# ABSTRACT

Title of Document: UNDERSTANDING THE REPRODUCTIVE BIOLOGY OF THE PRZEWALSKI'S HORSE (*EQUUS FERUS PRZEWALSKII*)

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The Przewalski's horse (*Equus ferus przewalskii*) once roamed the Eurasian Steppe but is now considered Critically Endangered with only 1872 individuals remaining in the world, representing progeny from only 14 founder animals (Lee and Boyd, 2008). Genetic diversity needs to be optimal for long term survival of this species. Unfortunately, increasing genetic diversity of the captive population in North America has been hindered by a decrease in fertility. Therefore, the main focus of this research was to characterize reproductive parameters in Przewalski's horse, including estrus cycle in mares and seminal traits in stallions, and determining whether age or inbreeding had an impact on these traits. A secondary focus was to determine whether hormone manipulation of the estrous cycle in mares could be utilized for the long-term goal of using artificial insemination as a breeding management tool for this species. To facilitate these studies, a technique for palpation of Przewalski's mares was developed; the first application of such a procedure in a wild equid. Subsequently, we were able to describe follicular changes in relation to urinary hormone patterns. Fifty percent of the mares had either irregular or acyclic hormonal and follicular patterns. These patterns were directly correlated with inbreeding which is the first time such a correlation has been described in this species. Estrous manipulation was possible using an injectable biorelease form of the progestagen, altrenogest. In stallions, we developed a reliable method of semen collection for Przewalski's stallions and, as a result, describe

seminal traits from 98 semen collections from 14 stallions. Based on these collections, we were able to show that sub-fertility in this population could be due to the low percentage of normal spermatozoa. Based on variable analysis, seminal traits total concentration, volume and morphology showed variable changes through the year. Traits also varied on an individual stallion basis. Together, these studies demonstrated that inbreeding is detrimentally affecting the reproductive fitness of this species and that aggressive management is needed for long term sustainability of the captive population.

UNDERSTANDING THE REPRODUCTIVE BIOLOGY OF THE
PRZEWALSKI'S HORSE
(*EQUUS FERUS PRZEWALSKII*)

By

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2010

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2010

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# DEDICATION

This thesis is dedicated to my father, Del, who was my main support and source of inspiration throughout this long endeavor.

Also, to my husband, Jeff, who has been my best friend.

With all my heart, thank you.

In Memory of

Colonel Austin Bach

Thank you for all the support

ACKNOWLEDGEMENTS

This research would not have been possible without the support, friendship and guidance of Dr. Nucharin Songsasen, who served as my main advisor at the National Zoological Park. I was also fortunate to work with Dr. Carol Keefer, my main advisor at University of Maryland, who was a great guide especially through the final stages of my dissertation.

Of course, I would not have had the opportunity to work on this project without the ideas and support that I received from Dr. Steven Monfort and Dr. David Wildt. I would also like to thank the rest of my dissertation committee for their guidance and support: Dr. Tom Porter, Dr. Amy Burk, and Dr. Jim Dietz.

I am also extremely grateful to the individuals and organizations which provided funding for the project including: Morris Animal Foundation, Undersecretary for Science, Smithsonian Institution, and Shirley Sichel. I would especially like to thank Christine and Austin Bach for not only providing funding for the project but also for being such great friends and support when times were tough.

There were many people that assisted with this project, without which, I would have been unable to complete it. Many people at SCBI including Nicole Presley, Dr. Mandi Vick, Dr. Janine Brown, Dr. Mitch Bush, Dr. Luis Padilla, Lisa Ware, Dr. Budhan Pukazhenthi, and Linwood Williamson, assisted me with data collection and lab work.

A special thanks to the hoofstock team for all the extra work that they conducted: Ken Lang, Greg Peterson, Dolores Reed, Dave Shiflett, Shannon Hunter and Allyson O'Neill At the Wilds, I had unwavering support from Dr. Evan Blumer, Dr. Barb Wolfe, Dan Beetem, Dr. Rachael Weiss and the hoofstock staff. I was also fortunate to work with many great interns who helped me complete and interpret data, many of whom also provided me with great friendship and support.

I would like to thank my father for being an honorary committee member and keeping me focused on completion. Thanks to my stepmother, Patty, who had to suffer through discussions about science on the phone. Sincere thanks to my brother, Rob, and my sister, Lainey, for being able to commiserate with me as they complete their graduate degrees; my sister-in-laws: Laura, Jane and Sandy; and my nieces and nephews. Thanks to my mother for checking up on me and my in-laws for understanding when I could not visit.

Most of all, I would to thank my husband, Jeff, for sticking by me through the last few years and for helping me see the funny side of things. You complete me.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| AI | Artificial Insemination |
| ANOVA | Analysis of Variance |
| ART | Assisted Reproductive Technology |
| BSA | Bovine Serum Albumin |
| CL | Corpora Lutea/Corpus Luteum |
| Cr | Creatinine |
| CV | Coefficient of Variation |
| DCD | Distal Cytoplasmic Droplets |
| $DH_2O$ | Distilled Water |
| EDSO | Estimated Daily Sperm Output |
| EIA | Enzyme Immunoassay |
| eLH | Equine Lutieinizing Hormone |
| FSH | Follicle Stimulating Hormone |
| GnRH | Gonadotrophin Releasing Hormone |
| HAF | Hemorrhagic Anovulatory Follicle |
| hCG | human chorionic gonadotrohpin |
| IUCN | the International Union for the Conservation of Nature |
| LH | Luteinizing Hormone |
| NaOH | Sodium Hydroxide |
| PCD | Proximal Cytoplasmic Droplets |
| $PGF_2\alpha$ | Prostaglandin $F_2\alpha$ |
| PM | Progressive Motility |

| | |
|---|---|
| PMMN | Progressively Motile Morphologically Normal |
| reLH | Recombinant Luteinizing Hormone |
| SD | Standard Deviation |
| SEM | Standard error of the mean |
| SSP | Species Survival Plan |
| TM | Total Motility |
| TPMMN | Total Progressively Motile Morphologically Normal |
| TTV | Total Testicular Volume |

# CHAPTER ONE

## Introduction

### *The Asian Wild Horse*

Small and pony-like in stature, dun colored with a dark upright mane, the Przewalski's horse is a distant cousin to the domestic horse (*Equus caballus).* It is now believed that the Przewalski's horse shares a common ancestor to the domestic counterpart, but the former have 66 chromosomes compared to 64 in the latter (Ryder, 1994). Once thought to be the ancestor to the domestic horse, the Przewalski's horse is the only remaining wild horse in existence today (Groves, 1994).

The first visual account of the existence of wild type similar to Przewalski's horses was 20,000 years ago. Cave drawings in Italy, western France and northern Spain depicted small horses with upright manes, similar to the Asian Wild Horse (Bouman and Bouman, 1994). After the last ice age around 10,000 BC, the steppes in Europe changed to forests and many of the wild horses moved east (Azzaroli, 1966). It was not until 900 AD that the first written account of the Asian Wild Horse occurred in Tibet (Zevegmid and Dawaa, 1973). In 1226, while on a campaign against Tangut, Genghis Khan spotted a herd of wild horses which resulted in his horse getting spooked (Bőkőnyi, 1974). The first western account of the Asian Wild Horse was in 1719, when John Bell, a doctor

working under Czar Peter the Great, described wild horses in an account of his travels from St. Petersburg to China (Mohr, 1971).

In 1878, Colonel Nikolai Przewalski, a Polish explorer under Czar Alexander II, received a horse skull and hide as a gift during his travels in China (Bouman and Bouman, 1994). On his return to Russia, the conservator of the Zoological Museum of the Academy of Science, I.S. Poliakov, concluded that the hide and skull belonged to a wild horse. As a result, the Asian Wild Horse was given the official name *Equus przewalskii* (Bouman and Bouman, 1994).

At the time of their discovery in the late 1800s, horses were distributed throughout the entire Eurasian steppe belt, which extends from Hungary to Mongolia and the northeastern margins of China (Bouman and Bouman, 1994; Garrutt *et al.,* 1965). Unfortunately, by 1958, the population range had severely contracted to the Dzungarian Gobi in Mongolia (Mohr, 1971). In 1969, the last sighting of a Przewalski's horse occurred north of the Tachjin-Shar-Nuru (Paklina and Pozdnyakova, 1989).

**Species on the Brink**

Hunting by nomads, competition with domestic livestock and harsh weather resulted in rapid decline in the numbers of the Przewalski's horse during the 20$^{th}$ century (Paklina and Pozdnyakova, 1989), and the species was declared extinct in nature in 1969. The current captive Przewalski's horse population in North America is derived from 14 founders that were captured in the wild during the 1900s (Ryder, 1994; Monfort *et al.*, 2009). Starting in the 1980s, re-introduction sites were established in China, Mongolia and Kazakhstahn (Boyd *et al.*, 2008). Zoological institutions from Europe, North America

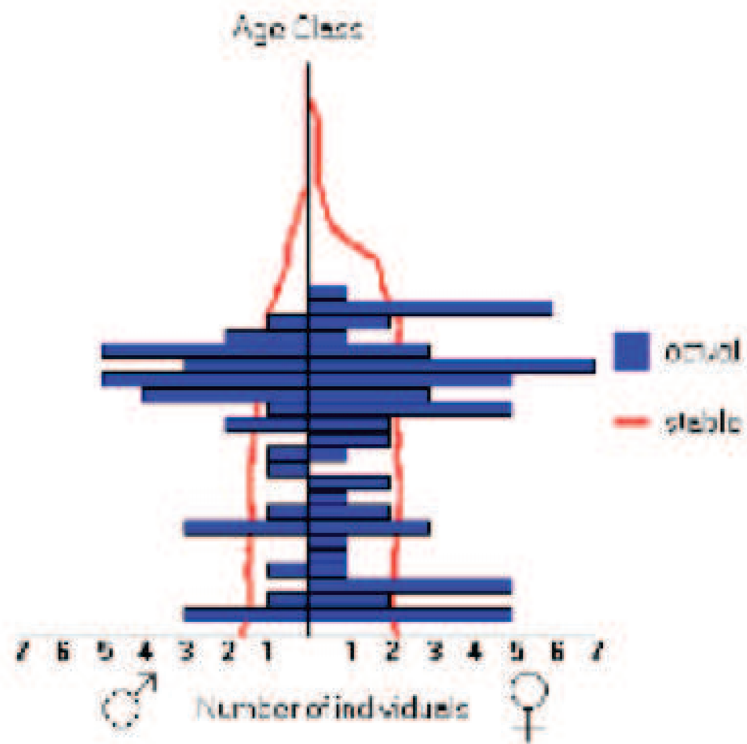



**Figure 1.1:** Demographics of current SSP population. Blue represents actual population. Red indicates a stable population (Monfort *et al.*, 2009).

and Australia contributed animals to re-introduction sites and there are currently over 500 animals living in their native habitat. As a result, the International Union for the Conservation of Nature (IUCN) changed the status of the Przewalski's horse from 'Extinct in Wild' to 'Critically Endangered' (Boyd *et al.*, 2008). To ensure the maintenance of a genetically healthy reservoir population, approximately 500 Przewalski's horses are managed by zoos in the USA and in Europe. Although reproduction in captive populations has been guided by mean kinship values, imperfect captive breeding management has resulted in inbreeding, loss of genetic diversity and reproductive capacity, and decreased fertility and fecundity (Monfort *et al.*, 2009). In 2004, the Association of Zoos and Aquarium's (AZA) Species Survival Plan (SSP) for the Przewalski's horse determined that the demography of this regional population (Fig. 1.1) was unstable due to an age structure skewed towards older horses (Monfort *et al.*, 2009). To alleviate this trend, urgent priority was given to organizing breeding pairs to initiate a coordinated research program and to make animals available for reproductive research. AZA institutions agreed to contribute facilities, and the SSP invited the Smithsonian's National Zoo's Department of Reproductive Sciences to lead studies in the evaluation of assisted reproductive technologies (ART) as a means to better manage the genetic diversity and demography of the Przewalski's horse population.

### *Artificial Insemination in the Domestic Horse*

Artificial insemination (AI) has been used successfully in many domestic species for over seventy-five years (Vishwanath, 2003). In cattle, sheep and goats it is used for the improvement of genetics in herds. In the horse (*Equus caballus*), AI offers many

advantages over natural mating, including: 1) more efficient use of valuable semen samples by allowing allocation of a single ejaculate into several insemination doses; 2) augmenting the number of offspring produced per stallion during each breeding season; 3) increasing availability of stallion semen; 4) decreasing venereal transmission of disease to mares due to use of antibiotics in semen extender; 5) reducing breeding injuries that occur with natural breeding; and 6) assessing semen quality prior to insemination, which assists in early detection of possible fertility problems (Blanchard *et al.,* 2004). Also, frozen-thawed semen allows for the production of offspring from a stallion post-mortem (Brinsko and Varner, 1992).

The success of AI in the domestic horse, however, relies heavily on a heightened knowledge of reproductive physiology of mares and stallions. Furthermore, because spermatozoa are very susceptible to environmental injury, proper semen collection, handling, processing, and insemination are necessary to produce higher pregnancy rates (Blanchard *et al.,*2004). For example, stallion spermatozoa are extremely sensitive to cold shock, therefore all equipment used for handling or processing sperm should be at 37° C (Brinsko and Varner, 1992; Loomis and Graham, 2008). Spermatozoa should also be placed in a suitable semen extender to maintain viability. Properly formulated semen extenders for the stallion are milk and egg based with added sugars and antibiotics (Blanchard *et al.,* 2004).

In addition to semen handling techniques, mare management is equally important for achieving pregnancy following AI. Successful AI (75% pregnancy rate) generally requires mares to be inseminated with 250 – 500 million progressively motile frozen or chilled semen (Brinsko and Varner, 1992; Fiala *et al.,* 2007) once during the 48-hours

interval preceding ovulation (Woods *et al.,* 1990). Overall, when proper semen handling and insemination techniques are used, optimal pregnancy rates are attainable, even in mares and stallions with marginal fertility (Blanchard *et al.,* 2004; Crowe *et al.,* 2008).

***The Application of Artificial Insemination in Wildlife***

There are several reasons why AI has tremendous potential for application to wildlife species, including: 1) overcoming incompatibility between designated breeding pairs; 2) decreasing the need to hold males at institutions only for breeding purposes; 3) preserving genetic diversity in captive populations; and 4) reducing risks associated with moving animals from one zoological institution to another for the purpose of natural breeding (Pukazhenthi and Wildt, 2004). AI has been used successfully in several wildlife species, for example, the giant panda (*Alluropoda melanoleuca;* Huang *et al.,* 2002), African (*Loxodonta Africana)* and Asian elephants (*Elephas maximus;* Hermes *et al.,* 2007; Thongtip *et al.,* 2009), Eld's deer (*Cervus elddi;* Monfort *et al*., 1993), scimitar-horned oryx (*Oryx dammah;* Morrow *et al.,* 2000) and black footed ferrets (*Mustela nigripes;* Howard *et al.,* 2003). Similar to the Przewalski's horse, these species either have rapidly decreasing numbers of or no new founders in the wild.

Reproductive research in Przewalski's horses is especially important because the North American population is comprised mostly of older animals that are greater than 10 yr old (96/149 horses total; 66% of mares and 53% of stallions; Spevak and Monfort, 2004; Powell, 2009). Decreased fertility in domestic horses greater than 10 yr old has dictated more intensive breeding management in these animals, and ARTs, such as artificial insemination and embryo transfer, have been used to overcome these difficulties

(Samper, 2000, van Buriten *et al.,* 2003). However, success requires a detailed understanding of the reproductive biology of Przewalski's mares and stallions, as well as developing optimal collection protocols and technologies for semen collection, cryopreservation, and AI.

Limited research has been conducted on the Przewalski's mare to establish temporal follicular (Durrant and Hoge, 1988; Durrant *et al.,* 1986) and endocrine patterns through milk (Zimmermann, 1985), urine (Monfort *et al.,* 1991), and fecal (Schwarzenberger *et al.,* 1992) steroid monitoring. Furthermore, only a small number of reports have described ejaculate characteristics in this species (Stover *et al.,* 1981; Durrant, 1990; Bader *et al.,* 1991). The Przewalski's horse is a flagship species that simply would not exist today without the contribution made by the international zoo community. However, nearly 30% of the genetic variation present in the original 14 founders has already been lost due to suboptimal reproductive management (Spevak and Monfort, 2004). Zoos face a daunting challenge: a century's long obligation to maintain genetically viable reservoir populations, including genome resource banks (GRBs) that will help to insure the survival of Przewalski's horses. This responsibility will remain even after the full complement of founder-based genetic diversity is represented in self-sustaining wild populations - an equilibrium that has yet to be achieved for this species.

***Reproduction in Przewalski's Mares***

Limited research has been conducted on reproductive physiology of Przewalski's mares. Behavioral research has shown that mares cycle in the spring and summer, and produce foals 11-12 months after breeding similar to their domestic counterparts

(Monfort *et al.,* 1991; Houpt and Boyd, 1994). Estrous cycle patterns have also been determined by assessing follicular structures using rectal ultrasonography (Durrant *et al.,* 1986) or by assessing hormone metabolites in milk (Zimmermann, 1985), urine (Monfort *et al.,* 1991) and feces (Schwarzenberger *et al.,* 1992).

Two mares that were examined seven times over a three month period exhibited follicular characteristics that were similar to the domestic mare (Durrant *et al.,* 1986). Each mare was examined under anesthesia using a linear array ultrasound and follicular size was noted as well as other structures. Follicles grew 3 – 5 mm per day and ovulated when they reached a diameter of 48 – 50 mm. While information on follicular changes was established, no data on hormonal changes or estrous cycle length were reported.

The most extensive longitudinal study of reproductive endocrinology in Przewalski's mares utilized non-invasive hormone monitoring in eight individuals over the period of 18 months (Monfort *et al.,* 1991). Analysis of estrogen metabolites using radioimmunoassay (RIA) revealed that the onset of hormonal activity occurred with increasing day length (from February through April), but that most mares did not conceive until after March (Monfort *et al.,* 1991), indicating the possibility that mares have a transitional period of hormonal activity prior to actual follicular development, which is similar to what is observed in the domestic horse (Ginther, 1992). It was also determined that estrous cycle length was about 24 days based on average length between urinary estrogen peaks. However, this study only looked at urinary estrogens in relation to estrous behavior and pregnancy.

An earlier study that analyzed progesterone metabolites in milk secretions from two post-partum mares revealed that milk progesterone levels increased and decreased 8 and

35 days post-ovulation, respectively, implying that progesterone is secreted post-ovulation (Zimmermann, 1985). Findings from this study suggest that, similar to most species, progesterone is important in maintenance of early pregnancy. In a subsequent study, fecal progestagen excretion revealed cyclical patterns in 3 of 4 non-pregnant Przewalski's mares (Schwarzenberger *et al.,* 1992). Although no study has yet defined the temporal patterns of progesterone excretion during pregnancy, it has been presumed that Przewalski's mares, like other mammalian species, require corpus luteum (CL)-derived progesterone to sustain early pregnancy.

### *Reproduction in the Domestic Mare*

#### Seasonality

The domestic mare is considered a long day breeder with cycles occurring in spring and summer (Northern Hemisphere: April – August; Southern Hemisphere: September – February). Similar to other seasonal breeders, photoperiod is the main factor driving seasonality in the domestic mare (Ginther, 1993; Blanchard *et al.,* 2004). Increases in day length trigger a decrease in melatonin secretion from the pineal gland, that in turn, causes an increase in gonadotropin releasing hormone (GnRH), which leads to resumption of cyclicity in late spring in most mares (Ginther, 1993).

In addition to increasing day length, opioids, a family of peptides secreted in the brain, may also participate in the regulation of seasonal reproduction by modulating luteinizing hormone (LH) secretion during winter anestrus (Turner *et al.,* 1995). Opioid levels were higher in mares during deep winter anestrus than during the breeding season, however, the use of opioid antagonists (naloxone) in mares did not alter onset of

reproductive activity in anestrous mares (Turner *et al.,* 1995). Specifically, anestrus mares treated with naloxone showed no significant increase in estrous activity compared to untreated mares.

More recent research also has shown that other factors such as body condition score (BCS) can have an impact on reproductive seasonality in mares (Waller *et al.,* 2006; Gentry *et al.,* 2002; Vecchi *et al.,* 2010). For example, mares with a BCS of 7.5 – 8.5 (out of 9) continued to ovulate or have follicular activity through the winter compared to mares of low BCS (3.0 – 3.5) (Gentry *et al.*, 2002). Similarly, Vecchi *et al.* (2010) showed that Standard bred mares in poor body condition and reduced adipose tissues were slower to return to estrus after winter anestrus than those with good body condition.

Leptin is an adipocyte-derived hormone that acts in some species to signal the brain regarding the body's nutritional status (Houseknecht *et al.,* 1998). In rodents, females lacking sufficient leptin display reduced gonadotrophin release, anestrus, or both (Barrash *et al.,* 1996; Carro *et al.,* 1997). In the horse, mature mares (6 – 12 years) showed higher leptin levels compared to younger individuals (2 – 5 years), suggesting that this peptide hormone may play an important role in regulating the reproductive cycle in this species (Fitzgerald and McManus, 2000). However, more recent studies, have shown that leptin is not consistently elevated in mares that cycle year round and that other metabolic factors may be confounding the length and depth of anestrus in mares (Gentry *et al.,* 2002; Waller *et al.,* 2006).

### ***The Estrous Cycle of the Domestic Mare***

In the domestic mare, the estrous cycle is defined as the period from one ovulation to the subsequent ovulation. The two periods of the estrous cycle in the mare are: 1) the follicular phase (estrus) – the mare is sexually receptive to the stallion and a dominant follicle is present on the ovary; and 2) the luteal phase (diestrus) – the mare is non-receptive to the stallion, and one or more corpora lutea are present on the ovary (Figure 1.2; Blanchard *et al.,* 2003). The average length of the estrous cycle ranges from 18 – 24 days (average 21 – 22 days). Length of estrus is variable (2 -12 days) based on time of year. It is believed that this variation could be due to a less prominent LH surge early in the breeding season (Palmer, 1978; Ginther, 1992). The length of diestrus remains relatively constant at 14 – 15 days and is less affected by season than length of estrus (Blanchard *et al.,* 2004).

Mares have one or two major follicular waves during the estrous cycle (Ginther, 1992). A major wave is defined by the emergence of several follicles that grow in synchrony until a dominant follicle is selected and subordinate follicles regress. In mares that have two waves during the interovulatory interval, the wave that first emerges is the secondary wave (Ginther, 1992). Emergence of this secondary wave occurs in late estrus or early diestrus and may result in an ovulation during diestrus or regression of the dominant follicle. The wave that results in ovulation during estrus is the primary wave, and emerges during late diestrus (Figure 1.3; Ginther, 1992).

The estrous cycle of the mare relies on a delicate balance of hormones produced by the pineal gland, hypothalamus, pituitary gland ovaries and the uterus (Figure 1.4). The

neurosecretory cells of the hypothalamus produce GnRH, which is episodically released into the hypothalamo-pituitary portal system and stimulates the anterior pituitary to produce the gonadotrophins, follicle stimulating hormone (FSH) and LH (Blanchard, 2004). FSH and LH act at the level of the ovaries: FSH is responsible for follicular

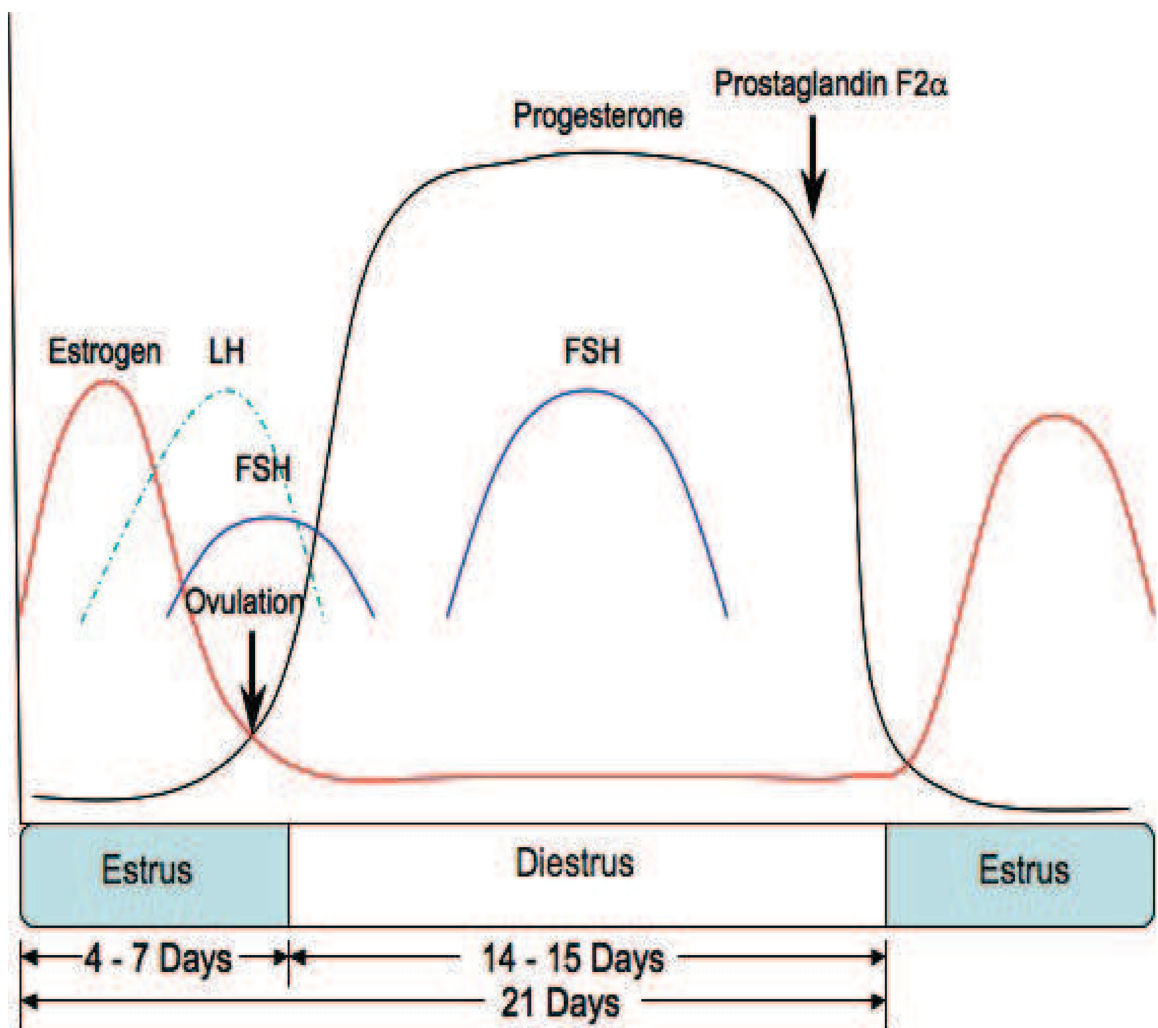



Figure 1.2: The estrous cycle of the domestic mare (Blanchard *et al.,* 2003).

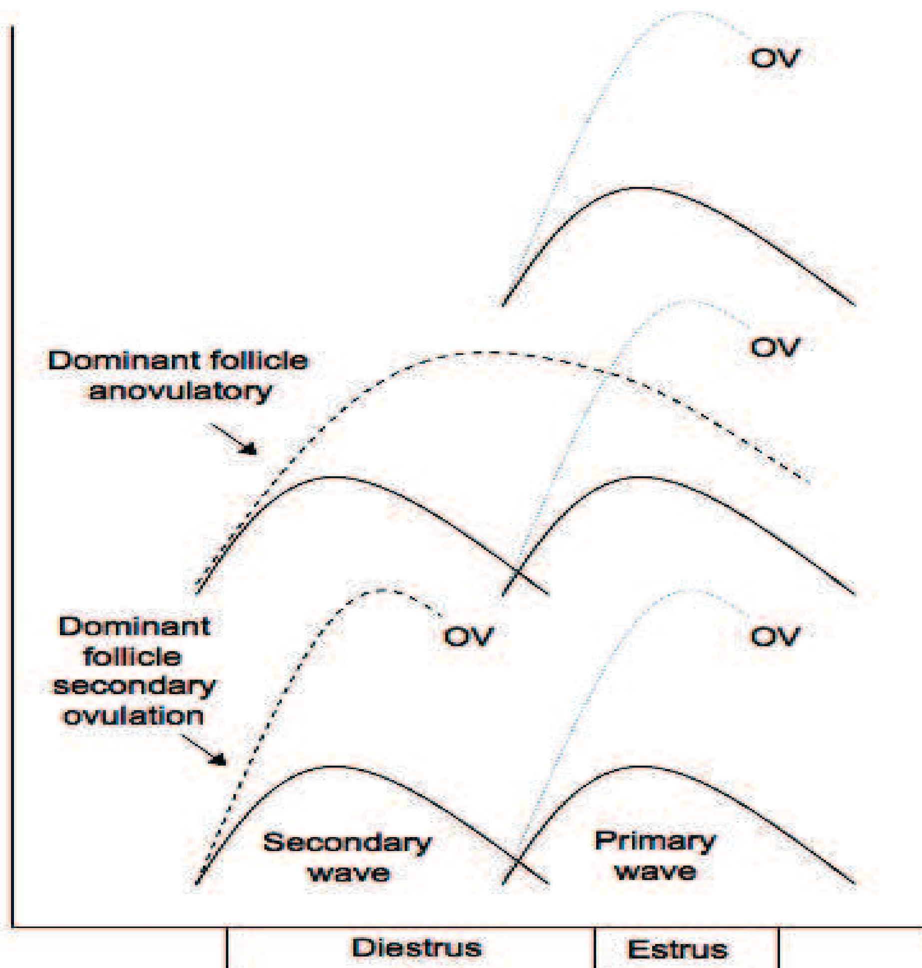



**Figure 1.3:** Follicular wave patterns in the domestic mare (Ginther, 1992).

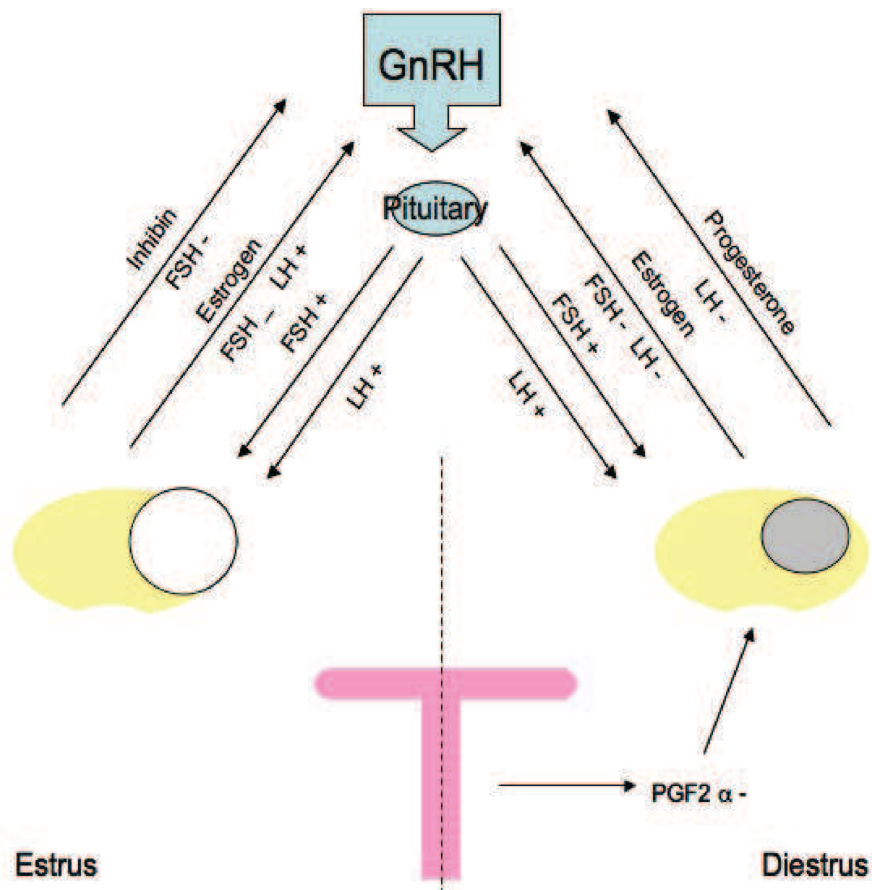



**Figure 1.4:** Hypothalamo-pituitary-gonadal axis in the domestic mare (Blanchard *et al.,* 2003).

recruitment and LH is responsible for follicular maturation, estrogen production, ovulation, and luteinization of the corpus luteum (Ginther, 1992; Senger, 2003).

