydig cells source the production of testosterone and estrogens (Eisenhauer et al., 1994; Eisenhauer and Roser, 1995; Roser, 1997). Due to its relationship with testosterone, LH may be associated with the maintenance of spermatogenesis and was evaluated in a group of hemicastrated miniature stallions. The alteration of LH following hemicastration may be moderated through a series of feedback loops forming a portion of the hypothalamic-pituitary-testicular axis (Roser, 1997). A normal range of LH in stallions is lower than the LH concentrations measured during this study (Burns et al., 1984). A possible explanation for the deviation in concentrations may be associated with the analysis of Miniature Horse concentrations during the current study.

In the current study, stallions exhibited an increase in plasma LH concentrations immediately after hemicastration. Plasma LH concentrations were significantly greater than pre-surgical values 12 and 48 h, and 14 d post hemicastration. When evaluated 30 d post hemicastration, however, plasma LH returned to concentrations quantified prior to hemicastration. Researchers have noted that hemicastrated rats and bulls have

concentrations of LH similar to those measured before hemicastration (Ramirez and Sawyer, 1974; Leidl et al., 1980). These results are in agreement with those from Hoagland et al. (1986), who reports that serum LH concentrations in stallions were elevated following hemicastration. Moreover, both studies find a return to pre-surgical values, with LH returning to basal concentrations by 30 d and 150 d (Hoagland et al., 1986).

Testicular growth in the bull has been linked to pulses of LH from the anterior pituitary (Schanbacher, 1981; Schanbacher, 1982; Thompson et al., 1985). It has also been suggested that compensatory hypertrophy is associated with an increase in LH and FSH (Hoagland et al., 1986). Mean plasma LH concentrations were greater following hemicastration and this coincides with significant increases in mean testis volume (from 28.21 ± 9.1 cm^3 to 39.37 ± 11.2 cm^3; $P < 0.05$) and mean testis weight (from 43.8 ± 33.4 g to 64.8 ± 14.9 g; $P < 0.05$). Thus, it can be expected that LH is at least one of the factors that may stimulate compensatory hypertrophy following hemicastration and thus promote testicular growth. Despite implementation of the study during the winter months of November to February, when day length is at its shortest, the remaining testis displayed an ability to increase in size following hemicastration.

Similar to testosterone, plasma LH concentrations rose considerably between 30 and 90 d post hemicastration (0.35 ± 0.04 ng/mL to 1.61 ± 0.21 ng/mL; $P < 0.05$). The pulsatile nature of LH is closely followed by the pulsatile manner of testosterone secretion (Sanford et al., 1974; Lincoln, 1976; Schanbacher, 1982). Therefore, it is not

surprising to see both LH and testosterone demonstrate simultaneous spikes in plasma concentrations between 30 and 90 d post hemicastration.

Estrone Sulfate

Endocrine activity in the stallion has been evaluated by the measurement of estrone sulfate in stallions (Raeside, 1978/1979). Stallions produce large quantities of estrogen and the sulfated form of estrone is the most abundant estrogen quantified in previous research (Nyman et al., 1959; Bedrak and Samuels, 1969; Raeside, 1969; Villani et al., 2006). The production of estrone sulfate is variable in each stallion as evidenced by wide ranges of previous estrone concentrations measured (2.447 ± 1.996 ng/mL to 180.85 ± 120.63 ng/mL) (Claus et al., 1992; Villani et al., 2006; Seale, 2009). The concentrations of estrone sulfate found in the current study (32.21 ± 2.10 ng/mL to 53.87 ± 3.58 ng/mL) are within concentrations previously reported. A clear and direct role of estrone sulfate in the stallion has not been established as evidenced by variable results in previous literature. It has been contemplated that estrone sulfate has a role in the appearance of sexual reproductive behavior in the stallion (Bono et al., 1982; Cavinder et al., 2010) and that the concentration of spermatozoa is correlated to the concentration of estrogens (Roser and Hughes, 1992). This claim is dissimilar to the findings of Cavinder et al. (2010) who described no correlation between concentrations of plasma estrone sulfate with seminal parameters. As a result, further research is needed to understand the function of estrone sulfate and other estrogens in stallion reproductive physiology. This study helps provide sound scientific knowledge by

assessing the endocrine response of the remaining testicle following hemicastration by noting the trends or changes to estrone sulfate production in the stallion.

In comparing plasma estrone sulfate concentrations prior to and post hemicastration, the results of the current study show a significant decrease 12 h post hemicastration (from 53.87 ± 3.58 ng/mL to 37.88 ± 2.68 ng/mL; $P < 0.0001$). This mirrors the response of testosterone concentrations when evaluating the same time periods. Furthermore, estrone sulfate continued to decline up to 48 h post hemicastration (Table 3). These observations show that the production of estrone sulfate from the remaining testicle following hemicastration does not compensate within 48 h following surgery. This is in contrast to the conclusions that the remaining testicle hormonally recompenses in the production of testosterone and stimulates the secretion of LH from the anterior pituitary by 48 h post hemicastration. This elevation of plasma LH concentration may be attributed to increased plasma concentrations of FSH, the gonadotropin which functions in synergy with LH. With the loss of one testis, the production of inhibin may be impaired, resulting in an increase in FSH release. Plasma concentrations of estrone sulfate steadily rose after 48 h post hemicastration as evidenced by values 14, 30, and 90 d post surgery. These concentrations, however, remained lower than pre-surgical values upon removal of one testis. Lowered plasma estrone sulfate concentrations may be explained by recounting the testicular origin of estrogens.