The mare, similar to most species, has a follicular pool at birth. Antral follicles are recruited from the pool under the influence of LH and FSH released from the anterior pituitary. LH binds to theca interna cells of antral follicles, which stimulates the production of testosterone. FSH then binds to granulosa cells of the follicle causing the conversion of testosterone to estradiol (Ginther 1992; Ojeda, 2004). Increasing estradiol stimulates granulosa cell proliferation, which increases follicle sensitivity to gonadotrophins. As the follicles continue to grow, more estrogen is produced, which in turn causes negative feedback to the anterior pituitary and suppresses FSH secretion, but induces an increase in LH secretion. Once follicle selection has occurred, the largest follicle produces more estrogen and inhibin, which further suppresses FSH and leads to the regression of smaller follicles (Senger, 2003). Rising estrogen triggers the preovulatory surge in LH, which induces a cascade of cellular events that result in ovulation. The specific endocrine and biochemical mechanisms involved in ovulation in the domestic horse are still unknown, but is assumed to be similar to other species whereby LH (via binding to theca interna cells), cyclic AMP, prostaglandins and steroids play a vital role in modulating a complex series of biochemical reactions leading to the preovulatory changes within the follicular wall that precede ovulation (Ginther, 1992; Senger, 2003; Ojeda, 2004).

The mare is unique compared to other domestic animals in that the preovulatory rise in LH occurs over several days and often reaches its peak 1- 2 days after ovulation of the dominant follicle (Palmer, 1978; Blanchard *et al.,* 2003). Follicular diameter at ovulation ranges from 30 – 70 mm (average 40 – 45 mm). After ovulation, theca interna and granulosa cells luteinize under the influence of LH, and turn into luteal cells (Senger 2003). The luteal cells develop into a CL on the ovary and secrete progesterone throughout the period of diestrus, which normally lasts 14 - 15 days (Blanchard *et al.,* 2003). The presence of tonic LH and cholesterol is required for progesterone to be produced by luteal cells via the steroidogenic pathway (Senger, 2003; Ojeda, 2004). Progesterone is important in that it provides negative feedback to the hypothalamus, reducing the episodic secretion of GnRH while a functioning CL is present on the ovary (Senger, 2003).

The life span of the CL in the horse is maintained until an endogenous release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterine endometrium that normally occurs between day 13 – 16 post-ovulation (Ginther, 1992; Senger, 2003; Blanchard *et al.,* 2004). Similar to other species, progesterone, estrogens and oxytocin control the release of $PGF_{2\alpha}$ from the endometrium (Ginther, 1992). However, the horse is different in that $PGF_{2\alpha}$ enters the systemic circulation after release from the uterus, rather than through a local countercurrent system as seen in ruminants (Ginther, 1992; Senger, 2003). This is because the mare does not metabolize $PGF_{2\alpha}$ as quickly as other species, and it is believed that the mare also is more sensitive to lower levels of prostaglandins compared to other species (Senger, 2003). $PGF_{2\alpha}$ binds to receptors on the plasma membrane of luteal cells and causes the activation of protein kinase-C, which inhibits progesterone

synthesis (Senger, 2003). Prolonged diestrus in the mare is often caused by failure of luteolytic mechanisms which results in continued progesterone production from the CL.

***Steroidogenic Pathway***

Steroids are a subclass of lipids that contain a basic structure of four fused rings referred to as perhydroxylopentanophenanthrene (Ojeda, 2004). All steroids are formed by the polymerization of an active isoprene unit, which is derived from acetyl CoA. Polymerization of six activated isoprene units results in cholesterol (Figure 1.5), which is the initial precursor for steroid biosynthesis (Ojeda, 2004). The regulatory step of steroid hormone production is the conversion of cholesterol (27 carbons) to pregnenalone (21 carbons), which involves two hydroxylations. Cytochrome P450 initiates this reaction, which occurs in the mitochondria of steroidogenic cells that are located in ovaries, testes, adrenal cortex, placenta and brain. Pregnenalone and progesterone represent the precursors for all hormonally active steroids (Figure 6). The type and amount of steroids being produced depends on the physiologic nature of the cell and the activity of the enzyme systems (Ojeda, 2004). For example, the testes and ovaries are able to produce all hormones shown in diagram 2, but the production of estrogens and dihydrotestosterone (DHT) by the testis is limited, whereas the production of DHT by the ovary does not occur (Ojeda, 2004).

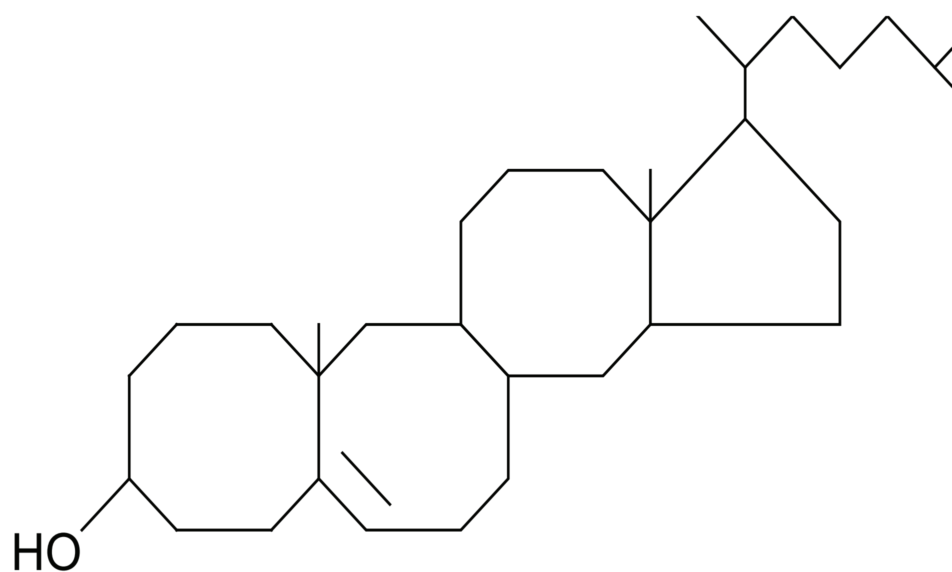



**Figure 1.5:** Cholesterol

**Steroid biosynthesis in the ovary**

The ovary produces steroid hormones that are important for reproductive functions (Ojeda, 2004). The main steroids produced by the ovary are progesterone and estradiol (Figure 1.6). Estradiol is produced mainly by granulosa cells of the follicle, whereas progesterone is secreted by all steroidogenic cells of the ovary (Ojeda, 2004). The ovary also produces androgens, such as androstenedione and testosterone, but a significant portion of these hormones is converted into estrone or estradiol (Ojeda, 2004).

Cholesterol, which is derived from dietary animal fats or from local *de novo* synthesis, provides the carbon backbone for steroidogenesis (Ojeda, 2004). Low-density lipoproteins (LDLs) transport cholesterol and bind to membrane receptors on cells causing the LDL complex to enter the cell by endocytosis (O'Malley *et al.,* 1991). Subsequently, cholesterol is transported into the mitochondria by Steroidogenic Acute Regulatory protein (StAR), which promotes the transfer of cholesterol from the outer to the inner mitochondrial membrane (Ojeda, 2004). Cholesterol, which has 27 carbons, is then converted to pregnenalone (21 carbons) by the mitochondrial enzyme cholesterol side chain cleavage (Ojeda, 2004). Pregnenalone is then converted to either progesterone or 17α - hydroxypregnenalone, both of which are transformed to 17α - hydroxyprogesterone. Androgens, estrogens and progestagen are derived from hydroxy-progesterone, which is converted to androstenedione, and then further reduced to either an androgen (19 carbons) or an estrogen (19 carbons) (Ojeda, 2004). Cleavage of 17α - hydroxyprogesterone by the enzyme 17, 20 lyase yields androstenedione, an androgen that is either directly metabolized to estrone or converted to testosterone by 17β - hydroxysteroid dehydrogenase.

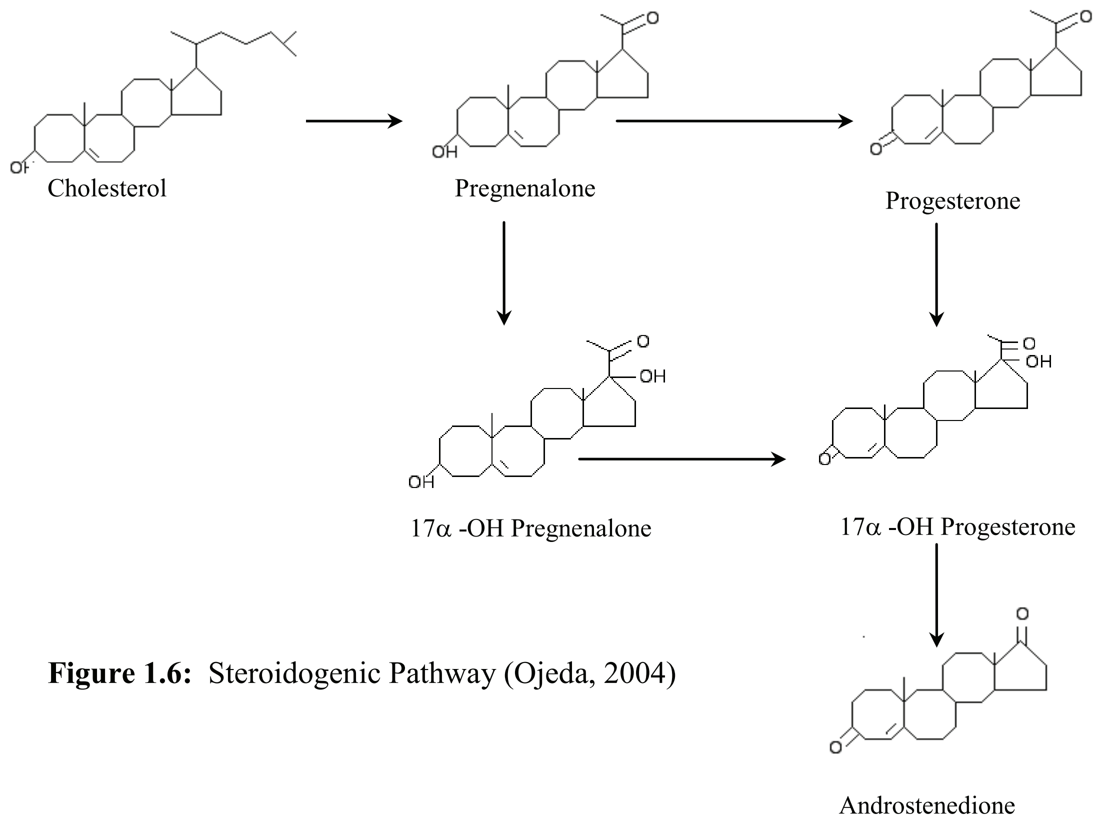



**Figure 1.6:** Steroidogenic Pathway (Ojeda, 2004)

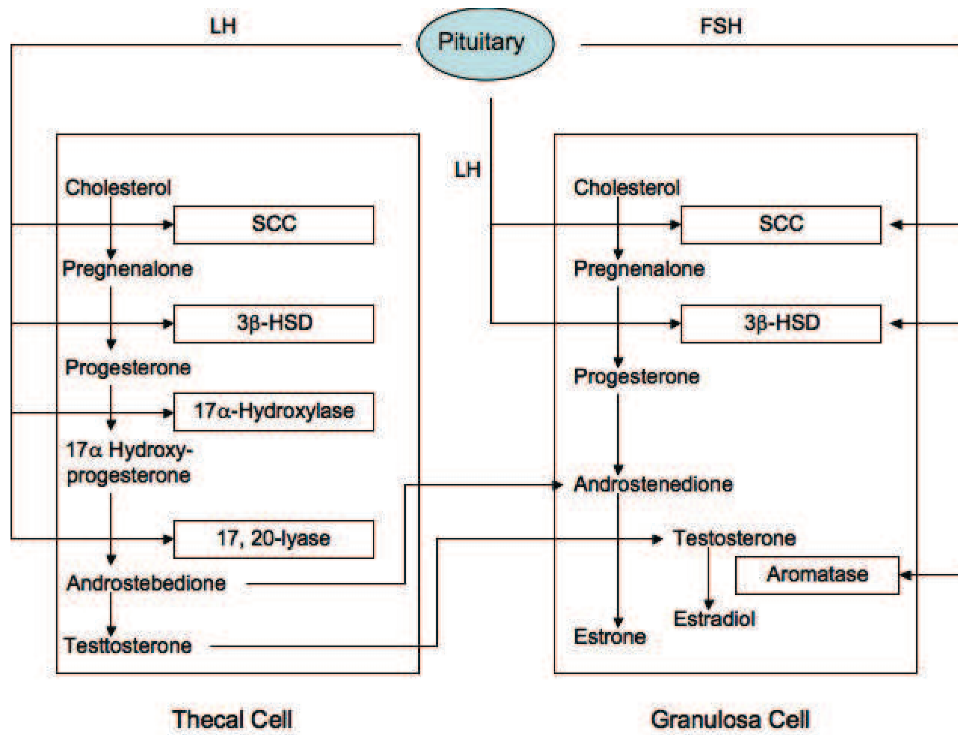



**Figure 1.7:** Two-cell – two gonadotrophin theory. LH acts on thecal cells to produce testosterone; FSH acts on granulosa cells to produce estradiol (Ojeda, 2004).

Testosterone is then converted to estradiol by an aromatase enzyme (Ojeda, 2004). For androgen and estrogen production to occur, ovarian granulosa and theca cells must work in tandem (O'Malley *et al.,*1991), which builds the basis of the two cell – two gonadotrophin theory (Figure 1.7). According to theory, LH dependent androgens derived from theca cells are converted to estrogen in the granulosa cells by FSH-inducible aromatase activity (O'Malley *et al.,* 1991). Studies of isolated granulosa cells revealed that only FSH stimulates estrogen synthesis (O'Malley *et al.,* 1991; Ojeda, 2004). In contrast, isolated theca cells did not produce significant amounts of estrogens, which supports the concept that granulosa cells are the principal site of estrogen production (O'Malley *et al.,* 1991).

Progesterone is produced by large luteal cells of the CL under the influence of LH (Figure 8; Senger, 2003). Esterified cholesterol forms a complex with LDL to facilitate delivery and binding to target cells. When LH binds with luteal cell surface receptors protein kinase C is activated this internalizes the cholesterol-LDL complex. Protein kinase also promotes entry of cholesterol into the mitochondria where it is converted to pregnenalone (Senger, 2003). Pregnenalone is then converted to progesterone, which enters the blood circulation and modulates GnRH production at the level of the hypothalamic-pituitary axis.

### ***Reproduction in Przewalski's Stallions***

Similar to the Przewalski's mare, there is very little known about the reproductive physiology of Przewalski's stallions. To date, there are no studies assessing endocrine rhythms and only a few reports that have described semen characteristics. Most studies

that have assessed semen characteristics were on anesthetized males using electro – ejaculation as the means for collection (Bader *et al.,* 1991; Durrant, 1990; Stover *et al.,* 1981) and most of these examined limited numbers of stallions. A study by Durrant (1990) showed that there were seasonal changes in semen parameters over a one-year period in five stallions. To date, no longitudinal studies have systematically assessed seminal parameters in the Przewalski's stallion.

***Reproduction in the Domestic Stallion***

**Seasonality**

Like the domestic mare, the stallion has been considered a 'long - day' breeder. Ejaculate volume, total sperm concentration, libido, testicular size, and plasma concentrations of steroid and protein hormones increase in summer months compared to winter months (Roser, 2008). However, it is important to note that stallions produce sperm throughout the year. The endocrine events that initiate an increase in testicular activity have not been characterized but appear to be based on photoperiods (Blanchard *et al.,* 2004). Similar to the domestic mare, longer day lengths cause decreased production of melatonin from the pineal gland, increased GnRH secretion from the hypothalamus, which in turn, stimulates spermatogenesis. In stallions, the shorter days of autumn are important to the photoperiod-induced increase in sperm production observed during the spring. Normally, increased day length would induce an increase in testicular size. However, stallions maintained on long days (i.e., 16 hours of light/day) for a 15-month period experienced testicular regression, demonstrating refractoriness to photoperiod (Clay and Clay, 1992). Based on these results, the decrease in day length in the autumn

may provide a critical trigger in resensitizing the stallion to the photoperiod of long days (Roser, 2008).

**Spermatogenesis**

Spermatogenesis is the sum of cell divisions and cellular changes that result in formation of spermatozoa from spermatogonia (Amann *et al.,* 1993). The entire process occurs in the seminiferous tubules, which comprises most of the testicular parenchyma in the stallion (Meyers, 2000). Sertoli cells comprise the myoid cell layer and, although their role in spermatogenesis is still not fully understood, probable functions include: 1) formation of the blood – testis barrier; 2) structural and nutritional support for the germinal cells, 3) facilitation of the movement of developing germinal cells within the seminiferous epithelium; 4) release of mature spermatozoa by a process of spermiation; and 5) cell-to-cell communication with developing germinal cells and Leydig cells (Amann *et al.,* 1993; Meyers, 2000). Leydig cells are located in the interstitial compartment and are critical to spermatogenesis due to their steroidogenic capabilities.

In the stallion, GnRH controls spermatogenesis through the production of FSH and LH from the anterior pituitary (Figure 1.8; Griffin, 2004). LH binds to Leydig cells and converts cholesterol to testosterone. Testosterone is then transported to Sertoli cells where it is converted to dihydrotestosterone and estradiol under the influence of FSH (Senger, 2003). Inhibin is also produced by Sertoli cells and is believed to modulate FSH production through negative feedback on the anterior pituitary (Griffin, 2004). The role of estradiol in male reproduction is still not understood but is believed to also play a role in negative feedback of FSH (Senger, 2003).

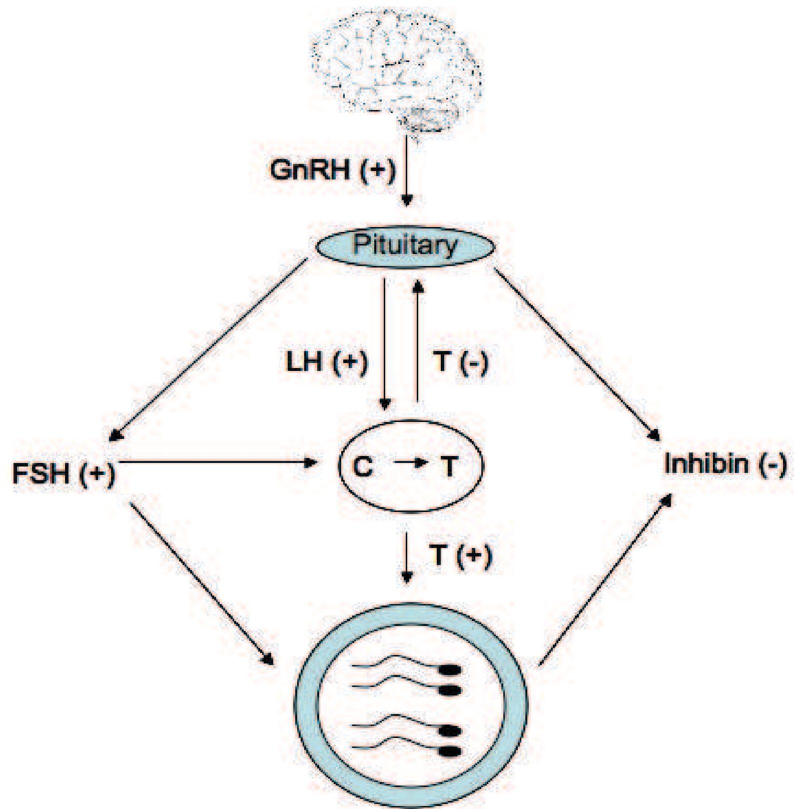



Figure 1.8: Endocrine control of spermatogenesis. T = Testosterone; C = Cholesterol (Griffin, 2004).

Spermatogenesis is comprised of three phases: 1) spermatocytogenesis, 2) meiosis, and 3) spermiogenesis (Meyers, 2000). The entire process takes 57 days in the domestic stallion (Amann *et al.,* 1993). The process starts with spermatogonia (Type $A_1$), haploid cells, which are the most primitive cells in spermatogenesis, located at the basal compartment of the seminiferous tubules (Senger, 2003). These cells divide to produce diploid cells that will either complete spermatogenesis or repopulate the stem cell population. The $A_1$ spermatogonia mitotically divide at regular intervals to enter the spermatogenic cycle and differentiate into at least five subtypes – $A_{1-3}$, $B_1$, $B_2$ (Meyers, 2003). The $B_2$ spermatocytes then enter the second stage of spermatogenesis by initiating meiosis as primary spermatocytes. After the first division of meiosis the primary spermatocyte becomes a secondary spermatocyte, which rapidly progresses to the haploid spermatid (Senger, 2003). The spermatid has an elongated shape, condensed nucleus and axoneme that are the characteristics of a sperm cell (Meyers, 2003). During maturation, mitochondria form the midpiece around the flagellum of the sperm and the postnuclear cap is formed (Senger, 2003). Once cells have reached this stage, spermiation occurs, which is a synchronous release of all spermatozoa into the seminiferous tubule and the epididymis for the final sperm maturation. Spermatozoa are continuously released into the seminiferous tubules throughout reproductive life in a male.

### *Assisted Reproduction in the Domestic Horse*

Assisted reproductive technologies, especially AI, are tools commonly used for breeding management in the domestic mare. Historically, studies on the collection, processing and insemination with stallion semen were initiated in the late 1800s in Russia. However, this technology was not used in the United States until the 1940s (Samper, 2000; Blanchard *et al.,* 2003). Since many breed registries now allow AI for the production of foals, this method has been increasingly utilized during the past decade. Artificial insemination in the domestic horse involves collection, dilution and preservation of stallion sperm, as well as timely placement of an adequate number of sperm cells in the mare's uterus (Samper, 2000).

## Semen Collection and Preservation

The most convenient method of semen collection is by use of the artificial vagina (AV). Although there are many different designs, all AVs work on the same principle, which utilizes a double rubber liner with warm water between the layers to provide appropriate temperature conditions (Samper, 2000). Stallions produce an ejaculate following proper stimulation that closely resembles natural breeding. The disadvantage of this technique is that stallions require training to mount a dummy phantom mare and also to adapt to collection with an AV (Blanchard *et al.,* 2003). Unskilled handlers can also be injured during the collection process.

More recently, another method of semen collection in the domestic stallion was developed using imipramine and alpha-2 agonists (xylazine or detomidine) that induce ejaculation in a standing stallion (McDonnell and Odian, 1994; Card *et al.,* 1997). While

the mechanism is not well understood, it is believed that imipramine lowers the ejaculatory threshold while $\alpha_2$ agonist induces ejaculation. This method requires a lengthy titration of drug combinations for individual stallions to effect and is less reliable than AV. For stallions that are not trained to collection by AV, condoms have also been used for the purpose of semen evaluation, but are rarely used for semen preservation due to the poor semen quality caused by bacterial contamination derived from the external sheath of the penis (Blanchard *et al.,* 2003). Electro-ejaculation under general anesthesia has also been attempted with limited success in the domestic stallion (Cary *et al.,* 2004).

In the domestic stallion, if semen is used for AI within 12 hours after collection, it can be stored at room temperature in the dark, but if semen is used 12 - 72 hours post-ejaculation, then semen should be extended and chilled to 4ºC (Samper, 2000). Most semen extenders used in the domestic stallion are milk-based products that contain antibiotics (Blanchard *et al.,* 2003). Some examples of common extenders are: Kenney extender (nonfat dry milk solids, glucose, penicillin, streptomycin and gentamicin); TAMU formula (nonfat milk solids, glucose, sucrose, penicillin, and amikacin); and VMD-Z™ Formula (egg-based extender with amikacin and penicillin) (Brinsko and Varner, 1992; Aurich, 2008). When semen is extended and properly stored, sperm cells usually are viable for up to 72 hours.

More recently, the use of frozen semen has grown in popularity because this technology allows transportation of samples obtained from genetically-valuable stallions throughout the world. The first foal born using frozen – thawed sperm was in 1957 (Samper, 2000). To date, fertility rates of mares inseminated with fresh-cooled sperm are comparable to natural breeding (60 – 70%, Samper, Vidament, 2005), whereas foaling

rates are much lower in mares inseminated with frozen-thawed sperm (30 – 65%, Vidament, 2005; Samper and Plough, 2010). There are large variations in the quality of frozen-thawed sperm among stallions. Specifically, spermatozoa from approximately 25 percent of stallions do not survive the freezing and thawing process (Graham, 1996; Adams *et al.,* 2009).

### *Manipulation of the Estrous Cycle*

Treatments aimed at synchronizing or manipulation the estrous cycle are used for management of many domestic and wildlife species for the purpose of fixed time AI, managing birth dates and for the synchronization of recipients in embryo transfer. It is commonly used in cattle, pigs, sheep, and white tailed deer. It has also been used in wildlife species, such as the scimitar horned oryx (*Oryx dammah;* Morrow *et al.,* 2000), Eld's deer (*Cervus eldii;* Monfort *et al.,* 1993) and killer whale (*Orcinus orca;* Robeck *et al.,* 2004). Ovarian synchronization schemes have benefited from an improved understanding of the endocrine system and the ability to objectively assess the success or failure of various regimens. In practice, however, factors such as cost, labor, time of year and personal preference have a strong influence on the methods chosen (Bergfelt, 2000). Most estrous synchronization systems utilize methods that control follicular wave development, which results in estrus and ovulation (Lucy *et al.,* 2004). Hormones used to pharmacologically control the estrous cycle have similar effects on the endogenous reproductive hormones found within the hypothalamus (gonadotrophins), ovary (estradiol and progesterone) and uterus (prostaglandins) (Bergfelt, 2000; Lucy *et al.,* 2004)

## Methods Used in the Domestic Horse

The extended estrus period in the mare, with ovulation occurring 1 – 10 days after the beginning of behavioral estrus, complicates reproductive management. Various combinations of reproductive steroids (progestagens and estrogens), $PGF_{2\alpha}$, human chorionic gonadotropin and GnRH agonists have been used to control follicular development and ovulation in domestic mares (Taylor *et al.,* 1982; Blanchard *et al.,* 2003). Despite much research in the area, reliable estrous synchronization of domestic mares remains challenging and research is - ongoing to determine the optimal method for this species (Bergfelt, 2000; Bergfelt *et al.,* 2007)

*Prostaglandins:*

Most synchronization methods in the domestic mare modify the luteal phase of the estrous cycle (Blanchard *et al.,* 2003). The administration of $PGF_{2\alpha}$ causes luteolysis of the CL in the domestic mare (Douglas and Ginther, 1972; Noden *et al.,* 1974). However, this treatment has low efficacy (52%; Burns *et al.,* 1979) and there is wide variation in the interval from when treatment is initiated to the subsequent ovulation. This variation relates, in part, to the stage of the reproductive cycle and follicular development at the time of hormone administration. Specifically, the CL is refractory to $PGF_{2\alpha}$ treatment until 5 days post-ovulation (Bristol, 1993). Also, if $PGF_{2\alpha}$ is administered more than 9 days post-ovulation the interovulatory interval will not be shortened (Blanchard *et al.,* 2003) due to release of endogenous prostaglandins. To increase estrous synchrony using $PGF_{2\alpha}$, two injections were given 14 days apart to 33 mares (Burns *et al.,* 1979). After the second injection, 26 (79%) of mares showed estrus within 4.4 ± 1.7 days and 31

(94%) ovulated in 7.2 ± 2.6 days (range: 2 – 10 days; Burns *et al.,* 1979). In further attempts to reduce variability associated with this two-shot $PGF_{2\alpha}$ protocol, human chorionic gonadotropin (hCG) was administered 4 – 6 days after the second $PGF_{2\alpha}$ injection (Palmer, 1975; Allen, 1976; Bosu *et al.,* 1983). While these studies showed improved synchrony, other studies showed varied response based on time of year (Squires, 1981; Holtan *et al.,* 1977). In one trial, two prostaglandin injections were given 18 days apart in pony mares from June to August. Six days after the second prostaglandin injection, hCG was administered and only 26.1 % of mares (6 of 23) ovulated within 48 hours of hCG (Holtan *et al.,* 1977)

*Progestagens:*

Progesterone treatment, which is intended to mimic the suppressive effects of endogeneous hormone on the hypothalamic-pitutiary axis, must be administered long enough to permit regression of any extant CL. Thus, exogenous progestagen treatment must typically last 12 – 18 days in the mare (Bristol, 1993, Bergfelt, 2000). Exogenous progestagen treatment, such as the use of oral altrenogest, is commonly used in the domestic mare to suppress endogenous LH secretion and ovulation (Bergfelt, 2000). Progestagen treatment alone has little, if any, effect on FSH secretion. This results in a large amount of variation in follicular growth during the treatment interval, which results in high variation in the degree of ovarian synchrony among females (Bergfelt, 2000). For example, 15 mares that were given oral altrenogest (0.044 mg/kg/day for 15 days) showed estrus 3.4 ± 1.9 days and ovulated 8.8 ± 2.2 days after treatment (Squires *et al.,*

1983). This degree of variation means that mares ovulated anywhere from 8 – 15 days post-treatment.

To improve synchrony, $PGF_{2\alpha}$ treatment at the end of progestagen treatment has been used to ensure regression of a primary or secondary CL. However, an 8-day treatment with altrenogest combined with $PGF_{2\alpha}$ administered on day 8 resulted in mares ovulating 8 – 15 days post treatment (Palmer, 1975). What is especially challenging is that while the degree of estrous synchrony can be relatively high using the progestagen/$PGF_{2\alpha}$ regimen, ovulation synchrony is poor (Lofstedt and Patel, 1989; Bergfelt, 2000). Because treatment with progestagens does not prevent follicular development and ovulation from occurring during treatment, $PGF_{2\alpha}$ may be only partially effective for inducing luteolysis (i.e., some younger CL would be refractory to treatment), and this has a detrimental effect on achieving ovarian synchrony (Bristol, 1993). Because follicular development is not fully inhibited by treatment with progestagens, a wide variation in follicular development – and hence the hormonal milieu – exists after termination of treatment (Lofstedt and Patel, 1989; Blanchard *et al.,* 2003). This results in a wide variation of intervals to ovulation in domestic mares.