The indication that concentrations of estrone sulfate are strongly associated with spermatozoa concentrations should be further investigated. Plasma concentrations of

estrone sulfate never recovered to pre-surgical values and were significantly lower 12 and 48 h, 14, 30, and 90 d post hemicastration. Though plasma concentrations of estrone sulfate were consistently lower after hemicastration, semen was collected from stallions by 14 d after surgery and a notice in a change in libido was not observed. Though libido was not formally following hemicastration, further research into the behavioral response after hemicastration would be of interest and provide valuable knowledge to stallion libido and its association with the endocrine response.

The current study provides better understanding of hormonal profiles and compensatory response of the remaining testicle. The endocrine response, along with the investigation of seminal parameters following hemicastration, provide a basis for future scientific use and are potentially beneficial to understanding reproductive efficiency in the stallion post hemicastration. A period of sexual rest (>60 d) assumed to be necessary for a stallion after hemicastration is not supported by changes in the profiling of seminal (McCormick et al., 2012) and endocrine parameters.

CHAPTER VI

CONCLUSION

The objective of this study was to measure blood plasma concentrations of testosterone, LH, and estrone sulfate following hemicastration in order to assess the response of the remaining testicle. In addition, this study stands as a companion piece to literature that evaluated the effects hemicastration have on semen quality and the size of the remaining testicle (McCormick et al., 2012). The results from the present study indicate the patterns that blood plasma testosterone, LH, and estrone sulfate concentrations fluctuate following hemicastration.

Immediately following hemicastration, blood plasma testosterone and estrone sulfate concentrations decreased from pre-surgical values. Estrone sulfate concentrations continued to decline and never recovered to values comparable to those assessed before hemicastration. Although the removal of one testicle may seem detrimental, stallions can respond to hemicastration by producing significantly greater amounts of testosterone and LH after stallion housing was modified. When pastured jointly in a harem group, hemicastrated stallions produced a dramatic increase in both testosterone and LH concentrations.

The endocrine response of the remaining testicle indicates that one functional testicle is able to produce significantly greater testosterone and LH concentrations than previously recorded values when two functional testicles were present. This hormonal alteration may be the cause of compensatory hypertrophy of the remaining testicle upon

evaluation. The exact cause and reasoning for hormonal adaptation is not completely understood; however, this response could be related to seasonal effects or to the alteration in housing of stallions, as stallions exemplified a greater production of reproductive hormones, with the exception of estrone sulfate, after being housed in a harem group.

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APPENDIX A

Seminal Response to Hemicastration

Table A.1 Effects of hemicastration on total sperm motility (TSM), viable sperm (VS), morphologically normal sperm (MNS), and total sperm numbers (TSN) before hemicastration (time period 0) and 14 d, 30 d, 60 d post hemicastration (mean ± SD).

	TIME PERIOD			
	0	14 d	30 d	60 d
TSM (%)	64±18	56±15	63±12	56±17
VS (%)	72±10[a]	77±10[b]	87±4[c]	85±6[c]
MNS (%)	63±11	59±17	62±23	56±15
TSN (x 10^9)	1.4±0.7[a]	0.58±0.3[c]	0.6±0.4[c]	0.9±0.6[b]

Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).

APPENDIX B

Testis Volume Measures

Table B.1 Testis volume (cm^3) pre-hemicastration, 30 d and 73 d post hemicastration as measured by ultrasonography.

	Testis Volume Measures (cm^3)		
Stallion	Pre-hemicastration	30 d post hemicastration	73 d post hemicastration
1	23.16	26.99	33.97
2	18.93	24.61	21.17
3	16.20	21.61	22.57
4	22.39	34.58	36.12
5	33.60	28.43	47.74
6	46.51	43.72	41.68
7	24.51	52.04	53.81
8	32.96	36.48	45.11
9	35.68	43.13	52.20
Overall mean (± SD)	28.21 ± 9.1^a	34.62 ± 9.6^b	39.37 ± 11.2^b

Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).

APPENDIX C

Testis Weight

Table C.1 Testis weight (g) of first and second testicles removed.

	Testis Weight (g)	
Stallion	First testicle removed	Second testicle removed
1	40.71	46.70
2	39.14	43.25
3	32.47	46.10
4	42.68	67.9
5	48.60	73.28
6	54.66	72.51
7	35.41	88.90
8	55.66	75.21
9	45.12	68.90
Overall mean (± SD)	43.8±33.4[a]	64.8±14.9[b]

Differing superscripts indicate significant difference from time points in a row ($P < 0.05$).