Recently, an injectable form of altrenogest (Biorelease altrenogest; Betpharm, Lexington, KY, USA) has been developed (Burns *et al.,* 2008). The use of this product has improved estrous suppression compared to the oral form (Burns *et al.,* 2006). Injectable altrenogest releases hormone into the circulation over a 10 to 12-day period in domestic mares and has been shown to be effective at inhibiting production of progesterone. To date, limited research has been conducted to compare the injectable to

the oral form of altrenogest and to assess the use of injectable altrenogest as a method of estrus synchronization in the domestic mare.

*Progestagens and Estrogens:*

The hormonal regimen used most commonly in the domestic mare to control both follicular development and ovulation is a combination of progesterone plus estradiol (P&E). Typically, the protocol requires daily intramuscular injections of progesterone and estradiol for 10 days beginning at any stage of the cycle. An injection of $PGF_{2\alpha}$ is administered on the last day of treatment and an ovulatory agent is given when a follicle > 35mm is detected by rectal ultrasound (Bergfelt *et al.,* 2007). The P&E treatment regimen, with or without an ovulatory agent resulted in ovulation in 54 – 68% of mares within 2 days of treatment cessation, and in 72 – 94% of mares within 4 days of treatment completion (Loy *et al.,* 1981; Taylor *et al.,* 1982). With hCG treatment, ovulations occurred within 2 days of treatment completion in 70 – 73 % of mares (Varner *et al.,* 1988). In a recent study by Bergfelt *et al* (2007)*,* 90% of treated mares ovulated within 4 days of treatment cessation. Pregnancy rates following progesterone and estradiol, with and without hCG, have ranged from 62 to77% (Taylor *et al.,* 1982; Varner *et al.,* 1988). While the results of progesterone and estradiol are very reliable compared to oral treatments, to date, the only method for administering estradiol is through daily injections, which is often not practical and may cause physiological stress and/or side effects such as anemia and abscess reactions to frequent painful injections (Bergfelt, 2000).

***Reasons for Success/ Failure***

There are many examples of how extensive research in wildlife reproduction has been necessary for the successful use of AI as a genetic tool. Assisted breeding was first attempted in the cheetah in 1980 using AI protocols adapted from cows, with little success (Pukazhenthi and Wildt, 2004). After extensive studies of *in situ* and *ex situ* populations, which included investigation of the reproductive cycle, time and type of ovulation, methods for inducing ovulation and sperm collection and processing methods, AI using fresh semen has been successfully applied in cheetah (*Acinonyx jubatus*; Howard *et al.,*1997).

The black footed ferret (*Mustela nigripes*) faced a rapidly declining population in the 1960s and 70s. The current population is based on 18 individuals that were discovered in Wyoming in 1981. Through reproductive research, a successful AI technique was developed and 6,000 kits have been produced since research was initiated in 1986 (Howard *et al,* 2003*;* Comizzoli *et al.,* 2009). Artificial insemination has also been successful in ungulate species such as the scimitar-horned oryx (*Oryx dammah;* Morrow *et al.,* 2000), Eld's Deer (*Cervis eldii;* Monfort *et al.,* 1993), and fallow deer (*Dama dama;* Jabbour *et al.,*1993).

Despite many of these successes, there have also been setbacks. In many species, such as the white rhinoceros (*Ceratotherium simum*) the Asian and African elephant(*Loxodonta africanus*), it is difficult to collect semen, limiting AI potential in these species (Hildebrandt *et al.,* 2000; Brown *et al.,* 2004; Hermes *et al.,* 2007). While there is limited research showing that electroejaculation works in Przewalski's stallions (Bader *et al.,*1991; Durrant, 1990; Stover *et al.,*1981), there is also evidence that semen

collection by electroejaculation does not work reliably in the domestic stallion (Cary *et al.,*2004).

Estrus synchronization of females can also present a challenge. In gazelles (*Gazella dama*), research on synchronization of females resulted in poor results (Holt *et al.,* 1996; Pickard *et al.,* 2001). In the scimitar horned oryx (*Oryx dammah*), treatment of females with CIDRs resulted in a delay in luteal development, whereas treatment with two injections of $PGF_{2\alpha}$ resulted in pregnancies (Morrow *et al.,* 2000).

Because there is limited understanding of reproductive physiology in many species, great gaps in knowledge that are necessary to produce pregnancies by AI exists. In the Przewalski's horse, for instance, there are only limited studies on female reproductive patterns and seminal characteristics. To date, there is no data on of ovulation timing or estrus synchrony, information which is critical for achieving AI in this species.

Therefore, it is necessary to assess reproductive patterns and seminal characteristics in Przewalski's mares and stallions in order for AI to be an effective method of genetic management. The overall goal of this dissertation research was to obtain a more comprehensive understanding of the reproductive parameters in the Przewalski's horse for application towards improved species conservation. Specific objectives include: 1) assessing female reproductive patterns using urinary hormone and follicular data to further characterize female estrous patterns; 2) determining whether the estrous cycle can be manipulated using exogenous progestagens and gonadotrophins; and 3) describing seasonal variation in seminal traits of Przewalski's stallions. Before these objectives could be accomplished it was first necessary to design a facility that would allow frequent ultrasound examination of Przewalski's mares without sedation or anesthesia. At SCBI, a

hydraulic tamer was placed at previously built chute to make manipulation of mares simpler. The chute was actually modified with the addition of extra doors so that movement of mares was slower to lower the chance of injury during manipulation. Mares were then trained to enter the chute system throughout the year as part of their daily routine. Animal care staff members were also trained in manipulation of the hydraulic tamer so that animals were lifted quickly during research periods. At the Wilds, a purpose built facility was already incorporated into the veterinary hospital facilities. As a result, we were able to examine mares 3-times/week without sedation or anesthesia. Frequent observations of follicular changes could be made throughout the estrous cycle. To get an accurate endocrine profile for all mares, urine samples were collected 4 – 7 days/week. Mares were trained to urinate each day through a reward system. Once each mare urinated, they were given a treat (apple biscuit, fruit or carrot). As a result, we collected a total of 8,284 urine samples from 22 mares over a 63 month period. After collection, urine samples were frozen at -20° C until analysis was complete. By combining the endocrine and follicular characteristics of mares, we have a comprehensive understanding of estrous cycle patterns in Przewalski's mares. By utilizing these techniques, we also described endocrine and follicular responses to hormone therapies that may be used to manipulate the estrous cycle.

Since semen collection in domestic stallions using electro-ejaculation has shown poor results, a reliable method of collecting semen from Przewalski's stallions had to be established. Using techniques that have been established in other wildlife species, we were able to develop a semen collection technique that was successful in 84 of 98 semen collection attempts from 14 Przewalski's stallions over a 4 year period. Thus, we were

able to get an accurate description of seminal traits in Przewalski's stallions due to the high number of ejaculates characterized.

Overall, we have a more comprehensive understanding of reproductive patterns in Przewalski's mares and seminal traits in Przewalski's stallions. As a result, we have determined that there are a high percentage of mares with abnormal reproductive patterns that are related to inbreeding. We have also shown that mares with normal cycles respond to exogenous hormone therapy, which could be utilized in breeding management of mares. In stallions, we have determined seasonal patterns in seminal traits and that there is a predominant percentage of abnormal spermatozoa in this population, a factor that is important to consider in long term breeding management and preservation of this species.

# CHAPTER TWO

## Abnormal Reproductive Cycles are Prevalent in Przewalski's Mares

### INTRODUCTION

The Przewalski's horse (*Equus ferus przewalskii*) is the last remaining wild horse in existence today. Once native to the Gobi Desert in Mongolia and China, the Przewalski's horse is now considered critically endangered with 1,872 animals living in captivity and re-introduction sites in Mongolia, China and Kazakhstan (Lee and Boyd, 2008). The current population is descended from only 14 founders captured from the wild over a period of time ranging from 1899 to 1947. The species was declared extinct in the wild in 1970 by the International Union for Conservation of Nature (Lee and Boyd, 2008). Recent conservation and re-introduction efforts have increased the number of individuals in the wild to 325 in Mongolia and 123 in China. As a result, the status of this species has been upgraded to 'Critically Endangered' (Boyd *et al.*, 2008).

Because there are no new founders available in the wild, the captive population serves as a genetic reservoir, or source population, for the re-introduced populations in Asia (Monfort *et al.,* 2009). The population in North America (123 individuals) is

managed by a Species Survival Plan (SSP; Monfort *et al.,* 2009). The Asian Wild Horse SSP uses mean kinship to prioritize breeding pairs that maximize gene diversity in the population (Monfort *et al.,* 2009). The pedigrees of all Przewalski's horses in captivity are known, which facilitates genetic management; however, current gene diversity in the population is only 78.8%, which is significantly lower than the average for all mammal species managed by SSPs (93%; Monfort *et al.,* 2009). When gene diversity falls below 90% of that in the founding population, it is expected that reproduction could be compromised due to neonatal mortality and decreased birth weights (Monfort *et al.,* 2009).

One challenge associated with the SSP-managed population is that some founders are overrepresented (Figure 2.1). In this population, the average mean kinship is 0.2161, which indicates that each individual is related to 21.6% of the population (Monfort *et al.,* 2009). As a result, gene diversity is decreased. This is further complicated by the small effective population size for this population (0.1867) (Monfort *et al.,* 2009). Ideally, all animals in a population should be able to reproduce and produce offspring; however, the effective population size is the actual percentage of animals that are contributing genetics to a population (Wright, 1933). This contributes to loss of genes and genetic drift, which can further impact reproductive fitness of the species. To date, there has been no research on the effect of gene diversity on reproduction in the Przewalski's horse.

| Genetics | Current | Potential |
|---|---|---|
| Number of Founders | 14 | 14 (0 additional) |
| Founder Genome Equivalents (FGE) | 2.36 | 4.64 |
| Gene Diversity (GD) Retained (%) | 78.80 | 89.23 |
| Population Mean Kinship (MK) | 0.2120 | |
| Mean Inbreeding (F) | 0.1935 | |
| % Pedigree Known | 98.3 | |
| $N_e/N$ | .3447 | |
| Years to 90% | Below | |
| Gene Diversity at 100 years from Present | 59.52% | |

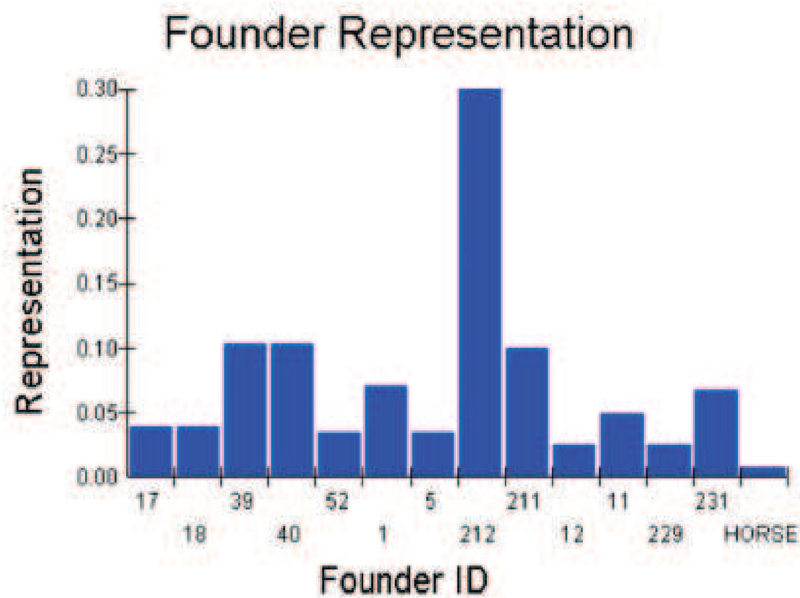
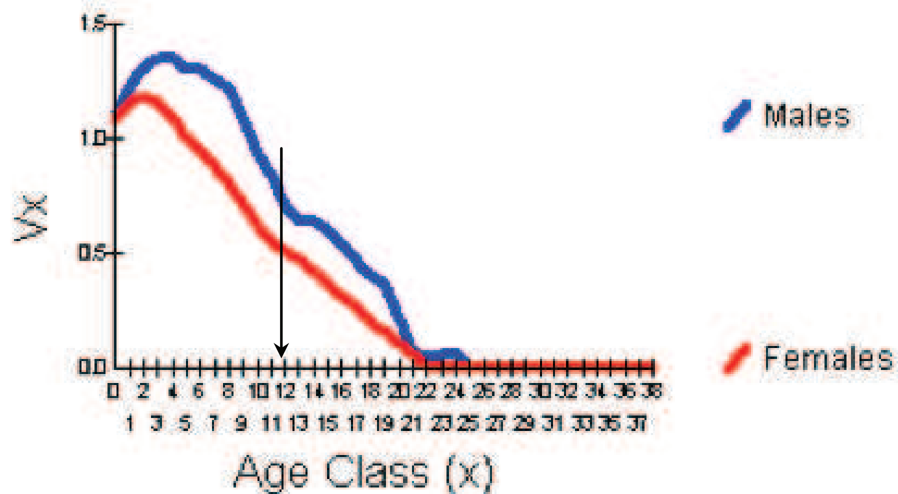



Figure 2.1: Founder representation in the North American SSP population for the Przewalski's horse. Table lists population mean kinship and current gene diversity for the population (Monfort *et al..,* 2009).

Because the current captive population in North America is skewed towards older animals, it is suspected that age-related fertility issues may be prevalent. Currently, the average age of breeding mares in the North American population is 12.6 years (range, 2–23 years) and 54% of breeding mares are over 10 years old. According to data from the SSP on reproductive fecundity, a Przewalski's mare aged 12 years would be expected to produce one foal every two years as compared to a 6-year old mare that produces one foal every year (Figure 2.2, Monfort *et al.,* 2009). During the past five years, an average of 8 live foals have been produced from 40 breeding pairs per year (20% foaling rate) – a rate significantly lower than the live annual foaling rate of 50 - 60 % observed in the domestic horse (Ginther, 1992; Blanchard *et al.,* 2004).

Assisted breeding, especially artificial insemination (AI) has been used as a tool to genetically manage *ex situ* populations of many wildlife species, such as the Giant Panda (*Ailuropoda melanoleuca;* Huang *et al.,* 2002), African elephant (*Loxodonta africanus;* Hildebrandt, 2000), Asian elephant (Brown *et al.,* 2004), Eld's deer (*Cervus eldii;* Monfort *et al*., 1993) and scimitar horned oryx (*Oryx dammah;* Morrow *et al.,* 2000), among others. However, success required preliminary efforts to establish baseline reproductive patterns in females such as seasonality, length of diestrus and estrus, and the timing of ovulation. To date, four approaches have been used to study reproductive physiology of the Przewalski's mare: 1) ultrasound evaluation of follicular structures (Durrant *et al.,* 1986); 2) milk progesterone excretion (Zimmermann *et al.,* 1985); 3) fecal progesterone metabolites (Schwarzenberger *et al.,* 1992) and 4) urinary estrogen metabolite excretion (Monfort *et al.,* 1991). Follicular growth patterns were assessed by




Vx, Reproductive Value – The expected number of offspring produced this year and in future years by an animal of age x

similar to what has been reported for the domestic horse (Durrant, *et al.,* 1991).

Figure 2.2: Predicted number of offspring produced based on age (years). For a 12-year-old mare (indicated by arrow), only 1foal is produced every 2 years (Monfort *et al.,* 2009).

rectal ultrasonography in two mares that were each evaluated seven times over a three month period. Based on their observations, follicular development and ovulation were However, because animals had to be anesthetized, animal numbers and the frequency of examinations were limited. Additionally, no endocrine measures were available for correlating hormones with observed ovarian structures.

Przewalski's mares are still managed as wild animals, which makes it difficult to collect repeated blood samples for longitudinal endocrine assessments. As a result, previous research has focused on the development and application of non-invasive techniques for analyzing steroid hormone and/or their metabolites in this species. In one study, milk progesterone metabolites assessed in two lactating Przewalski's mares revealed progesterone patterns that were similar to their domestic counterparts (Zimmermann *et al.,* 1985). After mating, the two mares exhibited a five to ten-fold increase in milk progesterone metabolites, which remained elevated until day 35 post-ovulation. After day 35, an additional two-fold increase in milk progesterone metabolites was observed, which was similar to the secondary rise in progesterone secretion in domestic mares (Ginther, 1992; Blanchard *et al.,* 2004). Although this study revealed important information about reproductive physiology in Przewalski's mares, numbers of animals were limited (n = 2), in part, because sample collection was restricted to mares that would allow milk samples to be collected.

A second study (Schwarzenberger *et al.,* 1992) assessed fecal progesterone metabolites in non-pregnant Przewalski's mares (n = 5). Periodic increases in fecal progesterone metabolites during the breeding season were detected in four of five mares evaluated indicating the potential of fecal progestagen assessments for tracking ovarian

activity in this species, but data were insufficient for elucidating the endocrine features of the estrous cycle.

The most extensive longitudinal assessment of reproductive endocrinology of Przewalski's mares was accomplished by assessing urinary estrogen metabolites in eight Przewalski's mares over a 19-month period (Monfort *et al.,* 1991). Estrogen excretion patterns confirmed reproductive seasonality for Przewalksi's mares maintained in North America, and the onset of estrous cycles occurred coincident with increased day lengths. Additionally, most copulations coincided with the peak of mid-cycle excreted estrogens across a 24-day ovarian cycle, and estrogen excretion was effective for diagnosing pregnancy.

Combining progesterone, estrogen, and follicular data would give the best estimation of ovulation timing, mainly because the horse has a longer period of estrus compared to other species (Koskinen *et al.,* 1986). Therefore, the objectives of this study are to: 1) characterize longitudinal profiles of urinary estrogen and progestagen metabolites; 2) describe follicular changes in relation to urinary hormone metabolites; and 3) assess changes in reproductive parameters based on age and mean kinship in Przewalski's mares. The hypotheses of this study were as follows: 1) differences exist in follicular changes and endocrine profiles between fertile and subfertile mares; and 2) mare age and genetic factors impact reproductive cycles.

## MATERIALS AND METHODS

*Animals:*

Nineteen Przewalski's mares (3-27 years of age) were included in the present study. Fourteen mares were housed at the Smithsonian's Conservation Biology Institute (SCBI) in Front Royal, Virginia (38.88° W, 78.17° N) and the remainder (n = 5) were kept at the Wilds in Cumberland, Ohio (38.82° W, 81.75° N). All mares were kept in enclosures ranging in size from 0.5 to 50 acres. Table 2.1 shows ages, mean kinship, location and number of foals produced (up to 2007) for all mares used in this study.

*Urine Collection:*

Urine samples were collected three to seven days per week using the technique described by Monfort *et al.* (1991). Briefly, freshly voided urine was aspirated with a clean syringe and kept cool until all samples were collected from all mares. Samples were then placed in labeled glass tubes and centrifuged at 1,500 G for 15 minutes (centrifuge) to remove sediment and dirt. Urine supernatant was then stored in plastic tubes at – 20 °C until analysis was completed. All samples (n = 7,517) were collected in the morning hours (7 AM to 12 PM).

*Enzyme Immunoassay*

**Creatinine**

Creatinine is secreted in urine and is an indication of glomerular filtration rate, therefore, to normalize all urinary samples based on dilution, all samples were indexed for creatinine (Cr) (Taussky, 1954). For Cr determinations, raw urine was diluted to

| Mare | Age at Start of Project | Mean Kinship | Number of Foals | Location | Category |
|---|---|---|---|---|---|
| 4890 | 3 | 0.500 | 0 | NZP | Normal |
| 4887 | 3 | 0.500 | 0 | NZP | Normal |
| 2861 | 11 | 0.178 | 1 | NZP | Normal |
| 3925 | 3 | 0.191 | 2 | NZP | Normal |
| 2156 | 15 | 0.197 | 1 | NZP | Normal |
| 2056 | 16 | 0.230 | 0 | Wilds | Normal |
| 4350 | 4 | 0.181 | 1 | NZP | Normal |
| 1363 | 21 | 0.183 | 1 | Wilds | Normal |
| 4176 | 6 | 0.207 | 3 | Wilds | Normal |
| 1866 | 17 | 0.227 | 2 | Wilds | Normal |
| 1830 | 17 | 0.201 | 0 | NZP | Acyclic |
| 1851 | 17 | 0.226 | 0 | NZP | Acyclic |
| 2168 | 15 | 0.225 | 0 | NZP | Acyclic |
| 3495 | 5 | 0.247 | 0 | NZP | Acyclic |
| 1893 | 17 | 0.278 | 2 | Wilds | Acyclic |
| 2050 | 16 | 0.200 | 0 | NZP | Abnormal |
| 1035 | 24 | 0.221 | 1 | NZP | Abnormal |
| 952 | 23 | 0.213 | 1 | NZP | Abnormal |
| 3516 | 8 | 0.245 | 0 | NZP | Abnormal |

**Table 2.1:** Mares included in study. List shows age, mean kinship, number of live foals, location, and category of reproductive cycles based on endocrine or follicular data.

1:50 concentration in BSA – free phosphate buffer. Diluted samples were added to 96-well, flat bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA, USA), combined with 0.05 mL each of $DH_2O$, 0.4 N picric acid, 0.75 N NaOH and incubated at room temperature (25°C) for 30 min. Optical density (OD) was measured at 490 nm (reference 620 nm) using a microplate reader (Dynex MRX; Dynex Technologies, Chantilly, VA, USA). Urine were run in duplicate and compared to reference Cr standards (0.00625 – 0.1 ng/mL, Sigma Aldrich, St. Louis, MO, USA). Samples that had concentrations < 0.1 ng Cr/mL were considered too dilute and were run at a higher concentration (1:10 or 1:25). Samples that were < 0.1ng/mL Cr at a 1:10 dilution were discarded (2% of samples). Hormone mass in a urine sample was divided by Cr concentration and expressed as mass of hormone/mg Cr (ng/mg Cr).

**Estrogen EIA:**

Urine samples were diluted (1:5 – 1:2000) in phosphate buffered solution and assayed in duplicate using the protocol outlined by Munro *et al.* (1991). The antiserum (R522, Coralie Munro, University of California, Davis, CA, USA) cross-reacts with estrone-3-glucoronide (100%), estradiol-3-sulfate (66.6%), estrone (238%), estradiol-17β (7.8%), estradiol-3-glucoronide (3.8%) and estradiol-3-sulfate (3.3%). Sensitivity of the assay at maximum binding is 0.78 pg/well. The inter assay coefficient of variation (CV) for the two internal controls was 11.49% (mean binding, 30.33%) and 7.14% (mean binding, 65.59%) and intra-assy CV was < 10%. Serially diluted urine samples demonstrated displacement curves parallel to those of standard hormone

preparations. Recovery of added standard to urine (y = 0.82x + 1.07, $r$ = 0.99) demonstrated significant recovery ($P$ < 0.05).

**Progestagen EIA:**

Urine samples were diluted (1:10 – 1:400) in phosphate buffered solution and analyzed in duplicate using progestagen protocol described by Graham *et al.* (2001). The antiserum (CL425, Coralie Munro, University of California, Davis, CA, USA) cross-reacts with 4-pregnen-3,20-dione (100%), 4-pregnen-3α-ol-20-one (188%), 4-pregnen-3β-ol-20-one (172%), 4-pregnen-11α-ol-3,20dione (147%), 5α-pregnan-3β-ol-20-one (94%), 5α-pregnan-3β,20-dione (64%), 5α-pregnan-3,20-dione (55%), 5β-pregnan-3β-ol-20-one (12.5%), 5-pregnan-3,20-dione (8%), 4-pregnen-11β-ol-3,20-dione (2.7%), and 5β-pregnan-3α-ol-20-one (2.5%; Graham *et al.,* 2001). Sensitivity of the assay at maximum binding is 0.78 pg/well. The inter-assay CV for two internal controls was 12.99% (mean binding, 30.32%) and 6.95% (mean binding, 72.59%) and intra-assay CV was < 10%. Serially diluted urine samples demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to urine (y = 0.99x – 0.48, $r$ = 0.99) demonstrated significant recovery ($P$ < 0.05).

*Training for Ultrasound Examinations:*

At SCBI, all females were subjected to an initial three-month training period to adapt to the chute and hydraulic tamer system that was designed to allow handling of large hoofstock species without the use of immobilizing drugs. Briefly, all mares were run through a custom-built chute system with an incorporated hydraulic tamer system

(Fauna Researh, Inc, Red Hook, NY, USA) that was positioned within the interior of the horse barn. For a one-month period mares were worked through the chute system three days per week without being restrained. During the second month, mares moved through the chute system three days per week, were briefly restrained between the padded squeeze walls, and then released. In the third month, mares were restrained and lifted in the hydraulic tamer once per week, but allowed to pass through the tamer without restraint on the other two days. To lift the mares, the padded walls of the tamer were closed to secure the animals sufficiently to permit them to be raised off the ground (~ one foot) using the hydraulic lift. This procedure provided sufficient immobilization to permit rectal examination. Only personnel specifically trained in operating the tamer were allowed to restrain/lift mares for this project. During the study period (four – six weeks), mares were restrained three days per week. Mares that were not successfully restrained after three attempts, or mares that appeared too stressed, based on keeper and operator assessments, were released without examination. On the off days (two days per week), mares went through the chute system as part of their morning routine before being let out to pasture. On weekends, mares were not run through the system. All mares received apple biscuits after leaving the tamer. Mares were worked through the chute system at least one – two days per week as part of their daily routine throughout the entire period of reproductive studies (2006 – 2009).

The hydraulic tamer and chute system at the Wilds is incorporated into their veterinary facility. Mares used for the study were moved from their pasture to the veterinary hospital 14 days before study onset. Without preconditioning, these animals were moved through the chute system on days of ultrasound examinations (three days

per week). All mares received morning feed and alfalfa after the ultrasound procedure was performed. Once the study was completed, all mares were returned to pasture with the rest of the herd. To decrease stress, there were always three mares involved in the study so that no animals were by themselves at the veterinary facility.

*Ultrasound Examination*

This study was conducted in June to July 2006 (five weeks) and May to June 2008 (five weeks). Transrectal ultrasound exam was performed three days per week on Przewalski's mares (SCBI: n = 7; Wilds: n = 3) using a portable B-mode ultrasound (SCBI: Sonovet 2000, Medison America, Inc, Cypress, CA, USA; Wilds: Aloka 500, Aloka America, Walingford, CT, USA) equipped with a linear transducer (4 to 7 MHz). Information on uterine edema, the presence or absence of endometrial fluid and ovarian structures (i.e., numbers of follicle, follicle size and the presence of corpus lutea [CL]) were recorded. Other ovarian structures, such as ovulations and abnormal follicles were also noted. To minimize stress, the total time during which each mare was in the tamer was held to less than five minutes.

*Statistical Analysis*

**Endocrine studies**

Yearly baseline concentrations of urinary estrogen conjugate and progestagen metabolites for each female were determined using an iterative process (Brown, *et al.*, 1994). For urinary progestagen metabolites, values in excess of mean ± 2.0 standard deviations (SD) were removed. The average was then recalculated, and the

elimination process repeated until no values exceeded the mean ± 2 SD remained. For urinary estrogen conjugate metabolites, values in excess of 2.5 SD of the mean baseline were removed until no values over 2.5 SD of the mean remained. Urinary estrogen conjugate metabolite values were considered elevated if they remained elevated above baseline for three days and urinary progestagen metabolite values were considered elevated if they remained elevated above baseline for four days.

To standardize profiles, steroid hormone metabolites were aligned to the day of peak urinary estrogen metabolites. Data were analyzed -10 to +20 days from peak urinary estrogens to assess an entire reproductive cycle based on previous study (24.1 days; Monfort *et al.,* 1991). To avoid possible influence of the variable number of cycles per female, least square means were calculated for the population. Data were presented as mean ± SEM and graphed longitudinally using Delta Graph (Red Rock Software, Inc., Salt Lake City, Utah, USA). Length of reproductive cycle was determined by calculating the interval between two urinary estrogen and progestagen peaks. Days between urinary progestagen and estrogen peaks were then compared using a student's *t*-test. Mares were classified based on endocrine profiles as cyclic, abnormal and acyclic. Mares were considered cyclic if they had an increase in urinary estrogens prior to increased urinary progestagens and discernible inter-estrus intervals. Abnormal mares exhibited cyclic patterns that were erratic with no regular inter-estrus pattern. Acyclic mares had little to no urinary hormonal activity through the breeding season (March – August). Data between groups of mares (mean urinary estrogens and progestagens) were then compared using Kruskal-Wallis one-way ANOVA and a multiple comparisons Dunn's test.

To determine whether independent factors influenced estrous cyclicity (as assessed using urinary estrogen and progestagen levels), linear regression was used to determine whether the independent variables age, mean kinship, or number of foals produced had an impact on levels of urinary estrogen or progestagen metabolites. All analyses were conducted with SAS (version 9.2, Cary, NC, USA).

**Follicular-Endocrine Comparison:**

Endocrine data for intervals time-matched with ultrasound examinations were analyzed as described above with slight modification. For cycling mares ($n = 5$), three-day rolling means were calculated for urinary estrogen and progestagen and these values were compared to corresponding observations of ovarian structures observed by ultrasound. Likewise, three-day rolling means of follicular size (mm) were calculated for ultrasound data. Day 0 was defined as day of detected ovulation. Data were presented as mean ± SEM and graphed longitudinally using Delta Graph (Red Rock Software, Inc., Salt Lake City, Utah, USA). Because numbers of normal cycling mares were small, many characteristics were recorded descriptively.

## RESULTS

### *Endocrine Data*

Baseline endocrine profiles for the Przewalski's mares (number of mares = 14; n = 1,064 urine samples) were analyzed from March to September for one breeding season during which no rectal palpations were performed. Based on these profiles, mares were grouped into three categories: cyclic (number of mares = 7; n = 518 samples), abnormal (number of mares = 3; n = 228 samples) and acyclic (number of mares =4; n = 304 samples). Cyclic mares exhibited an obvious estrogen peak preceding an increase in urinary progestagen (Figure 2.3), and exhibited regular intervals between the two steroid peaks (peak estrogen interval: 25.2 ± 1.2 days; peak progestagen interval: 25.1 ± 1.2 days). Hormone profiles for mares that exhibited abnormal estrous cycles are depicted in Figure 2.4. Two of the three mares (Fig. 2.4a, b) showed no obvious rise in progestagen excretion after the estrogen peak. The peri-ovulatory estrogen peak was absent in the third mare, although a brief increase in progestagen excretion was observed (Fig. 2.4c). For the acyclic mares (n = 4, Fig. 2.5), there were no discernable trends in urinary hormone excretion, and steroid metabolite concentrations (both estrogens and progestagens) were baseline in 90% of the samples evaluated.

In mares with normal endocrine cycles, mean urinary progetagen metabolites increased 7.1 ± 0.7 days after the first detectable rise in mean urinary estrogen and 4.9 ± 0.9 days after the mean estrogen peak. The interval during which urinary

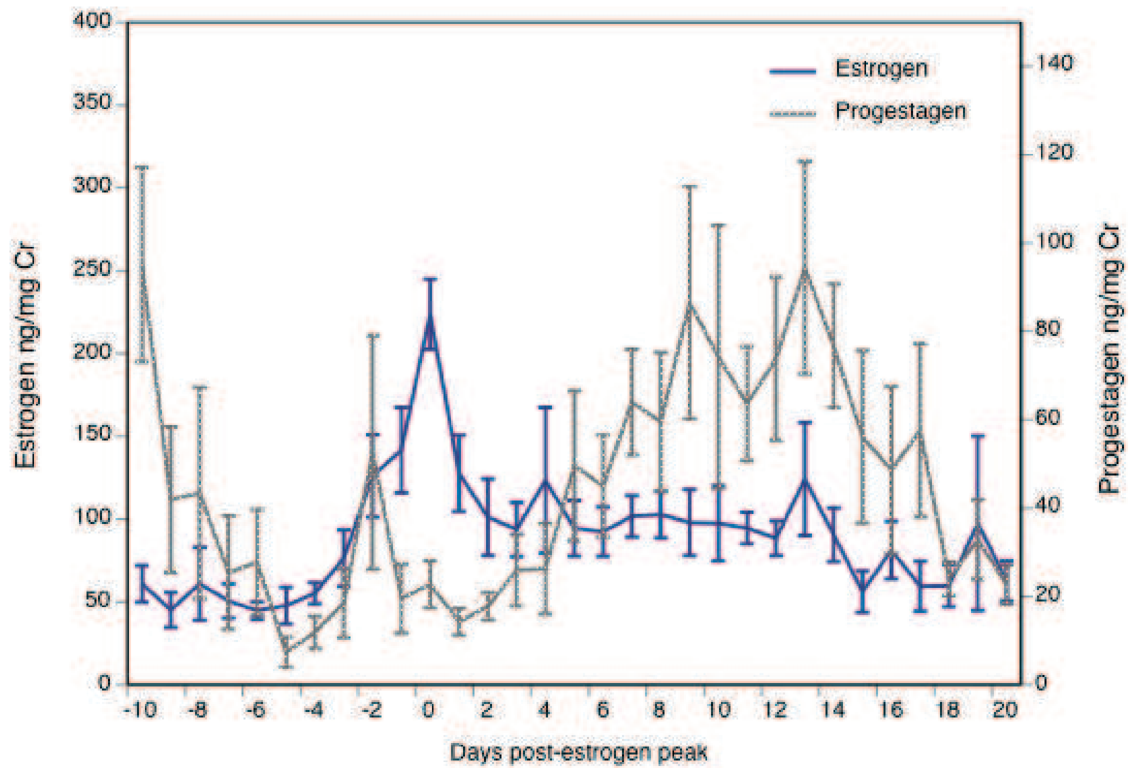



**Figure 2.3:** Mean urinary hormone metabolites (estrogens and progestagens) from Przewalski's mares (n = 7 mares; n = 518 urine samples). Data are shown as mean ± SEM. Data were aligned in relation to peak urinary estrogen (Day 0).

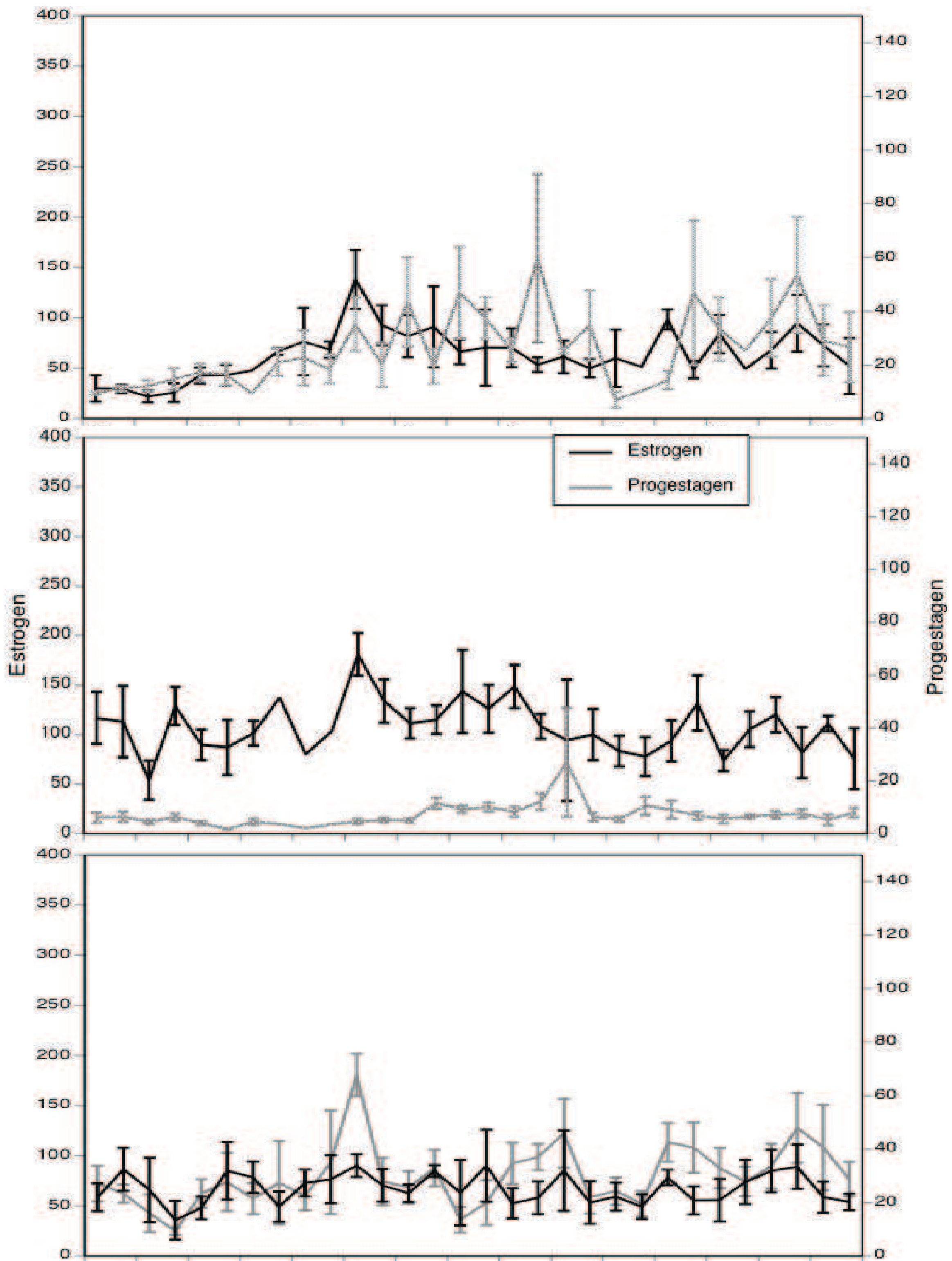



**Figure 2.4 a., b, and c**: Przewalski's mares (n = 3 mares; n = 228 urine samples) showing mean longitudinal profiles for n = 3 cycles. Error bars depict SEM.

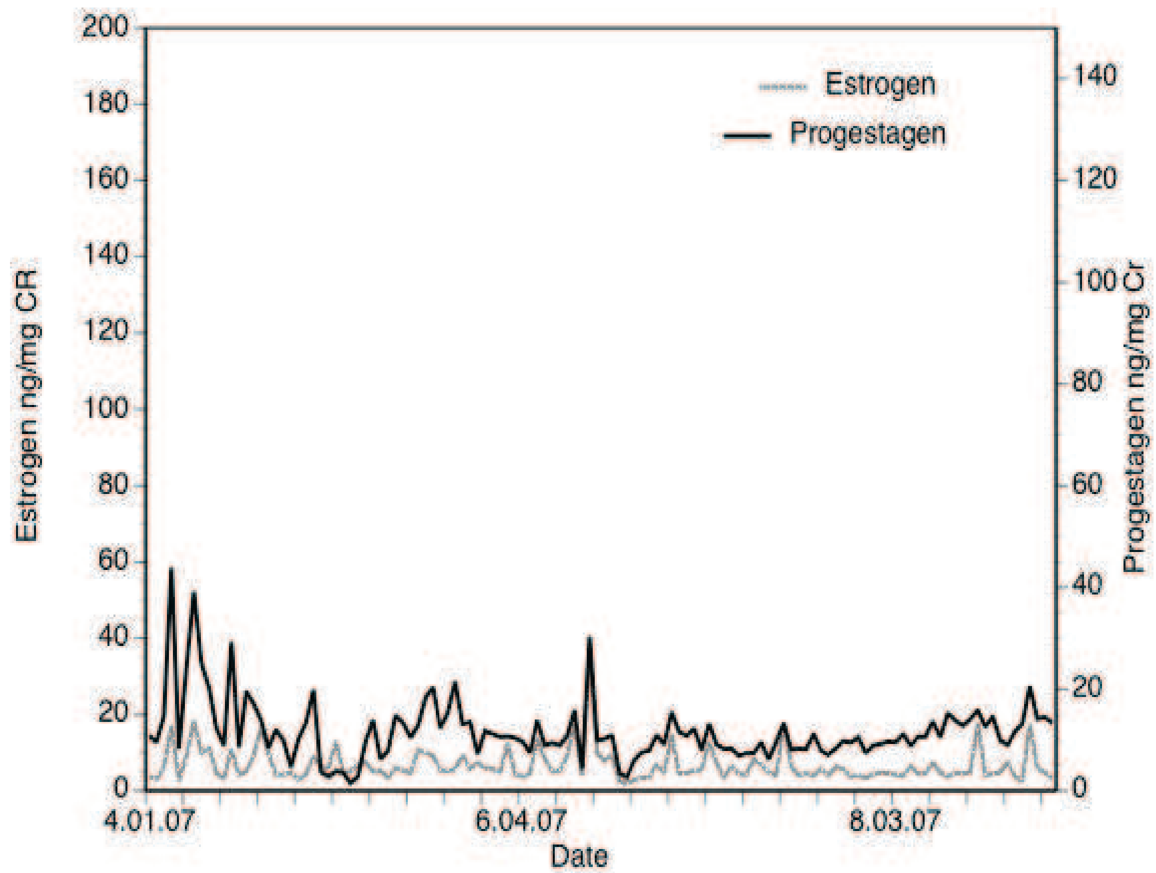



**Figure 2.5:** Representative longitudinal urinary hormone data for acyclic Przewalski's mare (n = 1mare; n = 304 urine samples).

progestagen was elevated was consistent among normal mares (range, 9.3 – 13.3 days), whereas there was considerable variation in duration of time that urinary estrogen remained elevated above baseline (range: 9 – 18 days). It was also noted that urinary estrogen was increased in three mares (3/7, 42%) coincident with periods during which urinary progestagen was also elevated.

Mean urinary estrogen and progestagen concentrations (Fig. 2.6) among cyclic, abnormal and acyclic mares were different ($P < 0.05$). Urinary estrogen and progestagen concentrations in cyclic mares were elevated (100.8 ± 4.1 and 44.7 ± 2.2 ng/mg Cr, respectively) compared to abnormal (77.7 ± 3.0 and 41.9 ± 3.3 ng/mg Cr, respectively) and acyclic mares (14.4 ± 1.2 and 11.7 ± 0.5 ng/mg Cr, respectively). Likewise, urinary estrogen and progestagen concentrations in abnormal mares exceeded ($P < 0.05$) those observed in acyclic females.

Based on linear regression analysis, mean kinship had a significant effect ($r^2 = 0.476$; $P < 0.05$) on mean levels of urinary estrogen metabolites in all mares analyzed (n = 13 mares). However, no other independent variables showed an effect on levels of either urinary estrogen or progestagen excretion.

*Ultrasound Data:*

A total of 268 ultrasound examinations were performed on nine Przewalski's mares in 2006 and 2008. Based on ultrasound data, mares were also classified into three groups: Cyclic, Abnormal and Acyclic. Cyclic mares had follicular development with regular patterns and depicted a normal ovulation. Abnormal mares

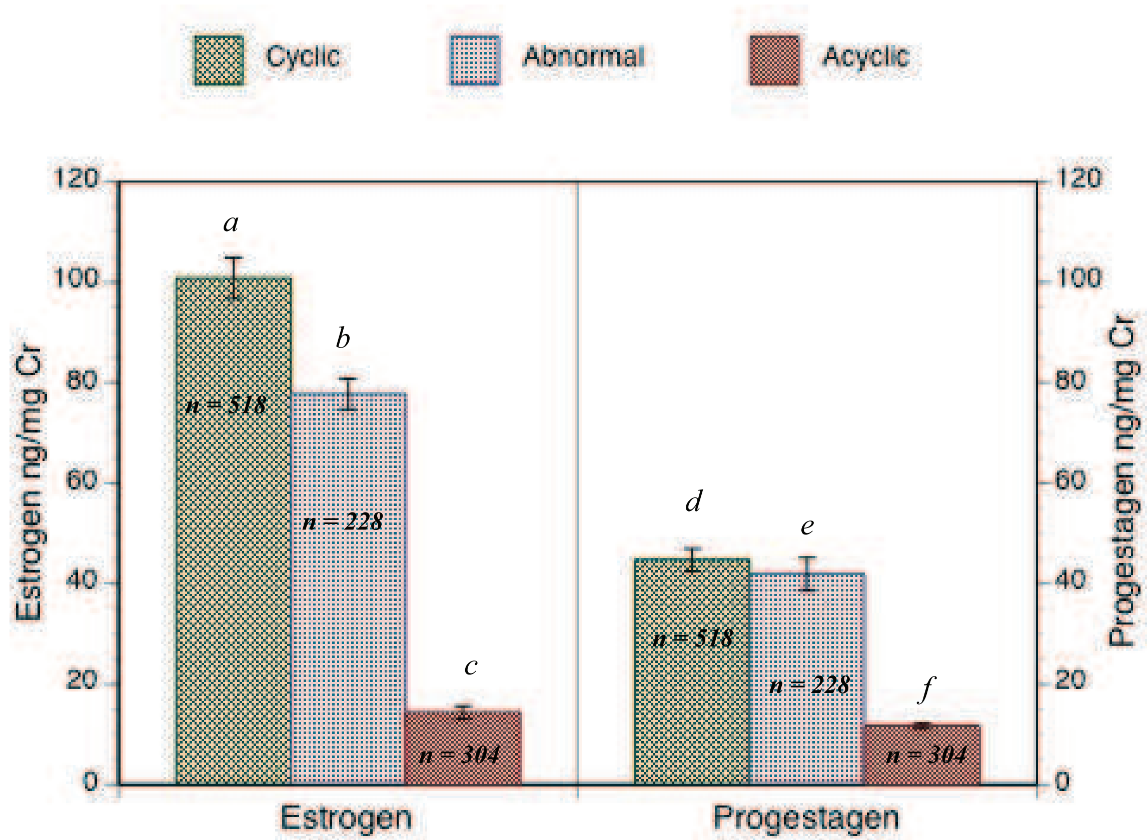



**Figure 2.6:** Bar chart showing the mean ± SEM of mares based or normal, abnormal, or acyclic endocrine profiles. Letters significant difference between groups ($P < 0.05$). Number of urine samples for each group are shown.

would also show follicular development; however, these resulted in the development of hemorrhagic follicles. Acyclic females showed no follicular development (4/9, 44.4%; n = 120 palpations) or ovulations. Regular ovarian cycle patterns were detected in four mares with normal follicular development; one had cyclic patterns with abnormal structures (1/9, 11.1%), one exhibited a normal cycle in one study period and was abnormal in the other period (1/9, 11.1%), and three mares (3/9, 33%, n = 12 palpations) experienced no follicular activity throughout the study period. Ovaries were noticeably smaller in acyclic mares (20 x 10 mm) with no detectable follicular structures and small flaccid uteri (tone 0/3). Normal mare ovaries varied in size, depending on follicular activity but ranged in from 50 - 60 mm x 25 - 40 mm. Ovaries in abnormal mares were comparable in size to normal mares.

Urinary estrogen concentrations increased with increased follicular diameter (Figure 2.7). In the nine cycles analyzed, 2 (2/9, 22%) of the mares had double ovulations: one synchronous and another asynchronous. Among all mares, a urinary estrogen peak was detected during the interval extending from two days before to four days after ovulation (mean, -0.4 ± 0.6 days), and urinary progestagen peaked from zero to six days after detected ovulation (mean, 1.9 ± 0.8 days). Inter-ovulatory cycles were detected in three instances whereby mares exhibited two separate ovulations across a five week interval. In these instances the ovulation-to-ovulation interval was 22.2 ± 0.9 days (range, 21 – 24 days). Average follicle size at ovulation amongst all mares was 40.4 ± 89.4 mm (range: 31 - 48 mm; n = 14 observations). Dominant follicles, defined as a follicle > 30 mm diameter in the absence of a CL, grew at a rate of 1.2 ± 0.6 mm/day (range: 0.5 to 2.7 mm) over a period of 9.2 ± 0.4

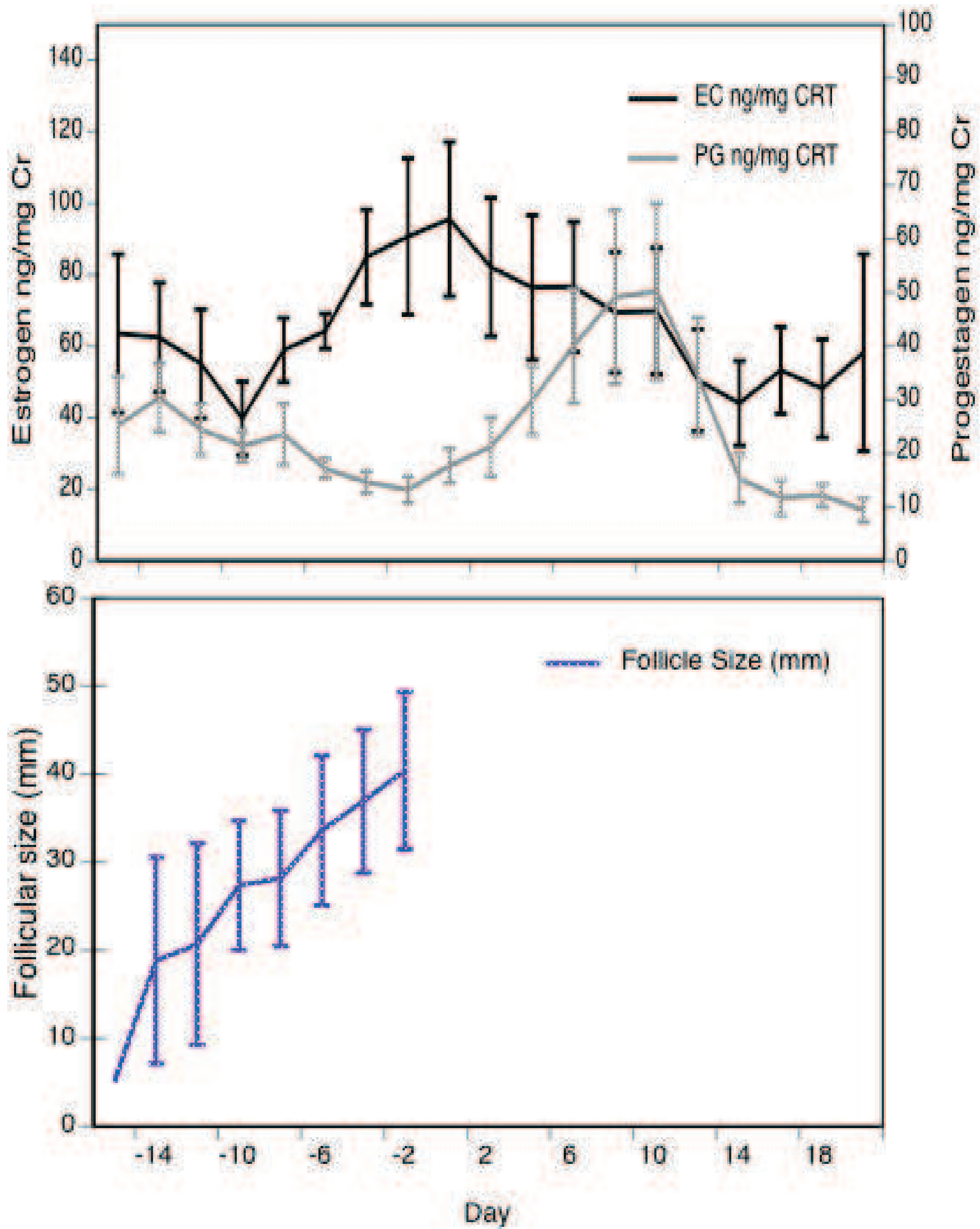



Figure 2.7: Changes in urinary estrogen metabolites in relation to follicular size in Przewalski's mares (n = 4 mares). Upper figure shows urinary estrogen metabolites (Mean ± SEM) in relation to ovulation day (Day 0). Lower figure shows follicular size (Mean ± SEM) in relation to ovulation.

days (range: 8 to 11 days) before reaching ovulation. The duration of a detectable CL on the ovary by rectal ultrasound was 12.2 ± 0.87 days.

There was no difference ($P > 0.05$) between the number of days a CL was detected by ultrasound and the number of days during which urinary progestagen was increased. There also was no significant difference ($P > 0.05$) between inter - estrous interval estimates using either hormones (i.e., urinary estrogen or progestagen peaks) or ovulation detection by ultrasonography.

**DISCUSSION**

This was the first study to combine assessments of reproductive steroids with direct observations of follicular dynamics to classify ovarian cycles in the Przewalski's horse. Results indicated that 50% of mares either exhibited abnormal estrous cycles or were acyclic. Of major signficance, we determined that abnormal cycles and acyclicity appear to be linked to loss of genetic diversity in this species. Longitudinal patterns of urinary estrogen excretion were previously assessed to establish that the Przewalski's mare is a seasonally polyestrous breeder (Monfort, *et al.,* 1991, Zimmermann *et al.,* 1985). Our additional longitudinal assessments of urinary progesterone excretion and serial ultrasonography revealed new insights into the estrous cycle of the Przewalski's mare. In particular, behavioral pre-conditioning for manual restraint permitted serial ultrasonography in unsedated mares, which provide a more comprehensive assessment of the ovarian cycle.

The inter-estrous interval estimates derived from this study using all three parameters (i.e., urinary estrogens, progestagens and rectal ultrasound) was similar to what has been previously reported (Monfort *et al.,* 1991). Likewise, the estrous cycle length averaged 25.2, 25.1 and 22.2 days based on urinary estrogens, progestagens and ovulation detection, resepectively, which was similar to the 24.1-day cycle length reported previously (Monfort *et al.* 1991). These estimates slightly exceed those reported for the domestic mare, which typically lasts 21 - 22 days (range: 18 – 24 days) (Blanchard *et al.,* 2003), although many pony breeds exhibit estrous cycles in the range of 24 – 26 days (Ginther, 1992). The variable range in domestic mares is often attributed to the length in estrus, whereas diestrus appears to remain relatively constant at approximately 14 days (Ginther, 1992). Similar to the domestic horse, the period of increased urinary progestagen metabolites was fairly constant in Przewalski's mares (9 – 13 days), whereas the period of increased urinary estrogen metabolites was quite variable (9 – 18 days).

In normal cycling mares, urinary progestagens increased 7.1 ± 0.7 days after an increase in urinary estrogens. Despite the limited numbers of mares assessed, there was little variation among individuals (range, 6.8 – 8.8 days). Ultrasound data were consistent with the estimates derived from endocrine assessments and the interval from the first significant increase in estrogen to ovulation was 6.4 ± 1.3 days. These data are especially important because they validate the use of urinary estrogen monitoring as a tool for pinpointing the most appropriate time for performing artificial insemination, a process that has been used successfully in other wildlife

species such as the Giant Panda (*Aiuropoda melanoleuca;* Huang *et al.,* 2002) and the bottle nose dolphin (*Lagenorhynchus obliquidens;* Roebeck *et al.,* 2005).

In a substantial number of mares (2/7, 28.6% with hormone monitoring only; 4/5, 80% with hormone monitoring plus ultrasound), urinary estrogen metabolites were increased coincident with periods of elevated progestagen excretion. For the subset subjected to ultrasound examination, a secondary wave of follicular growth was confirmed in the presence of a CL. These findings are similar to those previously found by Monfort *et al.,* (1991) where a secondary rise in urinary estrogens occurred four to eight days post peak in urinary estrogens. In the domestic mare, urinary estrogens also increase four to seven days post-ovulation (Ginther, 1992). It is believed that the secondary increase in estrogens observed in the domestic horse may reflect estrogen produced by a secondary follicular wave during the post-ovulatory interval.

Based on rectal ultrasound, the growth rate of follicles was less than that previously observed for this species (three to five mm/day; Durrant *et al.*, 1986) and also less than the domestic horse, in which follicles increase three mm per day from day -5 to day -2 and remain static until 12 hours pre-ovulation (Ginther *et al.,* 2004). In the present study, we found that follicles increased 1.2 ± 0.2 mm/day (range, 0.66 – 2.67 mm per day). For the final two to four days before ovulation, follicle size actually decreased in 33% (3/9) of mares, whereas size increased in 55% (5/9) of the cases. We speculate that the differences observed here relative to previous results (Durrant *et al*., 1986) may relate to the number of mares studies (five vs. two mares) and the fact that the current study included more frequent examinations (19 – 29

examinations for each mare in a five week period vs. seven examinations for each mare in a three month period). Nevertheless, the overall sample size for both studies is small, and additional study is needed to clarify the time course and dynamics of follicular growth in this endangered species.

In most species, a dominant follicle produces estrogen under stimulation of follicle stimulating hormone (FSH) and LH (Senger, 2003). In many ungulate species, such as the cow, ewe and sow, the period of estrus is short (15, 30, and 50 hours; Senger, 2003). However, in the domestic horse, estrogens increase over a period of at least five days coincident with follicular growth (Ginther, 1993; Ginther *et al.,* 2003). In the present study, urinary estrogens were elevated (7.8 ± 0.6 days prior to ovulation) in parallel with follicular diameter, which suggests that the follicular-endocrine dynamics in the Przewalski's mare are similar to the domestic horse.

Like the domestic horse (Ginther, 1986), ultrasound examinations indicated that ovulations in the Przewalski's mare were mono-ovulatory (7/9, 77.8%), whereas a minority of mares (2/9, 22.2%) exhibited double ovulations. While the sample size is small, 22.2% double ovulations would be considered high in the domestic horse, and is similar to the percentage observed in Thoroughbred mares (15 – 25%; Blanchard *et al.,* 2004). In this study, a synchronous double ovulation was observed in a single mare, with ovulation synchrony of < 1 day. Another mare exhibited an asynchronous double ovulation, with the second ovulation occurring 2 days after the first. Interestingly, elevated urinary estrogens excretion persisted until after the second ovulation. There are many factors that could be responsible for double ovulations in

mares, including increased sensitivity to gonadotrophins, genetics, age and season (Ginther, 1992). In the present study, both mares were over the age of 15 years, and this finding provides incentive for further exploring the relationship between age and ovarian function.

Our analyses revealed that urinary estrogen excretion peaked -0.37 ± 0.59 days from ovulation, although there was substantial among-mare variation (-3 - +4 days). By comparison, serum estrogen in the domestic mare peaked two days before ovulation, but among-animal variation was also reported to be high (Koskinen *et al.,* 1989). Species differences in timing of the pre-ovulatory estrogen peak may be an artifact of the time-lag (>24 hours in the horse) between the secretion of estrogen in blood circulation and the excretion of its metabolites in voided urine (Lasley and Kirkpatrick, 1991). Urinary progestagens increased 2.2 ± 1.9 days post urinary estrogen peak, but among-animal variation was substantial (range, zero to six days post-ovulation). Understanding timing of ovulation in relation to urinary estrogen peak could provide important information for attempting breeding by AI, especially in institutions that do not have handling facilities for large ungulates. Further studies need to be conducted on normal cycling mares to further confirm timing between urinary estrogen peak and ovulation in order to determine whether timed AI in relation to peak urinary estrogens is feasible.

This is the first study to demonstrate that an increase in mean kinship influences cyclicity in Przewalski's mares. Estrogen production was directly influenced by mean kinship of Przewalski's mares. Since estrogen is an indication of follicular development, this is an indication that inbreeding has an impact on reproduction in

this species. While the SSP recommends breeding based on mean kinship, these results indicate that continued genetic management is important in this population. While we were unable to look at founder effects on reproduction due to limited numbers, it would be important to see if particular founders have an impact on fertility. This is important because the SSP determines breeding pairs based on mean kinship, which only reflects heterozygosity, not possible deleterious genes from a founder line that may be impacting fertility. Future studies should assess whether females that are acyclic may have common founders to determine whether these animals should be represented less in the population.

Detailed descriptions of follicular and endocrine traits in Przewalski's mares studied across two locations revealed that a high proportion of mares exhibited abnormal ovarian cycles. Strikingly, mean kinship appeared to have a significant negative effect on ovarian function (i.e., hormone and ovarian morphology). These results should be a "wake up" call for the zoological community, which has only recently begun to come to grips with the fact that many of their managed breeding programs are unsustainable with current management and breeding strategies (Lees and Wickens, 2009). While Przewalski's horse reintroductions to-date have been successful, recent die-offs in the Gobi B Takhi Project in Mongolia reinforce the continued importance of captive horse populations as vital insurance against unanticipated catastrophes, and as a continuing source of animals for future reintroduction. However, zoo-based managed breeding programs are at risk, in part, because of failure to achieve their management goals. In a recent analysis of 87 managed populations, only 48% were capable of sustaining current population

numbers and only 55% were retaining gene diversity at or above 90% of founder stock (Lees and Wickens, 2009). The Przewalski's horse SSP is already depleted (i.e., 78% gene diversity), and concerted efforts are necessary to prevent further losses of gene diversity. We contend that there is a need for much more basic science to understand the relationships between gene diversity, age and fecundity in small populations. Our results clearly demonstrate it is possible to gain an improved understanding of reproductive mechanisms using existing, well-estabished tools such as noninvasive endocrine monitoring and ultrasonography. Equally important, however, is the need for specialized facilities and expertise in animal husbandry, management and behavioral conditioning that are dedicated to conducting science on some of the most under-studied, and endangered animals on the planet.

# CHAPTER THREE

## Manipulation of the Estrous Cycle in Przewalski's Mares

### INTRODUCTION

The Przewalski's horse (*Equus ferus przewalski*) is a wild horse that is native to Central Asia, which is now considered critically endangered (Boyd *et al.*, 2008). Because no new wild founders exist, genetic management of the *ex situ* population is critical for long-term survival of the species. Przewalski's horses are large mammals and many zoological parks lack sufficient space for appropriate breeding and genetic management, especially management of bachelor bands. As a result, these equids exist in small fragmented populations that are vulnerable to losses of genetic diversity, especially since it has been shown that Species Survival Plans (SSPs) do not always effectively manage captive populations due to failure of institutions to follow through with breeding recommendations (Lees and Wicken, 2009). Successful application of assisted reproductive technologies, especially artificial insemination (AI), has been shown to enhance breeding management of *ex situ* wildlife populations (Holt *et al.,* 1998; Wildt *et al.,* 1992; Pukazhenthi and Wildt, 2004). However, early studies in ungulates and great cats have shown that AI technology developed for domestic species is of little value for many wildlife species (Pukazhenthi and

Wildt, 2004). Developing AI as a routine tool for managing genetic diversity of *ex situ* Przewalski's horse populations requires pre-emptive research designed to understand the reproductive physiology of this species, including how to regulate ovarian activity.

In species such as the Przewalski's horse, AI requires anesthesia of stallions to collect semen, which carries risks for the animal as well as the personnel conducting the procedure. Additionally, the success of estrous synchronization and timed AI is enhanced when multiple mares can be managed in small enclosures connected to facilities that permit animals to be readily shifted and safely restrained for insemination. Timed AI has been developed in many ungulate species, such as the scimitar horned oryx (Morrow *et al.,* 2000), yaks (Zi *et al.,* 2006), dolphin (Robeck *et al.,* 2009) and the Indian blackbuck (Sontakke *et al.,* 2009). For many of these species, manipulation of the estrous cycle has been adapted from exogenous hormone treatments that were first applied to domestic species such as the cow, sow and mare. In the domestic mare, reproductive cycle management has been challenging, largely due to the long and variable follicular phase (2-18 days), which is also modulated by seasonal effects (Bergfelt, 2000; Ginther, 1993). In normally cycling mares, timing of estrus can be manipulated by shortening or lengthening the luteal phase of the cycle using several exogenous hormone treatments, including prostaglandins ($PGF_2\alpha$), progesterone (synthetic and natural forms) and estrogen, either alone or in various combinations (Bristol, 1993; Bergfelt, 2000). $PGF_2\alpha$ is very effective at shortening the luteal phase of the estrous cycle; however, it has very limited efficacy when used alone, particularly early (zero to five days post-ovulation) in the lifespan of the corpus luteum (CL, Bergfelt, 2000; Bristol; 1993). Single injections yield wide variation in the number of days from hormone administration to

detected estrus (5.1 ± 0.3 to 9.0 ± 0.7 days) depending on the size of follicle at time of injection (Loy *et al.,* 1979; Hughes and Loy, 1978).

Exogenous progesterone therapy has been used to manipulate timing of estrus in the mare (Bergfelt, 2000; Senger, 2003). Progesterone suppresses luteinizing hormone (LH) secretion from the pituitary gland, which in turn prevents final maturation and ovulation of dominant follicles (Bergfelt, 2000; Senger, 2003). Because of this mechanism, the response of mares to progestagens, in theory, is not affected by stage of the estrous cycle (Bristol, 1993). Progesterone is usually administered for 15 – 18 days, which artificially prolongs the luteal phase, thereby permitting additional time for the natural regression of any existing CLs while preventing subsequent ovulations (Bristol, 1993; Senger, 2003). The disadvantage of native progesterone is that it must be administered via daily intramuscular injections, and its use does not result in adequate synchronization of estrus (Holtan *et al.,* 1977). For example, the treatment of 21 pony mares with progesterone (18 consecutive daily injections) and human chorionic gonadotropin (hCG, administered 6 days after the final progesterone injection) resulted in a 52.4% response rate.

Because the use of injectable progesterone is labor intensive and resulted in poor estrous response, synthetic progestagens, including altrenogest and melegesterol acetate (MGA) have been developed and utilized with more promising results (Bristol, 1993; Bergfelt, 2000). Estrous synchronization can be achieved through the oral administration of these progestagens for as few as 14 days in the domestic mare. Estrus and ovulation in nine mares subjected to a 15-day oral altrenogest treatment regimen occurred three to six days and eight to fifteen days after treatment onset, respectively (Squires *et al.,* 1979; 1983). In the domestic mare, exogenous progestagens have little effect on follicle stimulating hormone

(FSH) secretion and the growth of follicles that exist before hormone treatment onset (Squires *et al.,* 1983). Therefore, among-mare differences in follicle size at the time of progestagen-treatment cessation are substantial, which results in wide variation in the interval of time from progestagen withdrawal to ovulation. Initial studies highlighted the positive benefits of altrenogest (Squires *et al.,* 1979; 1983; Turner *et al.,* 1981), but subsequent research demonstrated that altrenogest used alone did not consistently prevent ovulation (and CL formation) during the treatment period, which resulted in unacceptable variability in the interval from treatment cessation to ovulation (Lofstedt and Patel, 1989). As a result, $PGF_2\alpha$ administered at the end of the progestagen treatment interval, is now routinely used to regress any CL that may have developed during alternogest treatment (Bergfelt, 2000).

In the domestic mare, LH increases over three to five days during the peri-estrus interval, and typically peaks one to two days post-ovulation. Thus, estrus is prolonged in the mare relative to other species, which complicates efforts to pinpoint ovulation (Palmer, 1978; Senger, 2003; Gastal *et al.,* 2000; Ginther, 1992). Ovulatory agents are used in mares to enhance the predictability of ovulation timing, especially when AI is used. Currently, there are three commercially available ovulatory agents: hCG, Deslorelin (GnRH analogue), and recombinant equine LH (Samper, 2008). Ovulation occurs in mares 24 – 48 hours post-hCG, which is a protein that exhibits LH activity (Blanchard *et al.,* 2004; Samper, 2000). Because mares can develop antibodies to hCG, it is undesirable for repeated use in the same mare (Barbacinni *et al.,* 2000). Deslorelin is available in two forms: a biodegradable implant and a compounded liquid form (Samper, 2008). The implant form is very effective for inducing ovulation within 38 – 42 hours of administration. However, unless implants are removed, some mares experience delayed return to estrus due to the continued release of GnRH, which

results in negative feedback on the anterior pituitary (McCue *et al.,* 2002). The compounded biorelease form of deslorelin (Betpharm, Inc., Lexington, KY, USA) reliably induces ovulation within 40 – 60 hours of treatment without any evidence of delayed return to estrus (Samper, 2008, Burns *et al.,* 2006). Recombinant eLH is a single chain gonadotrophin that is a smaller protein than hCG. While eLH has only recently been available for ovulation induction in the mare, its efficacy has been comparable to hCG (Yoom *et al.,* 2007). In Przewalski's mares, it has been shown that hCG was not effective for inducing ovulation within 24 hours of injection (Durrant *et al.,* 1986). However, because there were limited ultrasound examinations, ovulation may have been missed or hCG may have been given at a suboptimal time in the estrous cycle.

To date, manipulation of the estrous cycle with hormone therapy has not been attempted in the Przewalski's horse. Similar to the domestic mare, the Przewalski's horse exhibits similar reproductive-endocrine patterns, including seasonally polyestrous cycles that last 24 – 25 days (Monfort *et al.,* 1991). The objectives of this study are to assess the efficacy of estrus induction using: a) the combination of oral alternogest and $PGF_2\alpha$; b) injectable altrenogest; and c) to determine whether ovulatory agents, deslorelin and eLH reliably induce ovulation in Przewalski's mares.

## MATERIALS AND METHODS

### *Animals*

Eleven Przewalski's mares from two institutions were used in this study. Three females (7 – 20 years of age) were housed at the Wilds near Cumberland, Ohio (39.82° N, 81.75° W). Eight mares (2 – 25 years of age) were housed at Smithsonian Conservation

Biology Institute (SCBI; 38.88° N, 78.17° W) near Front Royal, Virginia. All animals were housed in small groups (two to six animals per group) on dirt lots with access to barns or shelter. Animals had access to fresh water, good quality hay, and mineral blocks throughout the study period.

*Urine Collection:*

Urine samples were collected three to seven days per week using the technique described by Monfort, *et al.* (1991). Briefly, freshly voided urine was aspirated with a clean syringe and kept cool (4 to 6°C) until all samples were collected from all mares. Samples were then placed in labeled glass tubes and centrifuged at 1500 G for 15 minutes to remove sediment and dirt. Urine supernatant was then stored in plastic tubes at – 20 °C until analysis was completed. All samples (n = 965) were collected in the morning (0700 – 1100 h).

*Enzyme Immunoassay*

**Creatinine**

Creatinine is secreted in urine and is an indication of glomerular filtration rate, therefore, to normalize all urinary samples based on dilution, all samples were indexed for creatinine (Cr) (Taussky, 1954). For Cr determinations, raw urine was diluted to a 1:50 concentration in BSA – free phosphate buffer. Diluted samples were added to 96-well, flat bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA, USA), combined with 0.05 mL each of $DH_2O$, 0.4 N picric acid, 0.75 N NaOH and incubated at room temperature (25°C) for 30 min. Optical density (OD) was measured at 490 nm (reference 620 nm) using a microplate reader (Dynex MRX; Dynex Technologies, Chantilly, VA, USA). Urine were run in duplicate and compared

to reference Cr standards (0.00625 – 0.1 ng/mL, Sigma Aldrich, St. Louis, MO, USA). Samples that had concentrations < 0.1 ng Cr/mL were considered too dilute and were run at a higher concentration (1:10 or 1:25). Samples that were < 0.1ng/mL Cr at a 1:10 dilution were discarded (2% of samples). Hormone mass in a urine sample was divided by Cr concentration and expressed as mass of hormone/mg Cr (ng/mg Cr).

**Estrogen Conjugate EIA***:*

Urine samples diluted (1:5 – 1:2000) in phosphate buffer were assayed in duplicate using the protocol outlined by Munro *et al.* (1991). The antiserum (R522, Coralie Munro, University of California, Davis, CA, USA) cross-reacts with estrone-3-glucoronide (100%), estradiol-3-sulfate (66.6%), estrone (238%), estradiol-17β (7.8%), estradiol-3-glucoronide (3.8%) and estradiol-3-sulfate (3.3%). The inter assay coefficient of variation (CV) for the two internal controls was 11.49% (mean binding, 30.33%) and 7.14% (mean binding, 65.59%) and intra-assy CV was < 10%. Serially diluted urine samples demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to urine ($y = 0.82x + 1.07$, $r = 0.99$) demonstrated significant recovery ($P < 0.05$).

**Progestagen EIA:**

Urine samples diluted (1:10 – 1:400) in phosphate buffer were analyzed in duplicate using the protocol described by Graham *et al.* (2001). The antiserum (CL425, Coralie Munro, University of California, Davis, CA, USA) cross-reacts with 4-pregnen-3,20-dione (100%), 4-pregnen-3α-ol-20-one (188%), 4-pregnen-3β-ol-20-one (172%), 4-pregnen-11α-ol-3,20dione

(147%), 5α-pregnan-3β-ol-20-one (94%), 5α-pregnan-3β,20-dione (64%), 5α-pregnan-3,20-dione (55%), 5β-pregnan-3β-ol-20-one (12.5%), 5-pregnan-3,20-dione (8%), 4-pregnen-11β-ol-3,20-dione (2.7%), and 5β-pregnan-3α-ol-20-one (2.5%) (Graham *et al.*, 2001). Sensitivity of the assay at maximum binding is 0.78 pg per well. Sensitivity of the assay at maximum binding is 0.78 pg per well. The inter-assay CV for two internal controls was 12.99% (mean binding, 30.32%) and 6.95% (mean binding, 72.59%) and intra-assay CV was < 10%. Serially diluted urine samples demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to urine ($y = 0.99x – 0.48$, $r = 0.99$) demonstrated significant recovery ($P < 0.05$).

*Ultrasound Examination*

Transrectal ultrasound was performed three days per week (SCBI: n = 8 mares; the Wilds: n = 3 mares) using a portable B-mode ultrasound (SCBI: Sonovet 2000, Medison America, Inc, Cypress, CA, USA; Wilds: Aloka 500, Aloka America, Walingford, CT, USA) equipped with a linear transducer (4 - 7 MHz). To decrease variability associated with time of year, mares held at SCBI and the Wilds were subjected to rectal ultrasound examinations during the same period by two researchers that each have a minimum of two years of experience performing rectal ultrasound on domestic horses. The studies were conducted during five week intervals from May to August 2007, May to July 2008 and May to June 2009. Briefly, mares were manipulated through a chute system and briefly restrained within a padded hydraulic squeeze apparatus (Fauna Research, Inc, Red Hook, NY, USA). Researchers evacuated feces from the mare’s rectum and quickly palpated the reproductive tract to gauge ovarian structures, uterine and cervical tone. The ultrasound examination was then performed by introducing a linear

transducer into the rectum, which permitted visualizing uterine edema, presence or absence of endometrial fluid and ovarian structures. Follicles were assessed and measured electronically. Other ovarian structures, such as ovulations, anovulatory follicles or CLs were also noted. To minimize stress, the total time during which the mare remained in the tamer was kept below five minutes.

*Study 1:*

Rectal ultrasound was used to assess follicular structures in cycling females (SCBI, n = 3 mares; the Wilds, n = 2 mares) three time per week during a three-week pre-treatment interval. Altrenogest (Regumate®: DPT Laboratories, San Antonio, TZ, USA; Dose: 0.044 mg/kg per os) mixed in sweet feed was then administered to all mares for a period of 14 days (O14; Figure 3.1). On day 12, all mares received i.m. injections of $PGF_2\alpha$ (12.5 μg cloprostenol, Betpharm, Inc, Lexington, KY, USA) to lyse any remaining luteal structures on the ovaries. On days 15 – 20, mares were examined daily by rectal ultrasonography. Follicular structures were measured and any ovulations were noted. Once a follicle $\geq$ 35mm was observed, one of three treatments was administered by intramuscular injection: Treatment 1, saline (1 ml); Treatment 2, recombinant eLH (0.75 mg reLH, Dr Jan Roser, University of California, Davis, CA, USA); and Treatment 3, GnRH analog (1.5 mg Biorelease deslorelin, BETPharm, Lexington, KY, USA). The number of days from treatment administration to ovulation was recorded. Mares were then given a two-week period of rest before the study was repeated. All mares were rotated through treatment regimens one to three times (SCBI: three treatment cycles; the Wilds: one treatment cycle),

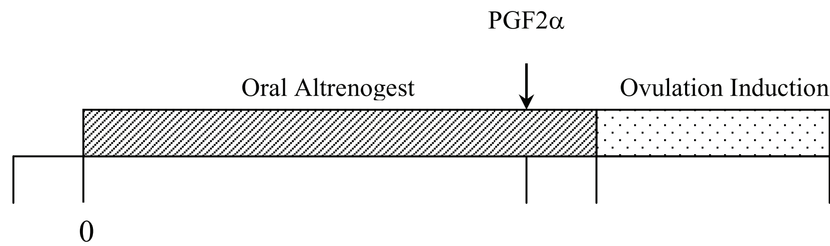



**Figure 3.1:** Schematic of hormone therapy in study 1. Altrenogest therapy was given for fourteen days. On day 12, PGF2α was injected. Mares were then checked for estrous follicle from days 15 – 21 and given an ovulatory agent (GnRH, reLH, saline).

totaling 11 treated cycles within the study period. Mares at SCBI received all treatments and, therefore served as their own controls. Urine samples were collected from all mares throughout the entire period (May – September 2007) and stored at -20 C until analyzed by EIA, as previously described.

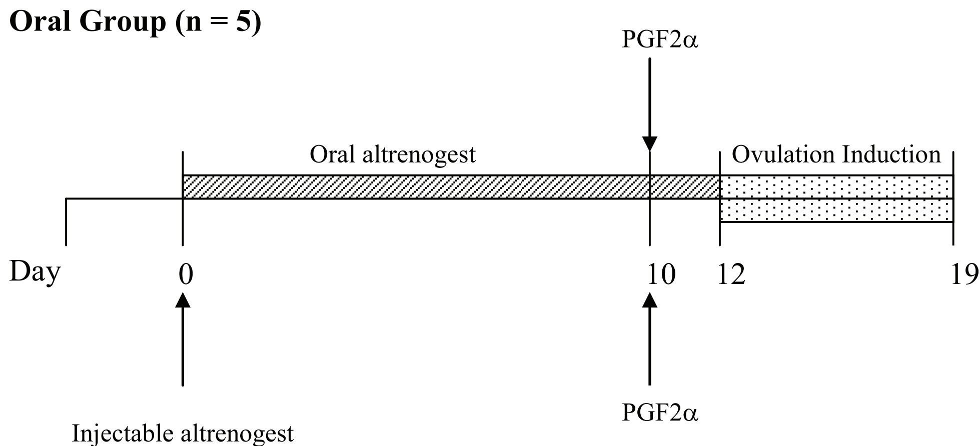
*Study 2:*

Cycling females (n = 10) at two institutions (SCBI, n = 8; the Wilds, n = 2) were used in this study. In 2008, all mares were examined three days per week by rectal ultrasound for follicular structures starting three weeks before treatment onset. In this study, two treatments were used on two groups of mares. Treatment 1 [O12 Treatment] consisted of oral Regumate (0.044 mg/Kg/day, DPT Laboratories, San Antonio, TX, USA) administered to five mares (SCBI, n = 3; the Wilds, n = 2) for 12 days. Treatment 2 [In12] consisted of a single

intramuscular injection of long-acting biorelease altrenogest (150 mg, Betpharm, Inc.) administered to five SCBI mares (Figure 3.2). On day 10 of treatment, all mares were injected with $PGF_2\alpha$ (12.5 μg cloprostenol, Betpharm, Inc). Mares were then examined daily by rectal ultrasound for follicular development or ovulation. When a follicle > 35mm was detected, a GnRH agonist (1.5 mg Biorelease deslorelin, BetPharm, Inc.) was given to induce ovulation. In 2009, mares were only examined by rectal ultrasound on days 13 – 20 after treatment by injectable altrenogest to minimize stress associated with handling. Once a 35 mm follicle was detected, GnRH was injected to induce ovulation. To compare the response to GnRH, data from mares treated with GnRH were compared to natural cycles from previous year where no treatment was given (n = 10 mares). Urine samples were collected throughout the study period (May to July 2008, 2009), which included a four-week pre-treatment interval. Samples were frozen and stored at -20 C until analyzed by EIA for urinary estrogen and progestagen metabolites, as described previously.

*Statistical Analysis:*

Baseline hormone values were determined using an iterative process (Moreira *et al.*, 2001). For urinary progestagen metabolites, values in excess of the mean ± 2.0 standard deviations (SD) were removed. The average was then recalculated, and the elimination process repeated until no values that exceeded the mean ± 2 SD remained. For urinary estrogen conjugate metabolites, values in excess of 2.5 SD of the mean baseline were removed until no values that exceeded 2.5 SD of the mean remained. Urinary estrogen conjugate metabolite values were considered elevated if concentrations were elevated above




**Injectable Group (n = 5) Year 1** **Year 2 (n = 5)**

**Figure 3.2:** Schematic of hormone therapy for study 2. Przewalski's mares received either oral altrenogest daily or one injection of biorelease altrenogest. Ovulation was induced by GnRH agonist, Deslorelin.

baseline for three days, whereas urinary progestagen metabolite values were considered elevated if they concentrations were elevated above baseline for four days.

Standard descriptive statistics including mean and standard error of mean (SEM) were used to summarize data. For comparative purposes, each study period was divided into three periods: pre-, peri-, and post-treatment. Pre- treatment was ten days before treatment onset; peri – treatment was the actual treatment period (12 to 14 days); and post – treatment was the ten day period after treatment cessation. Hormonal data among periods and treatments were analyzed using one-way analysis of variance (ANOVA) (GraphPad Prism, Version 5.03, La Jolla, CA, USA). Non-normally distributed data were interpreted using Kruskal - Wallis ANOVA. Differences in means among treatment periods were compared using Dunn's test. Student's t-test was used to compare differences in the timing of ovulation between treated and naturally-cycling mares. One-way ANOVA of mean urinary hormone concentrations

between treatment periods (pre-, peri-, and post-treatment) was used to evaluate the impact of initiating treatment during the follicular or luteal phase.

**RESULTS**

<u>*Study 1:*</u>

*Ovulation Induction*

When comparing three ovulatory agents, two mares received two treatments and one mare received all treatments. Mares at the Wilds only received one treatment (GnRH) and did not respond and were not assessed after one trial. There were no significant differences in the interval between the time of injection and ovulation among ovulatory agent treatment (i.e., GnRH and reLH) and control (Figure 3.3). For the one mare that received all treatments, the days for each treatment were: 1) reLH, 3 days; 2) GnRH, 4 days; and 3) saline, 5 days. The mare that received all treatments was also the only mare to ovulate normally with all treatments. The other mares formed luteinized follicles when treated with reLH (n = 1) and GnRH (n = 2).

*Endocrine Response:*

Because ovulatory agents had no effect on ovulation interval, data from all mares were combined to assess the efficacy of oral altrenogest therapy on estrous cycle management. Mares at SCBI (n = 3) received three treatments with altrenogest (n = 9 treated cycles) and mares at the Wilds (n = 2 treated cycles) received only one treatment of altrenogest (n = 2). Average ovarian steroid concentrations during the three treatment periods (pre, peri, and post) are shown in Figure 3.4. While there was no significant change in mean urinary

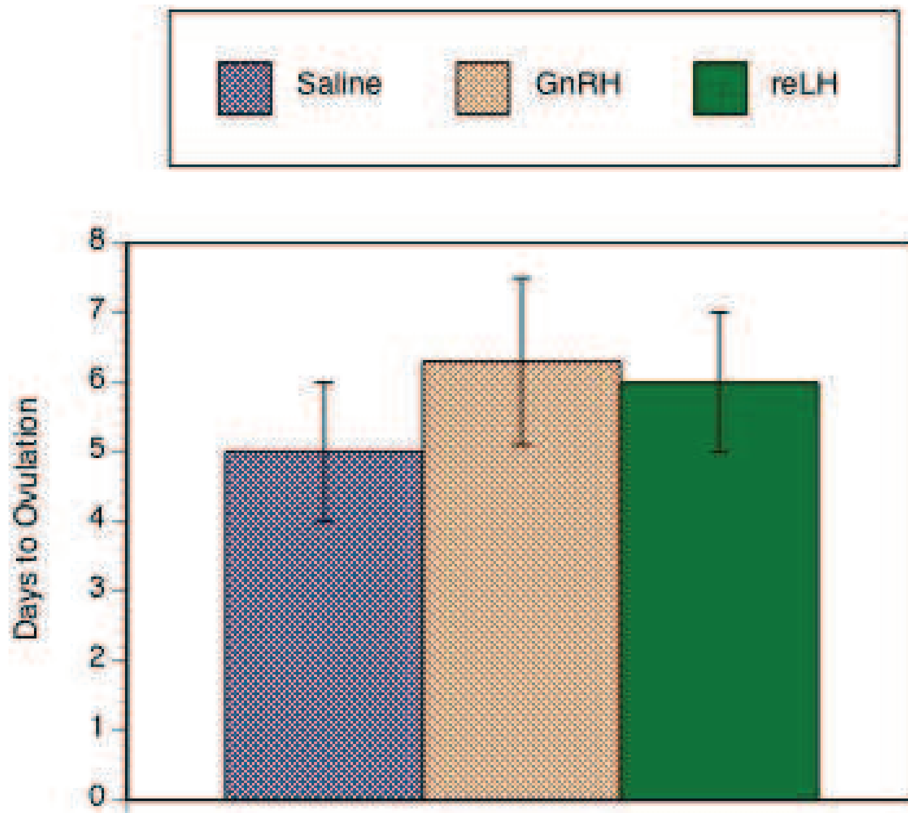



**Figure 3.3:** Days from treatment to ovulation in Przewalski's mares (n = 5) treated with saline (n = 2); GnRH (n = 5) and reLH (n = 2). Data are shown as means and error bars depict SEM. There was no significant difference detected between treatments ($P > 0.05$).

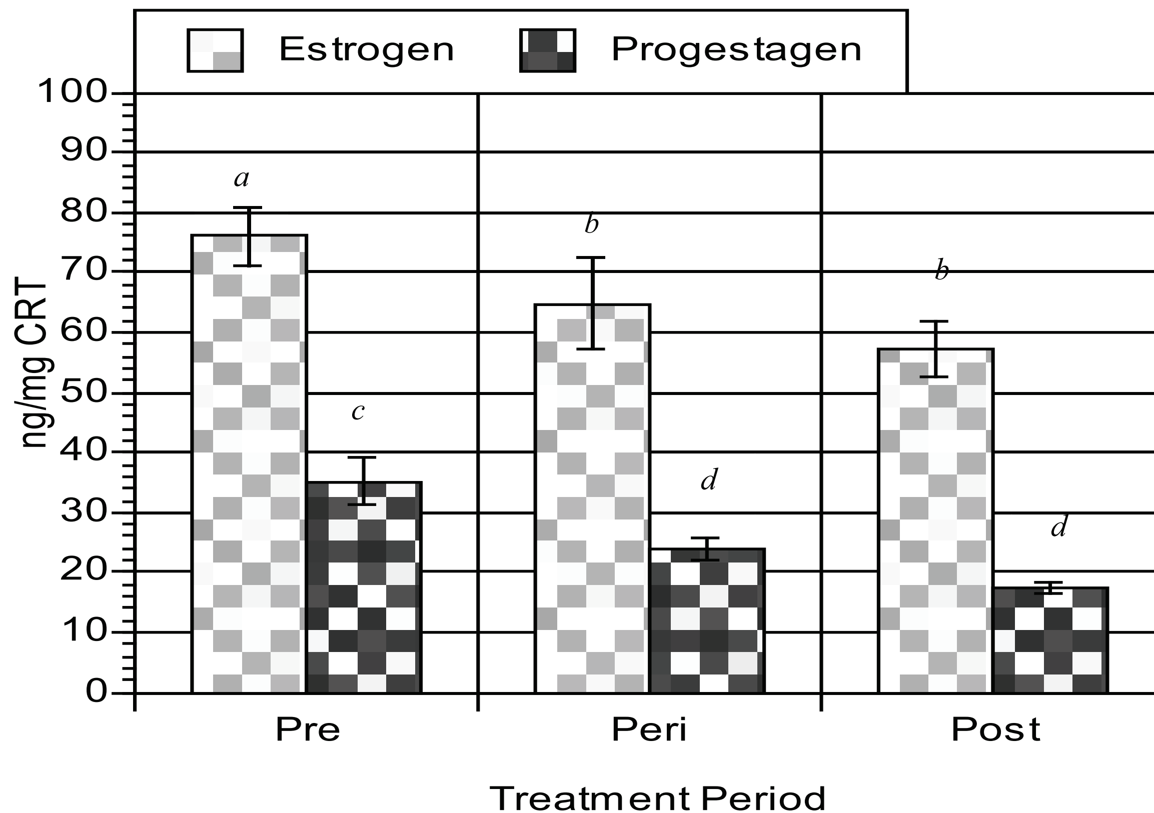



**Figure 3.4:** Mean urinary hormone levels for during treated cycles (n = 9) of Przewalski's mares with 14 days oral altrenogest. Data are shown as mean ± SEM and difference between groups was detected ($P < 0.05$).

estrogen metabolite levels among the three periods, there were differences in mean concentrations of urinary progestagen metabolites between pre- and peri/post-treatment ($P < 0.05$).

Of the 11 treated cycles, levels of urinary estrogen and progestagen metabolites were below baseline in five (45.4%) trials before altrenogest treatment onset. Four mares (80%) excreted baseline hormone concentrations during the treatment period. Urinary estrogen and progestagen excretion during the remaining six treated cycles were above baseline before altenogest administration, but hormone concentrations in four of the six mares (67%) declined to below typical baseline concentrations within 6.3 ± 1.2 days of treatment (estrogen: 5.7 days; progestagen: 7.5 days). Overall, urinary steroid hormone excretion was below baseline by the end of treatment in 9 of 11 cycles (91%).

Although estrogen and progesterone production appeared to be suppressed in the majority of the altrenogest-treated mares, there was wide among-female variation in hormonal response to treatment. Figure 3.5 depicts the overall mean response for all 11 treatments, whereas mean urinary hormone profiles for Przewalski's mares that received three treatments (n = 3 mares) are presented in Figure 3.6. Mare 1 exhibited a gradual increase in urinary estrogens near the end of the treatment period. There was wide variation in urinary progestagen excretion during treatment for this mare, which ovulated once during the treatment period. Mare 2, a mare that did appear to respond to two of three treatments, unexpectedly excreted increased urinary progestagen metabolites during the treatment period. Mare 3 excreted increased urinary estrogens during the late treatment period, but did not ovulate until after therapy was completed.

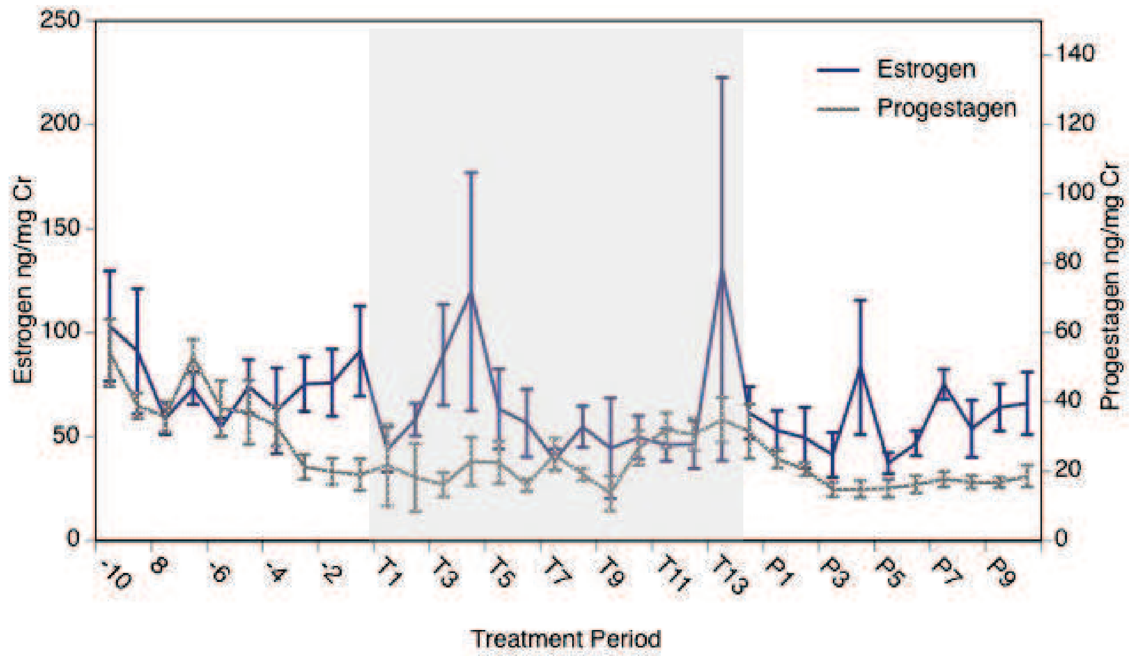



Figure 3.5: Longitudinal urinary hormone data for n = 11 cycles from Przewalski's mares treated with 14 day oral altrenogest. Gray area depicts treatment period. Data are shown as Mean ± SEM.

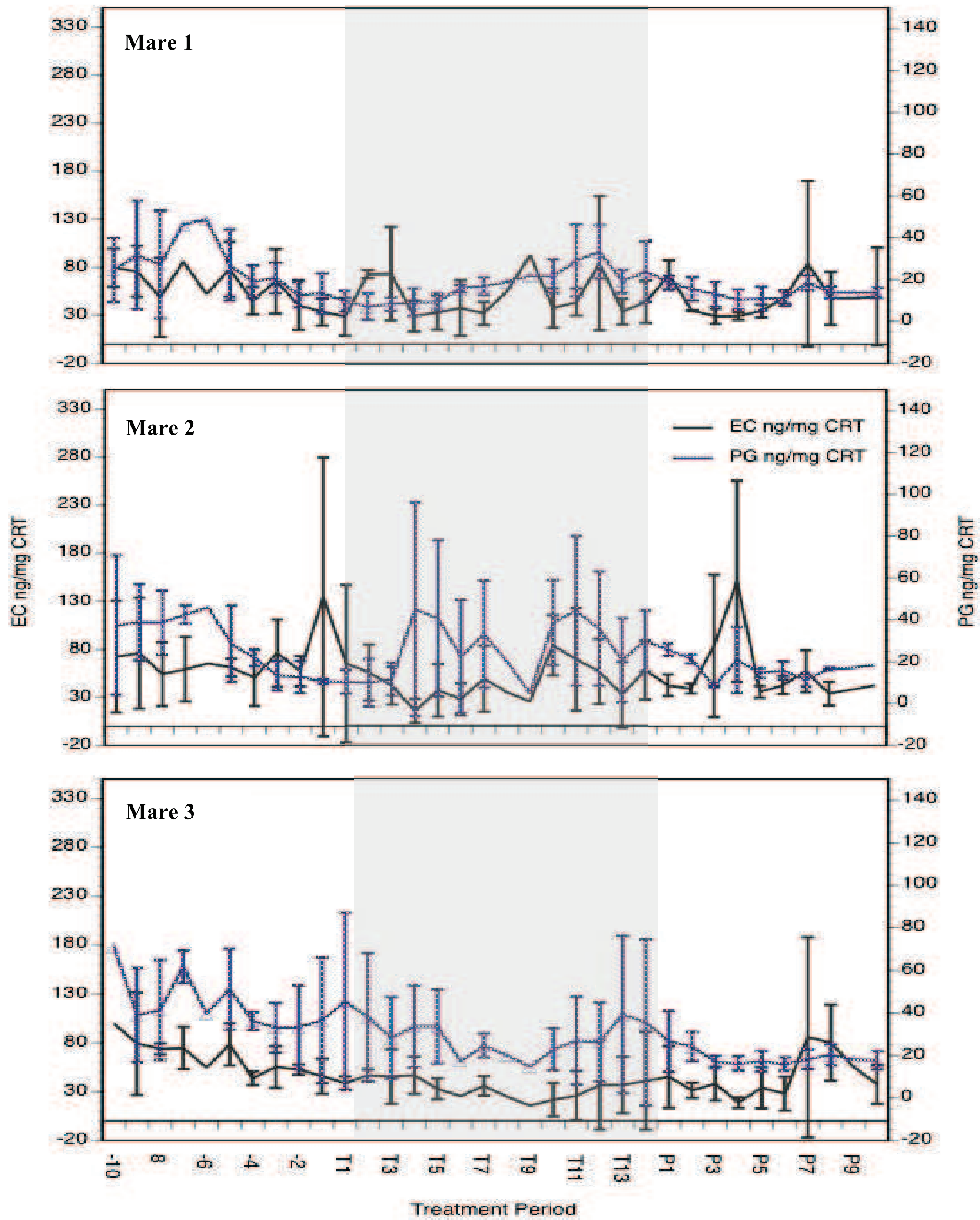



**Figure 3.6:** Longitudinal urinary hormone profiles for Przewalski's mares (n = 3) treated with 14 day oral altrenogest. Gray area depicts treatment with altrenogest. Graphs depict the variability of response to oral therapy.

*Follicular Response:*

Follicular changes also showed wide variation in response to oral altrenogest therapy. Of the 11 cycles monitored, two (18.2 %) ovulations occurred during treatment. In four cycles, a follicle > 30 mm was detected at initiation of treatment and three (75%) of these follicles regressed by the end of treatment. After treatment, 6 of 11 treated cycles (54%) exhibited normal estrous follicle development and ovulation 4.4 ± 0.8 days after treatment was completed. In contrast, luteinized follicles or no estrus was observed in 27% (3/11 cycles) and 9% (1/11 cycles) of estrous cycles, respectively.

<u>Study 2</u>

*Endocrine Response:*

Table 3.1 shows mean hormone levels for pre-, peri-, and post-treatment for both oral (O12) and injectable (In12) altrenogest therapies. There was a significant difference in mean urinary hormone concentrations among groups before ($P < 0.05$) but not after altrenogest treatment ($P > 0.05$). During treatment, mean urinary estrogen and progestagen metabolites were significantly lower ($P < 0.05$) in mares treated with injectable altrenogest (estrogen: 28.2 ± 2.5 ng/mg Cr; progestagen: 16.6 ± 4.1 ng mg Cr) compared to mares treated with oral altrenogest (estrogen: 43.1 ± 3.8 ng/ mg Cr; progestagen: 37.5 ± 4.9 ng/mg Cr). Figure 3.7 shows longitudinal urinary hormone data for mares in O12 and In12 treatment groups.

Urinary estrogen excretion at the time of treatment onset exceeded baseline in three of five mares (60.0%) treated with oral altrenogest (O12). Urinary estrogen and progestagen excretion declined below baseline 3.0 ± 0.6 days and 5.0 ± 0.7 days after the initiation of the treatment, respectively. Before treatment onset, four of the five mares (80.0%) treated with

| | Estrogen Conjugates | | | Pregnane | | |
|---|---|---|---|---|---|---|
| Treatment | Pre | Peri | Post | Pre | Peri | Post |
| Oral Altrenogest 12 D | 59.3 ± 5.0 | 43.1 ± 3.8 | 62.4 ± 9.2 | 56.0 ± 8.0 | 37.5 ± 8.0 | 28.3 ± 4.2 |
| Injectable Altrenogest | 43.9 ± 2.3 * | 28.2 ± 2.5 ** | 52.8 ± 4.3 | 21.2 ± 9.7 * | 16.6 ± 4.1 * * | 18.2 ± 7.8 |

**Table 3.1:** Mean urinary hormone concentrations during pre-, peri- and post-treatment periods. Data are presented as mean ± SEM and were compared among treatment periods. Injectable altrenogest showed a significant difference compared to oral altrenogest during the pre- and peri- treatment periods ($P < 0.05$). Significance is depicted by (*).

oral altrenogest (O12) excreted urinary progestagen concentrations that exceeded baseline; steroid concentrations fell below baseline 4.2 ± 0.7 days later (4/4 mares, 100%).

A total of 11 cycles were evaluated in six mares treated with injectable altrenogest (In12; n = 6 mares). Urinary estrogen and progestagen excretion was below baseline prior to therapy in 72.7% (8 of 11 cycles) and 81.8% (9 of 11 cycles), respectively. Of estrous cycles characterized by below baseline hormone concentrations before treatment onset (n = 8 cycles), urinary estrogen excretion increased 9.3 ± 0.7 days after altrenogest injection. In contrast, urinary progestagens were lower than baseline at the time of altrenogest administration in nine cycles, and progestagens exceeded baseline in only a single instance (8 days after altrenogest injection). Overall, urinary hormone excretion was below baseline within 12 days of treatment onset in 64% (7/11 cycles) of estrous cycles.

*Follicular Response:*

All mares treated with oral altrenogest (O12, n = 5 mares) had a CL present on the ovary at the initiation of treatment. Of five mares treated with injectable altrenogest, four (In12; 80%) had an active follicle present at the initiation of treatment (Mean: 37.5 ± 5.6 mm; Range: 25 – 50 mm). All follicles noted at the initiation of treatment regressed, and one of five (20%) mares developed a new follicle and ovulated during the treatment interval. Of the five mares treated with the oral altrenogest, one (20%) ovulated during treatment. During the second year (2009, n = 5 mares), all mares were treated with injectable altrenogest and ultrasound examinations were not performed prior to treatment. One of the six mares (16.6%) ovulated during treatment period, based on the presence of active CL detected after therapy and increased urinary progestagen metabolites during treatment period.

Overall, four or five mares (80%) treated with oral altrenogest developed an estrous follicle in 82% of cycles (9/11 cycles). Estrous follicles were detected in 83% (5/6 mares) of mares treated with injectable altrenogest. There was no significant difference between treatment and days to detection of dominant follicle (O12, 2.4 ± 0.9 days; In12, 2.3 ± 0.6 days; $P > 0.05$).

*Ovulation Induction:*

Figure 3.7 shows the difference between mares treated with GnRH compared to natural cycles from our previous study (n = 10 mares). These data revealed a significant difference in the number of days to ovulation between treated and untreated mares (Treated, 2.1 ± 0.1 days; Untreated, 9.9 ± 0.7 days; $P < 0.05$). Mares in the present study were treated with GnRH when follicle size averaged 39.0 ± 1.6 mm (Range, 31 – 43 mm) and ovulated at 2.1 ±

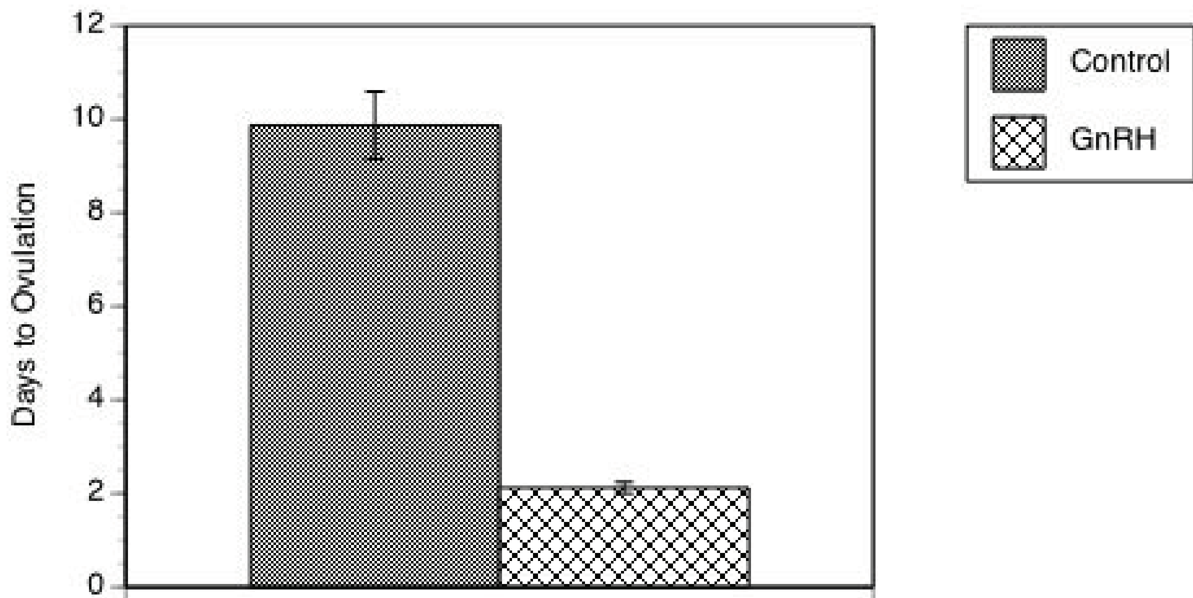



**Figure 3.7:** Days from detection of estrus follicle to ovulation in Przewalski's mares (n = 10) that had no treatment (natural cycle) and were treated with ovulatory agent (GnRH). There was a significant difference detected between groups ($P < 0.05$).

0.1 days post –treatment (Range, 2 – 3 days). Because mares were involved in an AI trial, two mares received two injections of GnRH in year 2 of the study. Because these mares failed to respond to the first dose of GnRH, a second dose administered 2 days later resulted in subsequent ovulation within 48 hours. All mares had uterine edema present at time of treatment (Range, slight – Grade 3). Of the ten mares treated over the two study periods, only one (10 %) showed no evidence of ovulation within four days of treatment. Urinary progestagen metabolites post- induced ovulation remained elevated for 13.5 ± 0.8 days (Range, 11 – 17 days), which is comparable to the luteal phase duration documented previously (11.0 ± .09 days; range: 9 – 11 days; Chapter 2).

*Comparison of Treatments:*

Table 3.2 shows mean urinary hormonal data for all treatments: oral 14 day (O14), oral 12-day (O12) and injectable 12-day (In12). There was no significant difference between mean hormone levels when comparing 12 - day to 14 - day oral treatments. However, urinary hormone concentrations in the injectable altrenogest group were different ($P < 0.05$) compared to the other treatments during both the pre- and peri-treatment periods .

There was no significant difference ($P > 0.05$) in days from completion of therapy to detection of a dominant follicle among treatments (O14, 4.4 ± 0.8 days; O12, 2.4 ± 0.9 days; In12, 2.2 ± 0.6 days). Treatment 1 (O14) had the lowest response post-treatment with only a 54.5% (6/11 treated cycles) normal response. These response rates were lower ($P < 0.05$) than the 80% (4/5 cycles) and 82% (9/11 cycles) levels observed in the O12 and In12 protocols. Ovulation was detected in 18% (2/11 cycles) of the O14, which was similar to the 20% (1/5 cycles) and 18% (2/11 cycles) ovulation rates detected in the O12 and In12 treatment groups. Luteinized follicles or no treatment response was observed in 27% (3/11 cycles) and 9% (1/11 cycles), respectively, of the O14 regimen.

*Treatment during the Follicular vs. Luteal Phase*

To determine whether stage of the estrous cycle impacted treatment outcomes, data were also analyzed based on whether treatment was initiated during the follicular or luteal phase. For treatment 1 (O14), 36% (4/11 cycles) were initiated during the follicular phase. Of these, 50% (2 of 4) resulted of treatments were successful in suppressing ovulation. Similarly, 51% (4/7 cycles) of treatments initiated during the luteal phase were successful at suppressing ovulation.

| | Estrogen Conjugates | | | Pregnane | | |
|---|---|---|---|---|---|---|
| **Treatment** | **Pre** | **Peri** | **Post** | **Pre** | **Peri** | **Post** |
| Oral Altrenogest 14 D | 76.0 ± 4.9 | 64.7 ± 7.6 | 57.1 ± 4.7 | 35.1 ± 3.9 | 23.7 ± 1.8 | 17.5 ± 0.9 |
| Oral Altrenogest 12 D | 59.3 ± 5.0 | 43.1 ± 3.8 | 62.4 ± 9.1 | 56.0 ± 8.0 | 37.5 ± 8.0 | 28.3 ± 4.2 |
| Injectable Altrenogest | 43.9 ± 2.3 * | 28.2 ± 2.5 * | 52.8 ± 4.3 | 21.2 ± 9.7 * | 16.6 ± 4.1 * | 18.2 ± 7.8 |

**Table 3.2**: Mean urinary hormone concentrations during pre-, peri- and post-treatment periods. Data are presented as mean ± SEM and were compared among treatment periods. Injectable altrenogest showed a significant difference compared to other treatments during the pre- and peri- treatment periods ($P < 0.05$).

Treatment 2 (O12) was initiated in all mares (n = 5) during the luteal phase, and one (20%) did not respond to treatment. For treatment 3 (In12), 36% (4/11 cycles) were initiated during the follicular phase. Fifty percent (2/4) of treatments initiated during the follicular phase were successful in suppressing ovulation, whereas 86% (6/7) of treatments administered during the luteal phase were successful. When all treatments (O14, O12, In12) were combined, altrenogest administered during the follicular phase was associated with a 50% (4/8 cycles) incidence abnormalities, including hemorrhagic follicles and ovulation during therapy. In contrast, similar abnormalities were observed in only 26% (5/19 cycles) of cycles in which treatment was initiated during the luteal phase.

## DISCUSSION

This is the first study to describe successful manipulation of the estrous cycle using hormonal therapy in a wild equid species. We demonstrated that altrenogest (injectable and oral forms) suppresses ovulation in a majority of mares, and, when combined with $PGF_2\alpha$,

causes reasonable estrous synchrony in Przewalski's mares. Similar to the domestic horse, altrenogest was more effective in manipulating estrous cycle when initiated during the luteal phase. These data revealed that Przewalski's mares may benefit from management practices commonly used to improve breeding management in the domestic horse industry.

In most mammals, follicles are recruited from an antral follicular pool during the luteal phase when progesterone is produced by the CL (Senger, 2003). At the end of the luteal phase, progesterone levels drop and the increased episodic production of FSH and LH cause selection and maturation of the dominant follicle(s), which then leads to ovulation. By mimicking the luteal phase, altrenogest, a synthetic progestagen, postpones the onset of follicular development by suppressing LH production from the anterior pituitary until treatment is stopped (Senger, 2003; Soede *et al.,* 2007; Blanchard *et al.,* 2004). Altrenogest has been used successfully for estrus synchronization. In gilts, 93 – 95% of animals treated for 18 days with oral altrenogest exhibit estrus within 5 – 7 days after withdrawal (Martinet – Botte *et al,* 1995; Koutsotheodoros *et al.,* 1998; Horsley *et al.,* 2005). Gilts treated with altrenogest develop more follicles (16.6 ± 1.7) after therapy compared to gilts that received no treatment (15.1 ± 1.2; Soede *et al.,* 2007). In the domestic queen, altrenogest therapy suppresses follicular activity and a normal follicular phase occurs after 38 days of therapy (Stewart *et al.,* 2010). In wildlife species, altrenogest has also been used with variable response. For instance, in the Pacific white-sided dolphin, 6 females were treated with altrenogest for 20 to 30 days over several years. Of the 57 treatments, only 17 (30%) produced a dominant follicle by 15.2 ± 5.5 days (Robeck *et al*., 2009). Likewise, in the killer whale, treatment with altrenogest resulted in a delay of follicular development after 30 days of treatment (Robeck, 2004).

In Przewalski's mares, all treatments of altrenogest were effective for manipulating the estrous cycle, although the injectable form and 12 day oral treatment showed better response rates compared to the 14-day oral treatment regimen (83.3% and 80% compared to 54.5%). A high prevalence (3 of 11; 27.3%) of hemorrhagic anovulatory follicles (HAF) was also associated with the 14-day oral treatment. One possible explanation for this observation is that this treatment interval exceeds the duration of the normal luteal phase (11.0 ± 0.7 days). Prolonged progesterone treatment in cattle results in development of persistent ovarian follicles and overproduction of estrogen (Ahmed *et al.,* 1994). Similarly, in the domestic mare, hemorrhagic follicles are associated with higher levels of peripheral LH compared to mares that have normal ovulations (Ginther *et al.,* 2008). In the domestic horse, cow and pig, altrenogest-induced LH suppression is weak. Thus, the hormonal milieu during the prolonged luteal phase in the Przewalski's mare may have resulted in the development of an increased number of HAF. Treatment with $PGF_2\alpha$ in the domestic horse induces a sudden increase in LH that can over stimulate immature follicles, leading to the formation of HAF (Cuervo-Arango *et al.,* 2009). However, because HAF were not observed in either of the other two treatment regimens (Oral 12 day and Injectable 12 day) that also employed $PGF_2\alpha$, we speculate that the length of altrenogest treatment was likely to have resulted in increased numbers of HAF.

Treatment with injectable altrenogest was more effective for suppressing ovarian steroid excretion during the treatment period. This may be because the biorelease system achieves high circulating levels of altrenogest that gradually subside to non-therapeutic levels over the 12-day period (Burns *et al,* 2006). In contrast, oral treatment results in a rapid increase in altrenogest within the bloodstream, but hormone concentrations decline rapidly within the

subsequent 12-24 hours; in some individuals, levels may even drop to non-therapeutic levels. Przewalski's mares demonstrated a response rate of 83.3 % (Injectable) and 80.0% (Oral 12 day), with estrus occurring 2.2 ± 0.54 (Range, 1 – 5 days; Injectable-12 day) and 2.4 ± 0.9 days (Range, 2 – 4 days: Oral-12 day) post - treatment. In constrast, response rates to the Oral-14 day treatment was 54.5%, with estrus occurring 4.4 ± 0.8 days (Range, 2 – 7 days). These results are comparable the domestic horse, whereby 80% of mares exhibit estrus 2 – 8 days after cessation of a 10-15 day oral altrenogest treatment regimen (Bergfelt, 2000).

Altrenogest administered during the first three days of the follicular phase significantly lowered the response to treatment (Squires, *et al.,* 1979; Lofstedt, *et al.,* 1989). This may be related to the fact that altrenogest only binds to receptors on the anterior pituitary with 60% of the binding affinity of natural progesterone, which results in incomplete suppression of LH secretion (Hughes *et al.,* 1972). In the present study, altrenogest treatments that commenced during the follicular phase resulted in a 50% response rate compared to 79% percent for treatments initiated during the luteal phase. These results provide incentive for additional research to better understand the relationship between altrenogest treatment and the dynamics LH secretion in the Przewalski's mare.

Prostaglandins ($PGF_2\alpha$) administered within 5 days of ovulation have been used to effectively induce luteolysis in the Przewalski's mares (Durrant *et al.,* 1986). In the current study, $PGF_2\alpha$ administration coincident with the cessation of progestagen therapy appeared to shorten the interval to estrus. Evidence for this included an absence of luteal tissue after treatment, as well as basal concentrations of urinary progestagen metabolites (20/26 mares, 77% overall) by the end of treatment and in 14 of 16 mares (87.5%) that received the 12-day altrenogest regimens (injectable and oral). Additional research should focus on comparing

altrenogest treatment with and without prostaglandins to determine which approach is most effective for achieving estrus synchrony.

Ovulation is a complex process that is induced by an increase in LH. The length of time from estrus to ovulation varies in many species from 15 hours (cow) to 9 days (bitch) (Senger, 2003). In the domestic horse, the length of estrus ranges from 2 – 12 days, depending on the time of year, and ovulation usually occurs 48 hours before the end of estrus, which makes ovulation timing difficult to predict (Palmer, 1978; Ginther, 1993; Blanchard *et al.,* 2003). For AI to be successful, semen needs to be deposited in the uterus within 12 – 24 hours of ovulation (Samper, 2008). In many species, ovulatory agents are used to enhance AI success. To date, only one study has tested the efficacy of an ovulatory agent, hCG, in Przewalski's mares (Durrant *et al.,* 1986). When hCG was administered 7 days after $PGF_2\alpha$ treatment ovulation did not occur within the subsequent 24 hours. Because hCG can take 24 – 48 hours to induce ovulation (Blanchard *et al,* 2003; Palmer *et al.,* 1993), failure to detect ovulation may have been due to the inability to examine mares daily due to anesthetic risk. While we were unable to determine a difference between reLH, GnRH and controls in the first study, in a subsequent study in which mares were treated with 12 days of altrenogest, we were able to determine a difference between days from detection of an estrous follicle to ovulation in natural cycles compared to cycles treated with a GnRH agonist. A significant difference ($P < 0.05$) was detected between length of time from gonadotrophin administration to ovulation between treated mares (2.12 ± 0.12 days) and untreated mares (9.87 ± 0.72 days). These data revealed that the GnRH agonist, deslorelin, was effective for shortening the time to ovulation, achieving treatment responses that were similar to the domestic mare. Most importantly, the post-treatment luteal phase was similar in

length to the luteal phase in untreated cycles, which indicated that induced that ovulations produced apparently normal CLs.

Previous studies in the domestic horse have shown that responsiveness to altrenogest varies depending on the reproductive stage at the time of treatment initiation (Lofstedt, 1988; Squires, 1979). In one study, only 1 of 4 mares treated with altrenogest during the follicular phase ovulated because altrenogest treatment during the follicular phase did not appear to shorten the CL life span (Lofstedt, 1989). In gilts, there is an increase in number of follicles produced when altrenogest therapy is initiated during the late luteal phase. One theory is that the high level of endogenous progesterone production during the luteal phase effectively suppresses follicular development, which is then extended by altrenogest therapy (Soede, 2007). This is consistent with the present study as there was a decreased response rate in mares treated with altrenogest during the follicular phase, even among treatments.

This was the first study to assess estrous synchronization in Przewalski's mares using a combination of altrenogest and $PGF_2\alpha$. We also demonstrated that GnRH effectively induced ovulation in when large ovarian follicles (>31 mm) and uterine edema were present in treated mares. Although ultrasound examination is still necessary to predict presence of an estrous follicle, we confirmed that injectable altrenogest, in combination with $PGF_2\alpha$, has tremendous potential for optimizing AI for genetic management in this endangered species. Additional studies focused on optimizing ovarian synchrony are necessary for the development of reliable fixed time AI with high conception rates.

# CHAPTER FOUR

## Characterization of Seminal Traits in Przewalski's Stallions

### INTRODUCTION

The Przewalski's horse (*Equus ferus przewalskii*) is the last of the wild horses, native to China and Mongolia. With only 1500 animals remaining in captivity and no new founders in the wild, genetic management is a key for long-term maintenance of this species. The captive population of Przewalski's horses in North America is currently managed by the Asian Wild Horse Species Survival Plan (SSP). Each year, breeding recommendations are made based on mean kinship values of mares and stallions in the captive population. After recommendations are made, mares or stallions must then be moved to the designated zoological institutions which can be expensive and risky to the animals being shipped. Because of this, zoological parks are less interested in movement of animals, which can result in failure to breed genetically valuable animals.

Poor breeding management and loss of animals in the wild have lead to increased inbreeding in the population of Przewalski's horses. In the SSP population, the coefficient of inbreeding is 0.216 with a gene diversity of 78.55% (Monfort *et al.,* 2009). It has been shown that reproduction can be compromised when the gene diversity is below 90% (Monfort *et al.,* 2009). Breeding recommendations from the SSP have

established 40 breeding pairs over the last five years, with only 8 foals produced on average each year (20%). This low foaling rate indicates that poor reproduction is a potential threat to the sustainability of the captive population of Przewalski's horse in North America. Studies in other ungulate species show that, seminal parameters, such as morphology and motility, and reproductive fitness are negatively affected by increasing homozygosity (Ralls *et al.,* 1979; Rolden *et al.,* 2006; Fitzpatrick and Evans, 2009). A recent study in Shetland pony stallions has shown a decrease in motility and percentage morphologically normal sperm when the coefficient of inbreeding was greater than 2% (van Eldik *et al.,* 2006)

Artificial insemination (AI) has been used as a breeding management tool for genetic management of wildlife species such as the Giant panda (*Ailuropoda melanoleuca*; Hori *et al.,* 2006), black-footed ferret (*Mustela nigripes*; Howard *et al.,* 2003) and Asian elephant (*Elephas maximus*; Brown *et al.,* 2004; Hildebrandt *et al,* 2000). While this procedure has been successful in some species, a foundation of basic reproductive physiology for each species, including characterization of seminal traits, was needed before AI could be incorporated into a management program. To date, there is limited information on semen parameters in Przewalski's stallions.

In the domestic stallion, semen evaluation is performed to: 1) predict the potential fertility of a given stallion and 2) determine whether a stallion's semen is able to tolerate handling procedures such as cooling or freezing (Blanchard *et al.,* 2003). Many reports confirm that semen parameters in domestic stallions can vary due to individual animal variation, age, and season of the year (Johnson and Thompson, 1983; Magistri *et al.,* 1987; Johnson *et al.,* 1991; Dowsett and Knott; 1996, Jannett *et al.,* 2003, Gamboa *et al.,*

2009). In the Przewalski's stallion, only limited work – mainly opportunistic and/or combined with other medical procedures – has been undertaken to examine semen parameters (Stover *et al.,* 1981, Durrant 1990; Bader *et al,* 1991); and no systematic serial semen evaluations have been performed. This is an important point because, in the domestic stallion, a single semen sample does not provide an accurate assessment; it is generally accepted that accurate semen assessments require 2-5 sequential collections (Blanchard *et al.,* 2003).

Domestic mares are long-day breeders and have a period of anestrus in the winter months (Blanchard *et al.,* 2003; Ginther, 1992). Stallions produce sperm throughout the year; however, effects of season on testicular function in the stallion have been well documented. During the physiological breeding season (May through August), stallions produce an elevated amount of sperm (Thompson *et al.,* 1977; Johnson and Thompson, 1983) and testosterone (Hoffman and Landeck*.,* 1999). Furthermore, it has been shown that numbers of spermatogonial cells increase in the testis during the breeding season compared to the non-breeding season (Johnson, 1985). Studies on the influence of seasonality on sperm motility have shown contradictory results; one study showed increased motility during breeding season (Hoffmann and Landeck, 1999), whereas another showed a decrease in motile sperm during this period (Blottner *et al.,* 2001). There also appears to be differences among horse breeds. For instance, a study in Warmblood stallions showed that volume, motility, total concentration and major morphological defects were highest in the summer (Janett *et al.,* 2003). However, it was shown that only total concentration was highest in summer compared to other seasons in Franches-Montagnes stallions (Janett *et al.,* 2003). Only one study in Przewalski's

stallions has shown that there is a seasonal effect on seminal traits such as morphologically normal sperm, volume and motility (Durrant, 1990). A total of 21 semen collections from ten stallions of varying age (2.5 – 22 years) were assessed over a 12 month period. Total volume was highest in spring and motility was highest in autumn and spring. While this study was important in characterizing seminal traits and seasonal variation in seminal traits in Przewalski's stallions, semen collections were only performed over a 12 month period. By performing a long-term study on more males, seasonality and seminal traits should be more defined.

Cryo-survival of stallion sperm has been shown to be influenced by seasonality (Magastrini et al., 1987; Blottner *et al.,* 2001; Janett *et al*, 2003; Gamboa *et al.,* 2010). In Franches - Montagnes and Warmblood stallions, post-thaw quality improved in samples collected in autumn. However, this finding is in contrast to an earlier study in which six mixed breed stallions showed improved post-thaw quality when spermatozoa were collected in winter (Magastrini *et al.,* 1987). Since long-term management of Przewalski's horses is reliant on preservation of valuable genetics, understanding the influence of seasonality on seminal parameters may indicate the optimal time to collect high quality samples for sperm preservation.

Therefore, the objectives of this study were to evaluate semen in Przewalski's stallions (n = 14; 4 – 23 yr old) and compare parameters among males with respect to individual stallion effect, season and inbreeding. This knowledge will inform decision making related to reproductive management in captive Przewalski's horses. We hypothesize that semen quality will show higher concentration and motility in the

breeding season and that individual stallion, age, and inbreeding will impact seminal traits.

## MATERIALS AND METHODS

### *Animals*

Przewalski's stallions (n = 14; Age: 4 – 28 years) from two institutions (Smithsonian's Conservation Biology Institute (SCBI): n = 7; The Wilds: n = 7) were used throughout this study. Stallions at SCBI were kept in individual paddocks except when in breeding situations (n = 1) during which mares were also placed in the enclosure. Stallions at the Wilds were kept either in a breeding herd (n = 2) or in a bachelor band. Animals were fed herbivore low protein pellets, hay and had access to grass and mineral licks.

### *Semen Collection*

Semen collections were performed during four different time periods each year so that all seasons were represented. Seasons were defined as: Spring: Feb22 – June 1; Summer: June 20 – Sep 20; and Fall: September 21 – December 21; Winter: December 22 - February). All stallions from both institutions were collected within a 2-week time period. At each collection, stallions were anesthetized using a combination of detomidine (Dormosedan®, Orion Corp., Espo, Finland), ketamine (Ketaved®, Phoenix Scientific, St. Joseph, MO), etorphine (M99®, Wildlife Pharmaceuticals, Fort Collins, CO), and guaifenisin (Guafenisin injection, Phoenix Scientific, St. Joseph, MO). A standardized electroejaculation protocol was used to collect semen (Howard, 1986). Briefly, a sine-wave

electro stimulator (AC, 60-Hz) and Teflon® rectal probe (7 cm; P.T. Electronics, Boring, OR, USA) was used to administer 90 incremental stimuli administered in a 3 sec on-off pattern in three series consisting of 30 (10 stimulations at 2, 3 and 4 Volts, respectively), 30 (10 stimulations at 3, 4 and 5 Volts) and 30 stimuli (10 stimulations at 4, 5 and 6 Volts). Each series was separated by a 5-min rest interval at which time seminal aliquots were assessed. At the end of each semen collection, a testicular examination was then performed by ultrasound (Sonovet 2000, Universal Ultrasound, Bedford Hills, NY, USA).

*Semen Evaluation*

Raw semen samples from each series were analyzed for volume, pH, osmolarity and total motility. One representative samples from the raw ejaculate (40 μL) was fixed in 400 μL 0.4% gluteraldehyde and stored at 4° C until morphological assessment was performed. Semen aliquots were then extended at a 1:4 dilution in skim milk based extender (INRA 96, IMV Technologies, Maple Grove, MN, USA) and assessed for percent total motility (%TM) and percent progressive motility (% PM) (Blanchard, 2003). Total progressively motile sperm numbers were also calculated for each stallion at each collection.

*Morphological Assessment*

At least 200 cells were examined by phase microscopy (100 X: Olympus BX40; Hunter Valley, PA, USA). Morphology was determined using the following categories: normal cells, head defects, mid-piece defects, proximal cytoplasmic droplets (PCD), distal cytoplasmic droplets (DCDs), tail defects, and loose heads. The number of total

progressively motile morphological normal (TPMMN) spermatozoa was then calculated for each ejaculate and each stallion.

*Testicular Examination:*

Testicles were assessed by digital palpation for any abnormalities in firmness or position. An ultrasound examination was then performed to assess structure of testes and the epididymides. Height and width (mm) of each testicle was then measured using ultrasound (Sonovet 2000, Universal Ultrasound, Bedford Hills, NY, USA) and length (mm) was measured using testicular calipers. Testicular volume for each testicle (TV) was then calculated using the formula for an ellipsoid structure (TV ($mm^3$) = $4/3\ \pi$ ((height x width x length)/2); Love, 1991). Total testicular volume (TTV) was calculated by adding TV for both right and left testicles together. Estimated Daily Sperm Output (EDSO) was then calculated using the formula: EDSO (billions of sperm/day) = (0.024 x TTV) – 1.26 (Blanchard, 2003).

*Statistical Analysis*

Seminal traits (concentration, pH, osmolarity, motility, extended motility, progressive motility, normal morphology, total testicular volume, and total progressively motile sperm) were compared by Analysis of Variance (ANOVA) by using PROC GLM (SAS, Version 9.2, Cary, NC, USA). To achieve normal distribution, data presented as percentage (motility, extended motility and progressive motility) were transformed using the formula: Log (100 – value). Stallion was set as the random effect, which corrected for the correlations between records of the same stallion. Fixed effects, included in the

model were season and year of collection. Age and genetics were not assessed due to the fact that these traits were heavily confounded with the stallion.

Due to the high variations in season, this effect was compared two ways: 1) standard season by comparing means; and 2) continuous variation throughout the year. Continual variation was assessed according to Fireman *el al.* (1997). Briefly, Julian dates were generated for each collection day (January 1 = day 1, December 31 = day 365) and then converted to radians (January 1, y = 0 radians, December 31, y = 2p radians). The trigonometric functions sine and cosine were applied to the measurements in radians corresponding to the collection days. The measures sin (y) and cos (y) allow detection of a yearly pattern (wave) in the dependent variables, with one peak (maximum value) and one valley (minimum value). In addition, the semi-annual wave was studied by using sin (2y) and cos (2y), which allows detecting important departures from the annual wave (e.g., two annual peaks or a seasonal plateau). The sine and cosine values were then used as independent variables in a covariance analysis. Significance was verified by t-tests since the independent variables were orthogonal and a regression coefficient was estimated for each sine and cosine regressor (Fireman et al., 1997). Predicted values were obtained using the mean value of the trait as the intercept and adding to that the products of the regression coefficients by their corresponding sine and cosine values.

## RESULTS

### *Seminal Traits*

A total of 98 semen collections were performed from September 2005 – June 2009 on Przewalski's stallions (n = 14) and semen samples were obtained in 86 collections (87.8 %). Semen parameters averaged from all collections are shown in Table 4.1. There was a large amount of variations among stallions in seminal traits, including volume, concentration, motility and progressive motility. Semen samples obtained in the present study contained large proportion of sperm with abnormal morphology (80%; Table 4.2). The majority of structural abnormality was defects to the head of the sperm, including folded acrosome, crater defects, size and shape of head (Figure 4.1)

### *Testicular Examination*

Testicles were examined at all collections and of the 14 stallions examined, one stallion (7.1%) was cryptorchid. On ultrasound, testicular parenchyma was fairly homogenous with a central testicular vein noted (see Figure 4.2). The cauda epidiymis was readily visible at the caudal pole of each testicle. In all examinations, no rotation of testicles was noted. Average total testicular volume (TTV) for all stallions was 212.31 ± 16.87 $cm^3$.

| Variable | Mean ± SEM |
|---|---|
| Volume (mL)<br>(n = 92) | 31.15 ± 2.14 |
| pH<br>(n = 91) | 9.2 ± 0.03 |
| Motility (%)<br>(n = 86) | 42.24 ± 2.27 |
| Extended Motility (%)<br>(n = 85) | 61.78 ± 1.76 |
| Progressive Motility (%)<br>(n = 85) | 52.09 ± 1.72 |
| Concentration (x $10^6$)<br>(n = 86) | 2.69 ± 0.27 |
| Normal Morphology (%)<br>(n = 56) | 19.13 ± 2.70 |
| Age at Collection (years)<br>(n = 94) | 14.36 ± 0.65 |
| Osmolarity (mOsm)<br>(n = 82) | 299.75 ± 2.76 |
| Total Testicular Volume<br>(n = 63) | 212.31 ± 6.87 $cm^3$ |
| TPPMN<br>(n = 56) | 0.74 ± 0.16 x $10^9$ |

**Table 4.1**: Seminal parameters for 14 Przewalski's stallions collected over a 45 month period. Data are presented as Mean ± SEM.

| **Sperm Morpholgy** | **Percentage** |
|---|---|
| Normal | 19.13 ± 2.70 |
| Head Defect | 27.09 ± 2.80 |
| Mid - Piece Defect | 17.81 ± 2.87 |
| Proximal Cytoplasmic Droplet | 21.04 ± 2.98 |
| Distal Cytoplasmic Droplet | 14.38 ± 1.58 |
| Tail Defect | 2.38 ± 0.45 |
| Round Cell | 0.83 ± 0.20 |
| Other | 2.63 ± 1.91 |

**Table 4.2:** Sperm morphology for Przewalski's stallions (n = 56 samples). Data are presented as mean ± SEM.

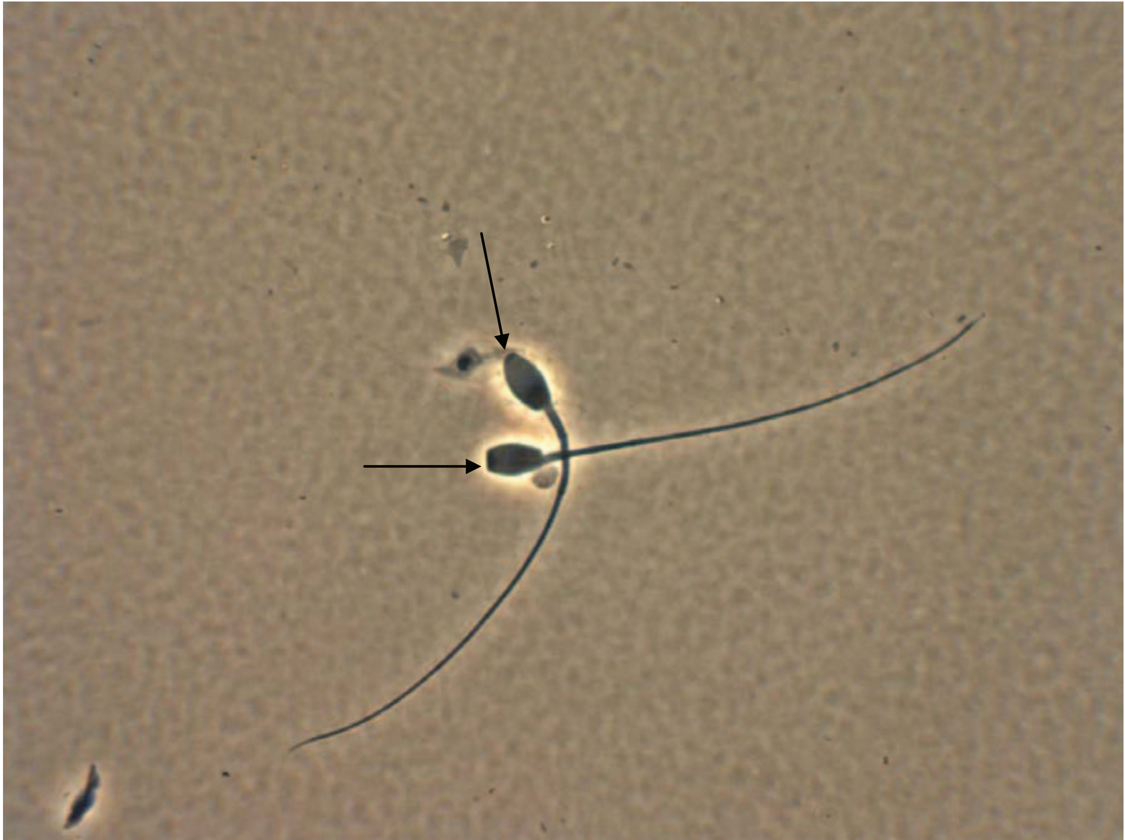


**Figure 4.1:** Two spermatozoa with folded acrosomes (depicted by arrow). Spermatozoa were examined by phase microscopy at 100X.

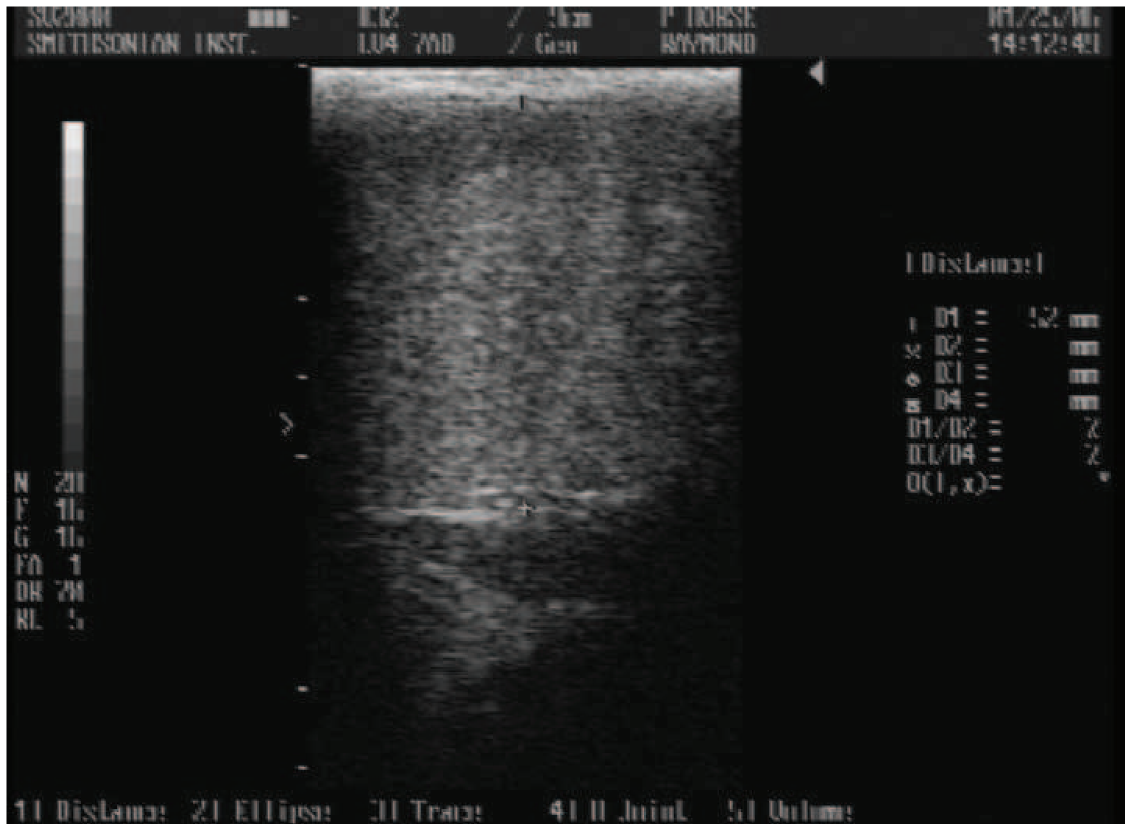


**Figure 4.2:** Ultrasound of testicle during examination of a Przewalski's stallions. Image shows cross section of testicle from the ventral aspect of the scrotum. This image shows height (mm) being measured (52mm).

*Seasonal Effects*

The results from ANOVA (Table 4.3) clearly demonstrate that the effect of stallion has a significant influence on all parameters except seminal pH ($P < 0.05$). Seasons did not affect seminal traits ($P > 0.05$) except volume and pH ($P < 0.05$). There were definite variations among individuals in total concentration (Figure 4.3), progressive motility (Figure 4.4), normal morphology, TTV (Figure 4.5) and total progressively motile sperm.

*Variable Seasonal Effects:*

Due to the high variation detected in seminal traits, over seasons, analysis was performed to determine whether there was continual variance in seminal traits. Table 4.4 shows that level of variation in seminal traits and significance for variable factors (sine, cosine, 2* sine, 2*cosine). Seminal traits that showed a high coefficient of variation were normal morphology, volume and concentration. Volume and pH were also analyzed because season had shown a significant effect on these values. Volume showed significance with independent variable cosine ($P < 0.05$), which indicates one wave in variation in relation to the Julian calendar (Figure 4.6). Interestingly, the variable curve shows a similar pattern to the mean values detected at each season, with peaks in spring and summer. Normal morphology showed significance with independent variable sin*2 ($P < 0.05$), which indicates that there are two waves in variation during the Julian calendar year in summer and winter (Figure 4.7). In this case, the curve is more variable than the mean values for each season. Concentration had a trend towards two waves in variation (cosine*2). However, due to a high coefficient of variation (80.25), this may be a true trend that is not detectable at low numbers (Figure 4.8). When comparing mean values for each season, the trend is similar to the variable predicted pattern based on cosine*2.

| *Variable* | *Stallion* | *Season* |
| --- | --- | --- |
| Volume | <.0001 | 0.001 |
| pH | 0.2353 | 0.004 |
| Motility | <.0001 | 0.4753 |
| Osmolality | 0.0011 | 0.0601 |
| Extended Motility | 0.0012 | 0.9866 |
| Progressive Motility | 0.0002 | 0.9374 |
| Concentration | 0.0071 | 0.6225 |
| Normal Morphology | <.0001 | 0.1928 |

**Table 4.3:** Seminal Traits of Przewalski's stallions (n = 14) in relation to variables stallion and season. Figures shown are significance of variable on each seminal trait.

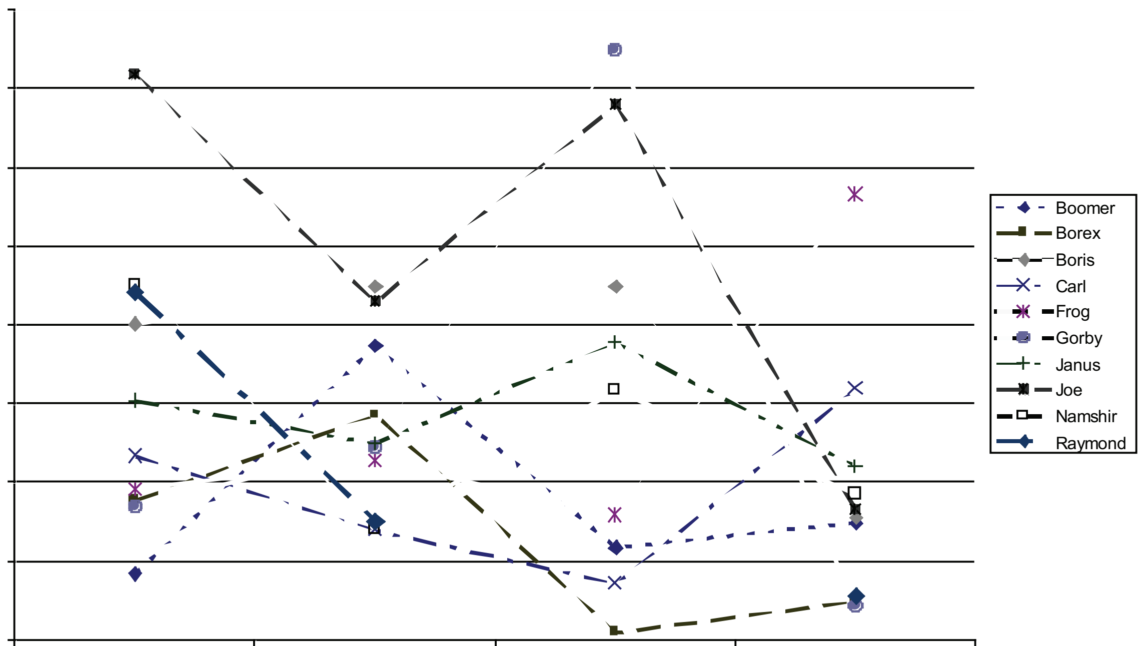



**Figure 4.3:** Total sperm concentration (x $10^{9}$) of Przewalski's stallions (n = 10) assessed at each season.

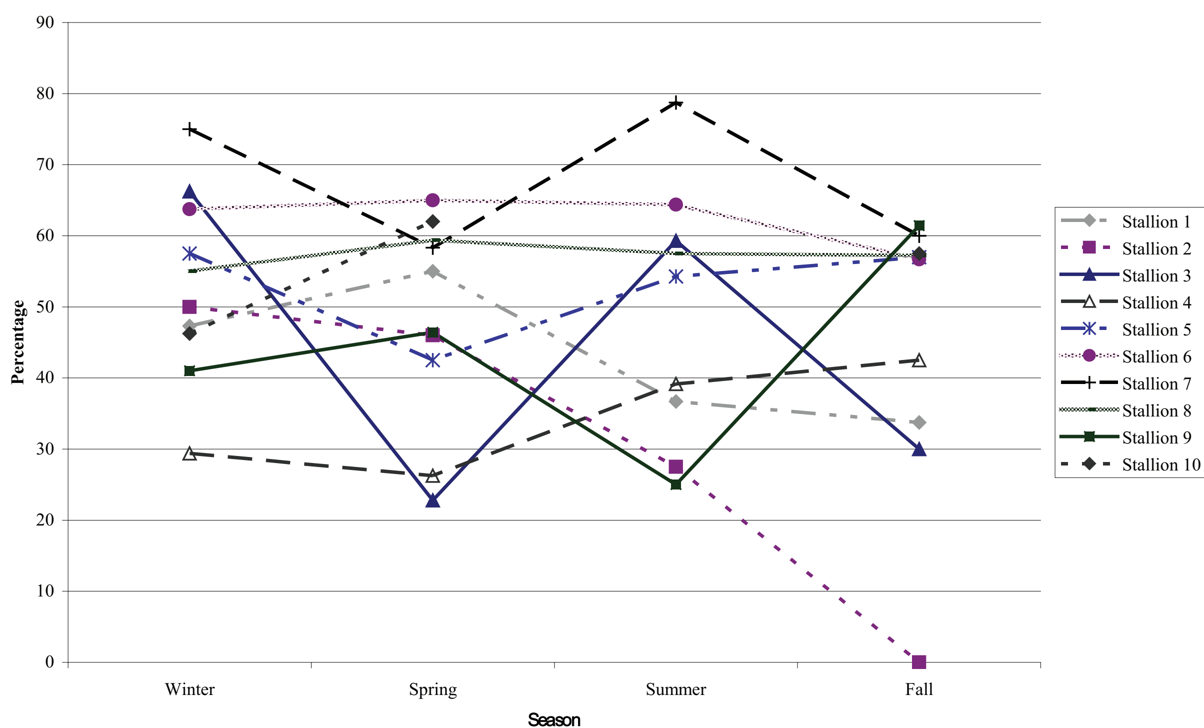



**Figure 4.4:** Progressive motility (%) over seasons in Przewalski's stallions (n = 10).

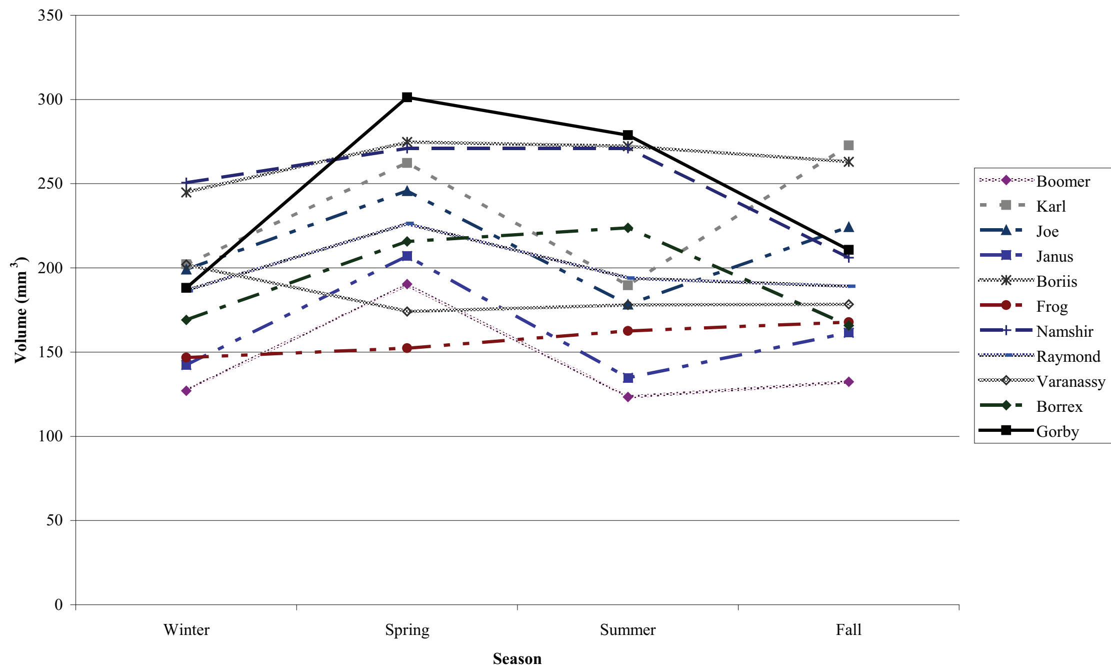



**Figure 4.5:** Total Testicular Volume over season in Przewalski's stallions (n = 10).

| Variable | Season | Coefficient of Variation | Variable |
|---|---|---|---|
| Volume | 0.001 | 49.939 | 0.004 |
| pH | 0.004 | 3.711 | 0.012 |
| Motility | 0.4753 | 7.292 | |
| Osmolality | 0.0601 | 7.404 | |
| Extended Motility | 0.9866 | 8.616 | |
| Progressive Motility | 0.9374 | 6.691 | |
| Concentration | 0.6225 | 80.25 | 0.143 |
| Normal Morphology | 0.1928 | 34.979 | 0.061 |

**Table 4.4:** Seminal traits of Przewalski's stallions (n = 14). Column 1 shows significance of season on each trait. Column 2 shows the coefficient of variation. Column 3 shows the significance of the variable sine, cosine, sine*2, or cosine*2.

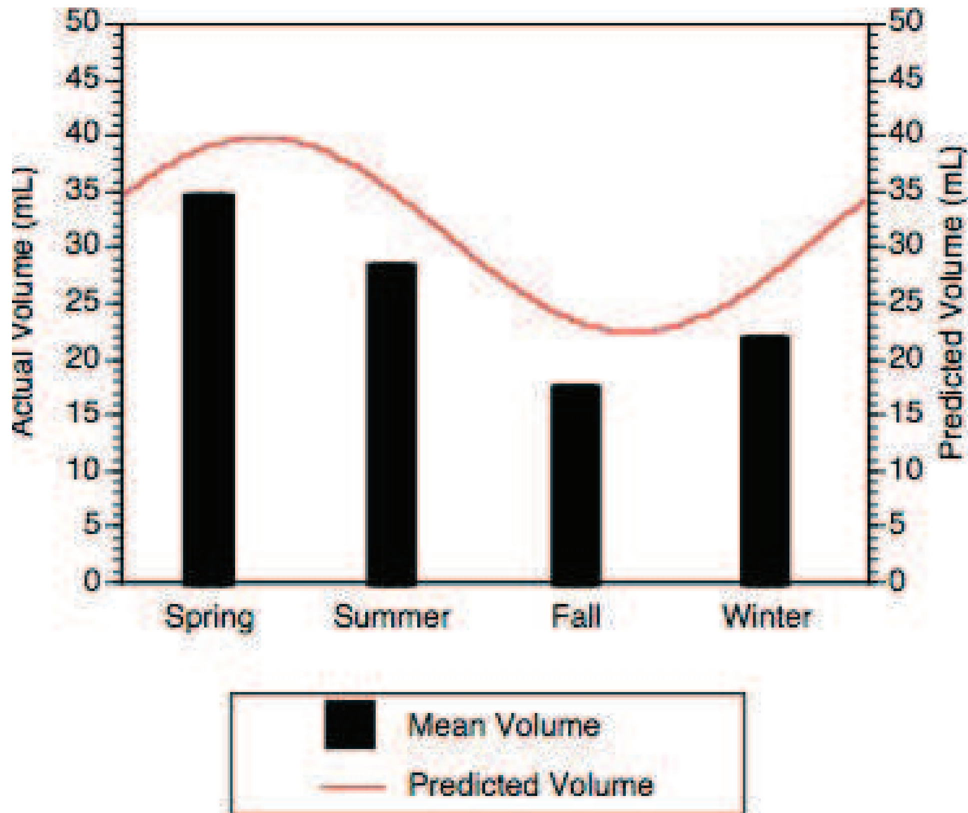



**Figure 4.6:** Variable volume of Przewalski's stallions (n = 10) compared to actual mean values at each season. Predicted values are shown by line and actual means are depicted by bars.

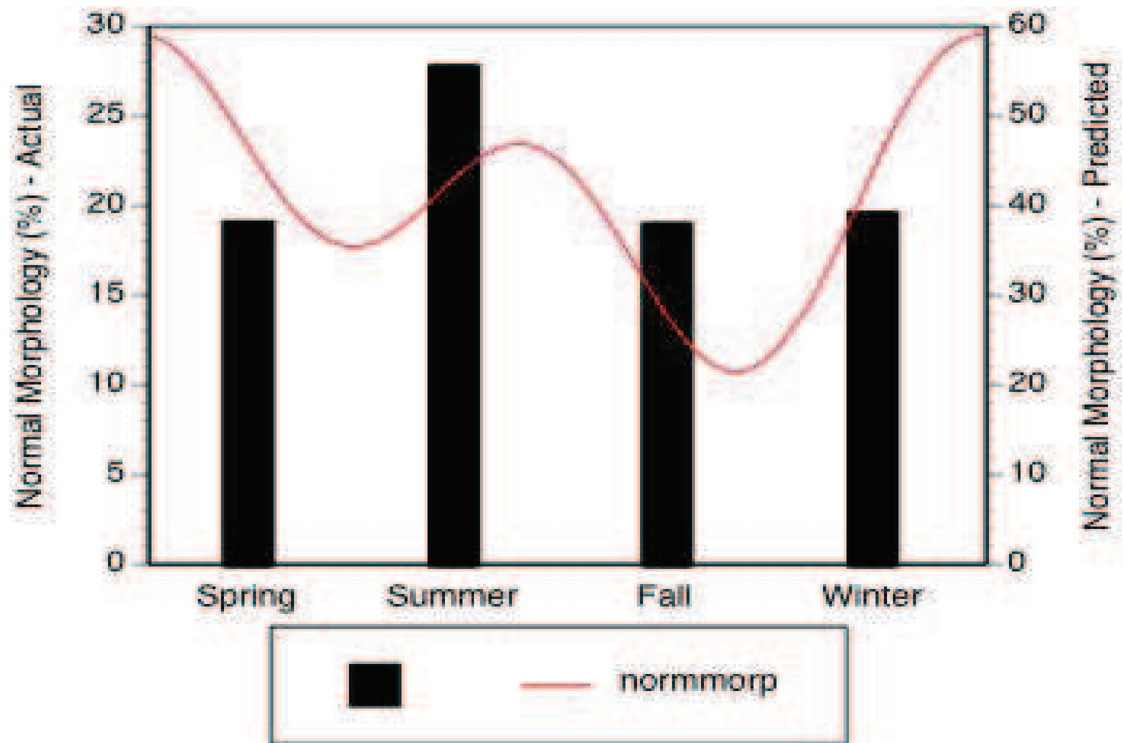



**Figure 4.7:** Predicted variable change in percentage morphologically normal cells (red line). Detected mean values for each season (n = 14 stallions) are shown by bars. Line shows expected data based on variable analysis.

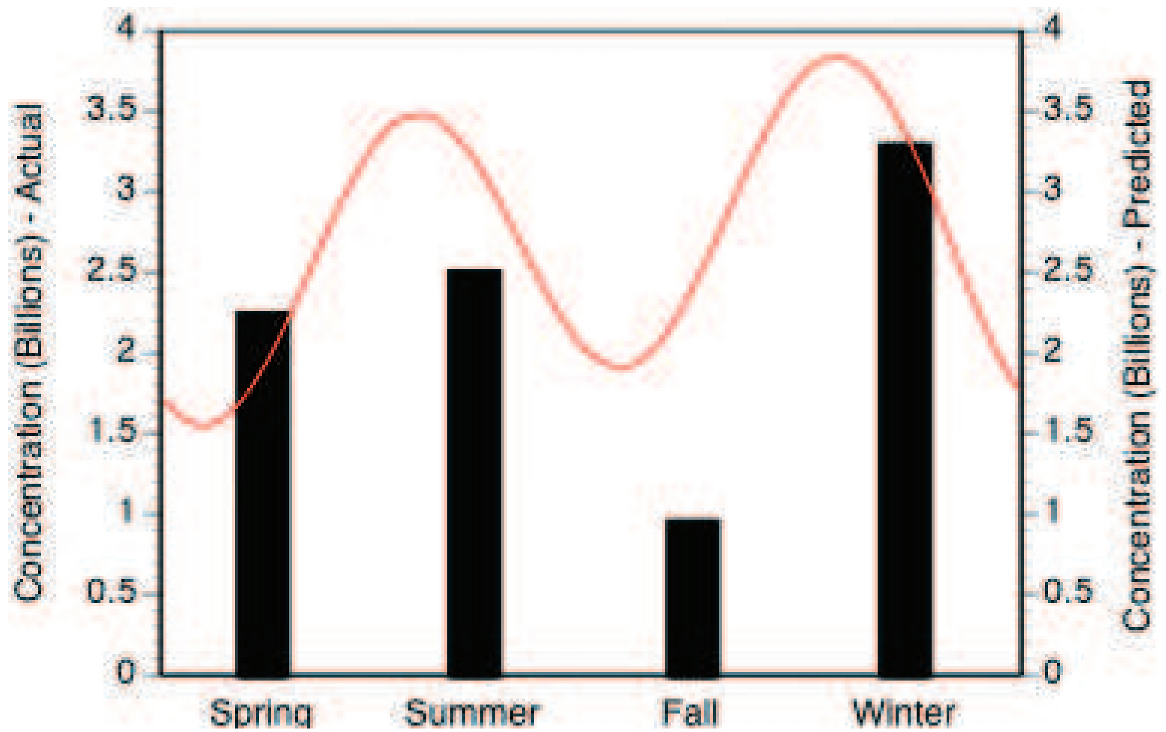



**Figure 4.8**: Predicted variable change in total sperm concentration in Przewalski's stallions (n = 14). Variable change is shown by line and actual mean values for each season are shown by bars.

## DISCUSSION

This is the first study to assess seminal traits and testicular changes in Przewalski's stallions over a two – year period. Eighty-nine semen samples from fourteen stallions were assessed to detect any seasonal changes. Our results indicate that there was high variation in seminal traits among stallions, which has made it difficult to clearly determine seasonality in most seminal traits. Because of this high variation, variable analysis methods were applied and showed that there were, in fact, seasonal variations in some seminal traits throughout the calendar year.

Prior to this study, there were limited reports on seminal traits in Przewalski's stallions. One study (Bader *et al.,* 1991) evaluated seminal traits in stallions that were considered sub- or infertile. Five stallions were collected by either electro-ejaculation (n = 10 collections) or by artificial vagina (n = 30 collections). One stallion was aspermic in all 5 semen collections using both techniques. In the present study, only one stallion (7.69%) failed to produce sperm after 4 attempts at semen collection. While several aspermic collections may indicate lack of spermatogenesis for an individual stallion, we were unable to collect semen from a stallion that produced several foals, indicating a potential issue with collection technique. Comparison in seminal parameters between samples collected from two Przewalski's stallions using artificial vagina vs. electroejaculation showed that the former technique yielded higher sperm concentrations ($6.72 \pm 3.0$ vs.$0.48 \pm 0.26 \times 10^9$ spermatozoa) and volume ($34.5 \pm 11.8$ vs. $18.0 \pm 7.5$ mL) than the latter (Bader *et al,* 1991), which had a 52% decrease in volume; a 90% decrease in sperm concentration; and a 17.5% increase in morphological defects when comparing samples collected by electro-ejaculation to samples collected by artificial vagina (Bader,

*et al.,* 1990). While electro-ejaculation is used in many species, there is still the possibility that this collection method may influence seminal parameters overall. Electro-ejaculation has been used extensively in non-domestic species and most species respond by producing seminal fluid containing spermatozoa (Howard *et al.,* 1986). Unfortunately, for many of these species, normal ejaculates have not been defined; therefore it is difficult to determine whether there is a real difference in seminal traits collected by electro-ejaculation compare to natural methods. For instance, in men, where electro-ejaculation is used to collect semen in paraplegic individuals, there are studies that state that there are damaging effects to sperm motility (Linsenmeyer *et al.* 1989; Sikka *et al.,*1994) and others that state that there is no effect on seminal characteristics in men (Hovav *et al.,* 2002) or dogs (Ohl *et al.,* 1994). Spermic sample can also be influenced by technique. In rams, the ejaculatory response is significantly affected by duration of stimulation and rest periods (Martin, 1986). Unfortunately, semen collection by electro-ejaculation has been unsuccessful in the domestic stallion (Cary *et al.,* 2004), so there is little information that could indicate a difference between collection techniques in equid species. Therefore, it remains possible that variation in seminal traits may have been caused by electro-ejaculation.

It was noted that there was a wide variation in sperm concentrations within and among individuals (Range: 0.56 – 16.8 x $10^{9}$ spermatozoa/ collection). This is similar to that reported in previous studies, in which total sperm concentration ranged from 0.48 – 7.18 x $10^{9}$ spermatozoa (Bader *et al,* 1991). In the domestic stallion, a wide range of total sperm numbers has also been observed among individuals (4 to 12 billion in a mature stallion; Blanchard *et al.,* 2003). Lower total numbers of spermatozoa in

Przewalski's stallions may also be a species characteristic as there has been variation in domestic stallions based on breed such as the Warmblood (Range: 9.5 – 10.6 x $10^9$ spermatozoa; Jannett *et al.,*, 2003), the Frances-Montagnes (Range: 5.4 – 8.3 x$10^9$ spermatozoa; Jannett *et al.,* 2003), the Throughbred (Mean: 5.03 ± 0.01 x $10^9$; Dowsett and Knott, 1996), and the pony (Mean: 1.12 ± 0.04 $10^9$; Dowsett and Knott, 1996). Based on mean TTV (212.31 ± 16.87 $cm^3$) for the population of Przewalski's stallions, the EDSO should be 3.49 x $10^9$ spermatozoa/ collection, which is higher than the actual sperm output for all semen collections (2.69 ± 0.27 x $10^9$ spermatozoa). The EDSO is also lower than TTV and EDSO recorded in the domestic stallion (Johnson and Thompson, 1983; Blanchard *et al.,* 2003). Specifically, TTV of domestic stallions (n = 7; mixed breeds) 13-20 years of age is 306 $mm^3$, which would result in an EDSO of 5.74 x $10^9$spermatozoa/ day (Johnson and Thompson, 1983). However, a more recent study (Dowsett and Knott, 1996) states that actual DSO for stallions that were 14 years of age (n = 81; mixed breeds) was 3.25 ± 0.03 x $10^9$ spermatozoa, which is lower than EDSO calculated in previous study, indicating a possible actual decrease in sperm production in aged stallions.

Data from the present study indicates that motility is higher for this population of stallions compared to those reported in previous studies (Bader *et al.,* 1991; Durrant, 1990), although previous studies do no indicate whether motility was assessed in extended or raw samples. Assessing motility in raw samples can be unreliable because sperm cells may agglutinate, causing the overall motility to be lower (Malmgren, 1997). Extending semen also reduces the influence of sperm concentration and pH, which was slightly basic in this study (mean, 7.72 ± 0.29). In the current study, there was an

improvement in motility in extended samples (61.78 ± 1.76%) compared to the raw ejaculates (42.24 ± 2.27%). Again, there was a wide range in motility parameters within and among stallions (Range, 0 – 85% motility). This variation is similar to motility seen in other studies conducted on Przewalski's stallions. For example, Bader *et al.* (1991) observed sperm motility from 5 – 73.5%, and Durrant *et al.* (1990) observed sperm motility from 15 – 56%.

In the domestic stallion, the relationship between sperm motility and fertility are contradictory. In many studies, percentage of motile spermatozoa did not influence overall stallion fertility (Dowsett and Knott, 1982; Voss *et al.,* 1981). However, it has been shown that the subjective appraisal of progressively motile sperm has a high correlation with stallion fertility (Jasko, 1992). In our present study, percentage progressively motile sperm in the Przewalski's stallion was 52.1 ± 1.7 %, and there were wide variations among individuals (0 – 78.75%). Overall, based on these data, progressive motility assessed in this study is comparable to that of the domestic stallion despite the different methods used (Jasko, 1992; Gambo *et al.,* 2009; Blanchard *et al.,* 2003).

Sperm morphology has become an important parameter for assessing overall semen quality associated with fertility (Jasko, 1992; Malmgren *et al,* 1997), because disturbances in spermatogenesis give rise to many morphological defects. This study has shown that there are a very high number of morphologically abnormal spermatozoa in this group of Przewalski's stallions (mean, 19.13 ± 2.70 %; range, 10 – 77%). This is much higher than numbers observed in the previous study (range, 38.3 – 52.3%; Bader, *et al,* 1991). The difference between the two studies may be due to the difference in

stallions and in number of observations (56 vs. 32) as well as observer subjectivity. However, overall numbers did not differ significantly from our initial reports conducted in nine Przewalski's stallions housed at three different institutions (Normal = 23.4 ± 2.7% morphologically normal spermatozoa; Collins *et al,* 2006). In the domestic stallion, a wide range of morphological sperm abnormalities may be acceptable for fertile stallions and the effects of specific morphological sperm abnormalities on the fertility of stallions has not yet been fully elucidated (Malmgren *et al.,* 1997), mostly due to conflicting findings. Jasko (1990) and Morrell *et al.,* (2009) demonstrated that sub or infertility was linked to sperm structural morphology, while others showed no relationship between the two parameters (Dowsett and Knott, 1982; Voss *et al.,* 1981). Jasko *et al.* (1992) performed the most comprehensive study of the relationship between semen quality parameters and fertility, and found reasonable correlations between percentages of progressively motile ($r$ = 0.46) and morphologically normal ($r$ = 0.36) sperm and fertility. As a result, the horse industry combine morphological data and motility into a single parameter, total progressively motile morphologically normal (TPMMN) spermatozoa/ejaculate, as a tool to assess potential fertility in the domestic stallions (Hurtgen, 1992; Blanchard *et al.,* 2003). Based on data collected, the TPMMN spermatozoa in this population of Przewalski's stallions is $0.74 \pm 0.16 \times 10^9$. This is lower than the acceptable level used in assessing the domestic stallion ($1.0 \times 10^9$ TPMMN spermatozoa; Hurtgen, 1992), however, further studies need to be conducted to determine whether Przewalski's stallions that are fertile require the same number of TPMMN spermatozoa as the domestic stallion.

By analyzing mean values for each season, we were able to detect seasonal variation in seminal pH and volume but not in concentration, motility, TTV or normal morphology. Using variable analysis, we were able to show that percentage normal morphology is improved in late summer and winter while total concentration is highest in late fall and late spring. Overall, these data indicate that improved sperm quality occurs during the breeding season as there is an increase in total numbers and percentage normal morphology. We were unable to detect seasonal changes in motility in our analysis. These findings differ from those of the previous study by Durrant (1990), which evaluated ejaculates from ten Przewalski's stallions over a 1-year period. According to Durrant's study, percentage motility was higher in fall and spring and numbers of morphologically normal sperm were highest in the winter. The study also showed that total volume was highest in the spring, which was similar to results found in the present study, although we found no significant difference in total volume between spring and summer ($P > 0.05$). Our study also found that seminal pH was significantly higher ($P < 0.05$) in the winter compared to all other seasons. Seminal pH in the domestic stallion is influenced by season, frequency of ejaculates, and spermatozoal concentration (Blanchard *et al., 2003).* More recent research has also shown that levels of the proteolytic enzyme, plasminogen activating factor, also vary throughout the year in the stallion (Zervos *et al.,* 2010). While it has not been linked to changes in pH, variations in seminal plasma could impact overall pH.

In the domestic stallion, it has also been difficult to determine whether there is seasonality in seminal traits. Despite the fact that there have been studies describing increased daily sperm production in domestic stallions during the breeding season

(Thompson *et al.,* 1977; Johnson, 1985), more recent studies have been showing that this is not always the case. For example, a study by Janett *et al.* (2003) states that motility of semen samples from Franches-Montagnes stallions is best in winter, spring and summer and that percent morphologically normal is highest in the autumn. However, the lower quality sperm traits in the summer are countered by the higher sperm concentration. A second study in Warmblood stallions contradicted previous results in that motility and total concentration were highest in summer and percentage morphologically normal sperm was highest in spring (Janett *et al.*, 2003). This conflict of defining seasonality in the stallion is further confounded by a recent study of spermatozoa from eight stallions of varying breeds that showed improved mitochondrial potential, intact acrosomes and membrane stability with increasing photoperiod (Gamboa *et al.,* 2010). Interestingly, low progressive motility and sperm concentration also occurred with increasing photoperiod, indicating that descriptive seminal traits (motility, morphology and concentration) are not always indicative of seasonal variation of fertility in stallions.

Because a high level of variation in many of the seminal traits was detected over seasons in our current study, analysis was performed to determine whether there were variable changes in seminal traits throughout the calendar year that may not have been detected in previous analyses. It was determined that there are variable changes in concentration, normal morphology, volume and pH. Based on variable analysis, our data showed some similarities to that of the previous study by Durrant (1990). Variable analysis revealed that morphologically normal sperm were highest in winter and volume was greatest in late spring. Durrant (1990), also described volume as highest in spring and morphologically normal sperm were highest in winter. In the domestic horse,

seminal volume is increased from May to August and shows a decrease in the winter months (Clay *et al.,* 1987). The reason for the decrease is still not understood but seminal volume is impacted by sexual stimulation, which is increased in the summer months (Pickett, 1993). Also, many stallions will have high sperm output in winter months despite lower total ejaculate volume; therefore, total volume is not considered important in relation to stallion fertility (Clay *et al.,* 1987). In the domestic stallion, there have been mixed results about when improved percentage morphologically normal sperm occurs in the year, but many report improved levels in autumn and/ or winter (Magistrini *et al.,* 1987; Blottner *et al.* ,2001; Jannett *et al.,* 2003). This could be impacted by seasonal changes in Sertoli cell population and hormone production in the testes (Johnson and Thompson, 1983; Johnson *et al.,* 1991). Because sperm maturation occurs in the epididymis, there are possible seasonal changes in the epididymis that have been reported in other seasonal breeders, such as the Roe deer, that may impact maturation and morphology of sperm (Schon and Blottner, 2009). Spermatogenesis is also a temperature sensitive process and slight increases in temperature can disrupt the process due to hypoxia (Lue *et al.,* 1999). However, in one study in the stallion, scrotal insulation, had little effect on sperm maturation in the epidiymis, but was more likely to impact primary spermatocytes (Love and Kenney, 1999). While there was no evaluation of morphological changes due to increasing temperatures, the higher temperatures in the summer months compared to winter months may have impacted percentage morphologically normal sperm cells in the Przewalski's stallions.

Because there was little variation in motility, progressive motility, total progressively motile sperm, and total testicular volume, variable analysis on these

seminal traits were not performed. Because stallions in this study were only collected once in each season, variation due to stallion and collection techniques may have made it more difficult to detect seasonal effects. In future studies, more frequent collections in each season should be conducted in order to determine seasonal effects on seminal traits in Przewalski's stallions.

More recent research (Scaramuzzi and Martin, 2008) has also shown that nutrition has a an impact on seasonality in that horses on a high nutritional plan in the winter months show fewer signs of anestrus or decreased reproductive activity. Przewalski's stallions in the wild survive in the Gobi Dessert where food is scarce in the winter months due to temperatures reaching -15° to -18° C (Groves, 1994). In the spring and summer months, grass is plentiful and condition of Przewalski's horses improves. At the SCBI and the Wilds, all horses were maintained on similar diets throughout the year, and, at the SCBI, weight is monitored regularly which influences diets of each individual animal. As a result, there is little fluctuation in weight of animals from winter to summer compared to animals in the wild. As there is limited research in nutrition of Przewalski's horses, especially in relation to reproduction, it is possible that condition of stallions may have masked seasonal effects more than would be expected of the *in situ* population.

It has been shown in the domestic stallion that frequency of collections influences both quality and quantity of spermatozoa due to extragonadal reserves (Picketts, 1985; Blanchard *et al,* 2003). These reserves are primarily stored in the tail of the epididymis and can be 3 -4 times higher than the actual daily sperm output for the stallion, thus impacting total sperm output (Pickett, 1993). Sperm reserves of sexually rested stallions also increase with age and approximately twice the number of spermatozoa are present in

the body of the epididymis of 10 to 16 year old stallions ($9.5 \times 10^{9}$) compared to 2 to 4 year old stallions ($4.2 \times 10^{9}$; Amann *et al.,* 1979). Because of this, estimation of total sperm numbers based on a single semen sample collected from a sexually rested stallion may be increased (Love, 1997). In the current study, stallions were collected once in a 3-month period. In future, more frequent collections from stallions that are not sexually rested may show less variability in seminal traits and be more representative of actual sperm quality in a breeding situation.

In this study, age and coefficient of inbreeding were heavily confounded in the overall stallion effect; therefore, we were unable to determine whether age or inbreeding had a significant impact in seminal parameters. Stallion effect did have a significant effect on all traits ($P < 0.05$) except seminal pH. One study (Durrant, 1990) did state that age did not have a significant effect on seminal traits. Contrary to this, in domestic stallions > 11 years of age, there is a reduced quality in seminal parameters (Dowsen, 1996). The population of *ex situ* Przewalski's horses in North America are composed of mostly older animals where the mean age is $13.62 \pm 1.08$ years and 26 of 35 total stallions (74.29%) in the SSP population are > 11 years of age (Powell, 2010). In the current study, 10 of the 14 stallions studied (71.4%) were > 11 years of age. Even though the population in this study is similar in age to the overall SSP population, having such a high number of older stallions in a study may have impacted the overall seminal traits. It has been shown in the domestic horse, that daily sperm output and testicular size decreases in stallions >13 years of age, although percentage motility and morphologically normal sperm were not impacted (Pickett *et al.* 1988). This is expected because it was determined that efficiency of sperm production is lower in older stallions (Amann *et al.,*

1979). Other studies have also determined that Sertoli cell numbers in the testes decrease in stallions with age (Johnson and Thompson, 1983). This would indicate that sperm production would be decreased due to not only decreased hormone production but also decreased supporting cells in the testicles. Future studies need to look at seminal traits stallions over time in order to assess seminal traits and whether they change in individual stallions with age.

In summary, we determined that individual stallion effect had a significant impact on seminal traits in Przewalski's stallions. Since the stallion effect includes individual, age and genetics, all of these factors could be impacting our data. Based on study design, we were unable to analyze age and inbreeding as separate effects from individuals, therefore we were unable to determine whether inbreeding has a significant effect on seminal traits of Przewalski's stallions. Also, this is the first study to assess seminal traits in Przewalski's stallions over multiple years, which is important in characterizing reproductive physiology in this species. Because of this we further defined seasonal variation in traits such as concentration, morphology, pH and volume. What is most concerning is the low number of morphologically normal sperm cells among all males, which may be one of the causes of sub-fertility in the *ex situ* population. Most importantly, we have determined that there is an increase in total sperm numbers and morphologically normal sperm cells during spring, which correlates with the breeding season. Since epididymal and testicular function has been shown to vary according to season in other seasonal breeders, these data could also indicate a change in spermatogenesis in the Przewalski's horse that could be related to photoperiod or nutrition. Future studies need to be conducted to determine whether cryosensitivity and

fertility also varies with season, which is very important for long term preservation of valuable genetics in this population.

# CHAPTER FIVE

## Overall Significance and Future Directions

The research in this dissertation has further characterized many aspects of male and female reproductive biology in the Przewalski's horse. Because we had unique handling facilities, we were able to monitor follicular development in Przewalski's mares, something that has yet to be accomplished in wild equids. By combining follicular and endocrine data, we have developed a greater understanding of reproductive physiology in Przewalski's mares by reaffirming previous data on the length of estrus and also defining the length of diestrus. We were also able to describe follicular changes in relation to hormonal changes which aided in the detection of sub-fertility in this population. It was determined that mean kinship is, in fact, impacting normal reproductive patterns in mares. This is especially concerning because there are no new founder animals that may contribute to improving heterozygosity in this population. Because the SSP population has an uneven representation of some founders, future research should be conducted in order to determine whether certain founder genetics are impacting reproduction in Przewalski's mares.

In our second study, we were able to demonstrate that the estrous cycle of Przewalski's mares can be manipulated by exogenous progestagens and gonadotroophins. Injectable altrenogest was as efficacious as the oral form, which is advantageous in wild

equids that are difficult treat with oral meds. This study also further confirmed the similarities between domestic mares and Przewalski's mares and their cyclic patterns. This is important because assisted reproductive technologies, such as AI, that are commonly utilized in the domestic horse for breeding management, may be applicable to the breeding management of Przewalski's horses.

In our third study, we were able to provide extensive information on seminal traits from Przewalski's stallions. We were also able to show, through predictive analysis, that there are seasonal variations in some of these traits. Because these changes may reflect testicular or endocrine function, it is important to understand these changes, especially when designing methods of cryopreservation. What was most alarming about this study was the high percentage of morphologically abnormal sperm detected among all stallions. While we were unable to show that inbreeding impacted seminal traits, research in other ungulate species has shown that inbreeding impacts percentage normal sperm.

This research is important because it is the first to demonstrate that genetic management of Przewalski's horses is important for long term survival of this species. Currently, breeding management of Przewalski's horses is managed by the mean kinship values, which indicates inbreeding of offspring of potential breeding pairs, yet this is not always a good indication of development of genetic defects. In other wildlife species, research is starting to show that breeding management based on mean kinship is not effective at preventing development of deleterious genes because it is only an indication of homozygosity. Because there is evidence that inbreeding is impacting infertility, future research should focus on whether over represented founders in the population are actually contributing deleterious genes that are impacting reproductive fitness in mares and

stallions. This could provide information that would encourage breeding of underrepresented founder genetics in this population.

While we were not able to determine that inbreeding or age had an impact on sperm output in Przewalski's stallions, we did determine that there is a high percentage of abnormal spermatozoa in this population of stallions. The most prevalent abnormality was the presence of proximal cytoplasmic droplets, which, in many species, indicates a possible sperm defect that occurs during spermiogenesis (Nöthling *et al.,* 1997). The fact that this defect was so prevalent in this population could indicate that there is an issue in the population. We did not assess inbreeding in relation to defects, so future research should focus on particular morphological defects in relation to genetics of the population. While sub-fertility has been described with these defects in the dog, there is no research to indicate whether the presence of PCDs impact fertility in the Przewalski's horse. Future research should be focused on whether the morphological defects that we have detected in this population can be overcome by assisted reproductive technologies, such as artificial insemination.

We were able to determine that Przewalski's mares have a similar reproductive cycle to the domestic horse. We were also able to show that mares have a similar response to estrous manipulation when given therapies that are used in domestic mares. This is important because ART in the domestic mare can be utilized for breeding management of Przewalski's mares. Future research should focus on describing LH in the Przewalski's mare, which would provide a better understanding of the physiology of ovulation. Development of artificial insemination, embryo transfer and *in vitro* fertilization in this species would also aid in better long term management of this species.

While development of ARTs is crucial, it is important to stress that genetic research in this species is necessary in the short term. Now that entire genome the domestic horse has been described, it has been shown that there are many similarities to the human genome. This makes the possibilities for looking at genetic changes in the Przewalski's horse limitless and, because they are an inbred population, they could serve as a model for genetic disease in the domestic horse and possibly, humans.

# APPENDIX

# APPENDIX A

In order to perform repeated follicular assessments of Przewalski's mares, a training program was initiated to acclimatize mares to repeated procedures without requiring frequent sedation or anesthesia. Both the Smithsonian Conservation Biology Institute (SCBI) and the Wilds had incorporated a hydraulic tamer system (Fauna Research, Red Banks, NY, USA) into handling facilities for large ungulates. At SCBI, the tamer was located in the hoofstock barn at the end of a chute system designed for movement of animals (Figure 1). At the Wilds, the tamer system was connected to the hoofstock hospital and was connected to a chute system which connected several enclosures (Figure 2).

The hydraulic tamer is a mechanized system that is designed to handle large ungulate species safely so that sedation and anesthesia is not required for repeated procedures (Figure 3). The hydraulic tamer actually immobilizes an animal by lifting it off the ground so that it is unable to move forward. Two padded walls hold the animal in place: One wall is fixed while the other is moveable. The moveable side is connected to hydraulics that is controlled by an operator. There is also a safety mechanism that releases the hydraulics when the pressure is higher than is deemed comfortable for the animal. Both the moveable and fixed sides are able to tilt at slight angles so that animals of different sizes may be lifted for procedures.

Mares at SCBI (n = 14) were trained four months prior to research procedures. At the start of the training period, mares were run, once per week for a three week period, through the chute system with the hydraulic tamer fully open as part of their movement from barn to pasture. Mares were then run through the chute system with the hydraulic tamer fully open three days a week as part of their movement from barn to pasture for one week. Each week after that, the hydraulic tamer was closed gradually so that the width between outer and inner pads decreased slightly. Once the tamer pads were at a diameter that was deemed satisfactory for lifting animals (Figure 4), each mare was then walked through the chute system and stopped in the tamer briefly by closing the front door of the

tamer. After 2 – 4 seconds, the door was opened and the mare was allowed out of tamer. This was conducted for one week. Mares were then kept in the tamer by closing both front and back door. Once each mare was quiet, the front door was opened in order to encourage mares to not struggle in the tamer.

The next stage of the training was to lift mares with the hydraulic tamer. Each mare was lifter for 2 – 4 seconds and then let down. After the mares were let down, the front door was opened to release the mare once she was quiet. Each mare was rewarded with an apple biscuit after each attempt. Mares were lifted once per week for a four-week period. Two other days per week, each mare was stopped in the tamer without being lifted. The other two days, mares ran through the tamer system without being stopped to acclimatize mares to tamer procedure and also so that animals would not anticipate procedures each time they were run through the chute system.

After the training period, mares were then examined three days per week (Monday, Wednesday and Friday) using rectal ultrasonagraphy. Each mare was moved into the tamer and lifted. Feces were evacuated from the rectum and the linear array probe was introduced in a gloved hand (Sonovet 2000, Medison America, Inc., Cypress, CA, USA). Both left and right ovaries were then examined for structures (follicles, anovulatory follicle, corpora lutea), which were noted. Both uterine horns and the uterine body were then examined for edema and uterine fluid, which was noted if present. Once the exam was complete, mares were placed on the ground and the front door was opened so that mares could leave the tamer. Each mare was rewarded with an apple biscuit after each examination.

Each examination performed was kept under a five-minute period. If a mare was not picked up correctly after three attempts, no examination was performed that day. Also, if a mare struggled or appeared too stressed, she was released and no examination was performed on that day. On days that examinations were not performed, mares were run through the chute system with the tamer fully open. When research studies were not being conducted, mares were run through the chute system 1 – 3 days per week as part of their movement from barn to pasture.

At the Wilds, mares used for the research study (n = 5) were moved to the hoofstock hospital 2 – 4 weeks prior to research trial. Because the tamer was used for other procedures, mares were only run through the chute system on days of examination. Rectal examinations were performed as described above on the same days as examinations at SCBI. Once the research study was complete, mares were then returned to pasture.

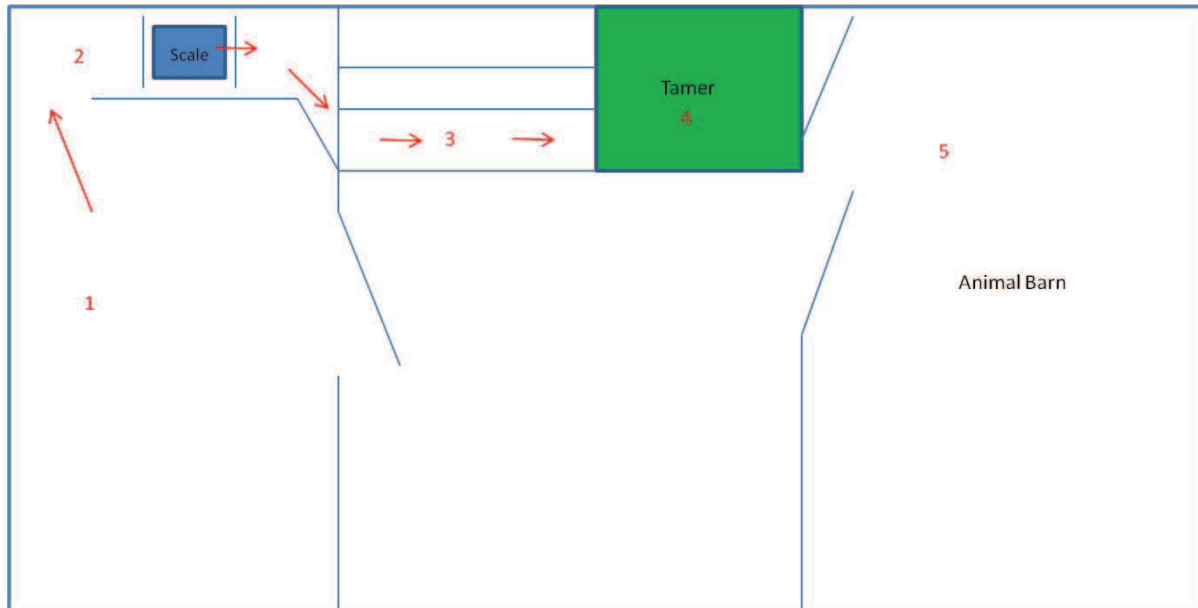



Figure 1: Schematic of chute and tamer in hoofstock barn (SCBI). Movement pattern is depicted by numbers: 1) mares brought into holding area in small groups (1 – 4 individuals); 2) individual mare led into chute system and weighed on scale if needed; 3) each mare was then run down one chute towards tamer; 4) examination in tamer or run through on off days; and 5) mares released to barn area after examination and given apple biscuit.

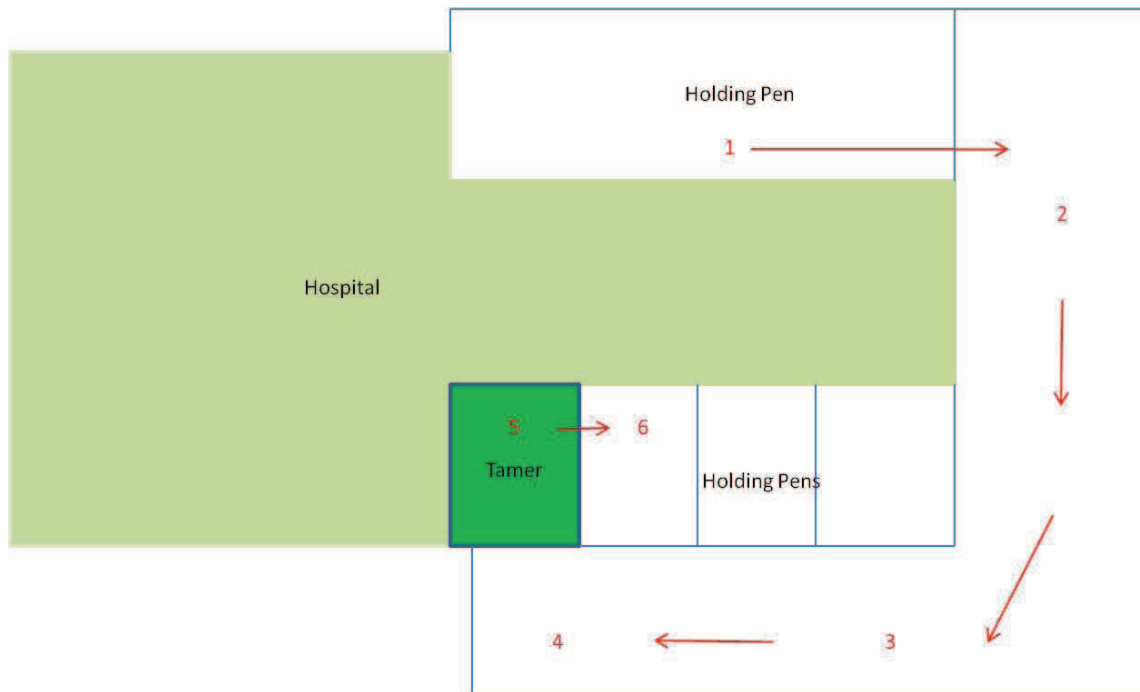



Figure 2: Schematic diagram of chute and tamer system at the Wilds. Mares for research were held in holding pen (1). Movement pattern was as follows: 1) Individual mare led out of holding pen; 2) mare led down the chute area with a series of gates that close once mare has passed through; 3) mare continues down chute area ; 4) push gate allows mare to enter tamer without backing up; 5) rectal examination is performed; and 6) mares are held in small holding pen until examinations are complete and are then lead back up chute to holding pen 1.

Figure 3: Photograph of hydraulic tamer used for rectal examinations of Przewalski's mares. The visible side is the moveable wall of the tamer that is used to widen the diameter between inner pads.

Figure 4: Photograph of tamer demonstrating the width of the pads when open for procedures. The pads were just wide enough to allow each animal through the tamer.

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