Methods of Reproductive Control in Domestic and Free-Roaming Horses and the Behavioral Implications

by

Christina Negretti

THESIS

submitted to

Oregon State University

Department of Animal and Rangeland Sciences

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the requirements for the

degree of

Baccalaureate of Science in Animal Science

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AN ABSTRACT OF THE THESIS OF

Methods of Reproductive Control in Domestic and Free-Roaming Horses and the Behavioral Implications

The current American free-roaming horse (*Equus caballus*) population far exceeds the appropriate population limit set by the Bureau of Land Management (BLM) to preserve resources on public lands. Several techniques have been tested to assist in decreasing repopulation rates. The purpose of the present study was to analyze two contraceptive techniques, one that is reversible and targets domestic mares and one that is irreversible and targets free-roaming stallions. Administration of exogenous progesterone or progestin have been shown to suppress estrous behavior in mares. Five regularly cycling domestic mares were treated with 2 g of long-acting progesterone two days after luteolysis. Daily stallion exposure was used to score estrous behavior, and blood serum progesterone concentrations were tested every other day. Serum progesterone concentrations were significantly elevated (>2 ng/mL) for 10 days following treatment with a long-acting injectable progesterone. Behavioral estrus suppression during this period was minimal and not significant. All mares developed local injection site reactions following treatment. Due to the side effects associated with this treatment, long-acting formulations of injectable progesterone are not appropriate for use in performance horses. In many species, male social rank has been found to correlate with testosterone concentration, with more dominant males having higher circulating testosterone. Fifteen free-roaming stallions gathered for the purpose of castration were behaviorally evaluated for two days to assess social hierarchy status. These males were categorized into three groups. Mane hair and serum samples were collected from each stallion and testosterone and cortisol concentrations were evaluated. There were no significant differences found between hair and serum hormone concentrations and observed behavior. There was also no correlation found between serum and hair testosterone or cortisol concentrations. The present study could be used across various free-roaming horse ranges where range managers are making decisions about population control tactics with the most regard to animal welfare.

Keywords: Cortisol, equine, mare, progesterone, stallion, testosterone

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I understand that my project will become part of the permanent collection of Oregon State University Scholars Archive. My signature below authorizes release of my project to any reader upon request.

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Christina Negretti, Author

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I. Introduction

I.A. Statement of problem

The growing population of American free-roaming horses (*Equus caballus*) currently inhabiting public lands has posed problems for many fields. When the Wild Free-Roaming Horses and Burros Act of 1971 was signed into law, the provisions assigned the Bureau of Land Management (BLM) and the Forest Service as responsible for the "management and protection" of these horses "in a manner that is designed to achieve and maintain a thriving natural ecological balance on the public lands" [1]. With a lack of natural predation, the free-roaming horse and burro population has increased annually by 20-25% [2]. The current population stands at about 81,951 free-roaming horses and burros and is confined to 10 states [3]. The BLM estimates that 26,690 individuals would be an appropriate population limit that would allow for conservation of the natural resources on the public lands [3,4]. Reducing the free-roaming horse and burro numbers has become a priority by the BLM in order to conserve natural resources and ecological integrity.

Population management used by the BLM includes removing thousands of free-roaming horses and burros annually into Herd Management Areas [5]. The animals are then adopted and sold into private care [6]. Housing, feeding, and caring for unadopted and unsold animals has cost the BLM about $80 million per year for the last six years [6]. With the BLM housing 50,020 animals in off-range facilities, an incentive program has recently been implemented where adopters can receive up to $1,000 when they adopt a horse or burro [7].

I.B. Contraception options for stallions

Regulating reproduction rates with fertility control in wildlife has been used across various species and provides a potential solution to managing free-roaming horse populations reducing the need to remove free-roaming horses and burros from ranges [8]. Harems of horses, like other equids, exhibit female-defense polygyny mating systems where dominant stallions are the only mating males in a harem and control females by defending them against bachelor males [9,10]. Irreversible contraceptive methods have been evaluated to disrupt this dominant breeding structure. Vasectomies and castrations were performed on dominant stallions that were then released back to their respective ranges. Initially, few castrations were performed, but has not been widely studied as a contraceptive tool for free-roaming horses due to concerns about behavior alterations and the resulting effects on social order within harems and bachelor stallions [11]. Surgical vasectomies have been found to moderately reduce birth rates in subsequent years following vasectomies, but bachelor stallion reproductive success extended the breeding and foaling seasons [8,9]. Chemical vasectomies failed to provide complete sterilization [11].

Few reversible contraceptives targeting stallions have been analyzed. Microcapsulated testosterone propionate is a reversible contraceptive identified to suppresses spermatogenesis and inhibit fertility for the duration of one breeding season [12]. Microcapsulated testosterone propionate did not affect libido or observed dominant stallion behavior, but only had a limited decrease in foaling instances and prolonged the breeding season [12]. Other reversible contraceptive compounds, such as testosterone cypionate, quinestrol, 17β estradiol, and α-chlorohydrin, have been tested on domestic pony stallions, but have not been applied to feral horse management [13].

The main concern regarding using stallions as targets for contraceptive, reversible and irreversible, is the possibility of lengthening the breeding and foaling seasons from the later success of bachelor stallions and interfering with harem social hierarchy by reducing testosterone, which alters stallion behavior [5, 11].

I. C. Contraception options for mares

Due to patriarchal hierarchy within breeding groups of horses, mares have been the major target of contraceptive methodologies both in free-roaming and domestic herds. Like the free-roaming stallion, few irreversible contraceptive methodologies for the mare have been extensively researched. Removing the ovaries (ovariectomy) is the main method that has been performed [14]. This procedure can be performed by making an incision through the flank (laparotomy) or through the vagina (colpotomy) [15]. Ovariectomy is not commonly performed because mares that undergo this procedure have to be gathered and housed in off range facilities. In addition, there has been limited large scale testing on the impact performing ovariectomies has on reproduction rates and social hierarchy [16].

Non-surgical reversible contraceptives have been more extensively researched for the mare than irreversible methods. Immunocontraceptives are one category of contraceptive that utilizes the production of antibodies against specific ovarian targets to inhibit reproduction [17]. Porcine zona pellucida (PZP) is an immunocontraceptive that stimulates the production of zona pellucida antibodies that prevent sperm from binding to the surface of the ovum and limits pregnancy [18]. PZP decreases fertility in free-roaming mares by up to 94% for 1 year with few

short-term behavioral changes observed. In addition, there is a general decrease in production of ovarian estrogen and ovulation rates as a result of PZP immunocontraception [18, 19, 20].

Immunization against gonadotropin-releasing hormone (GnRH) prevents endogenous GnRH from binding to GnRH receptors in the pituitary gland. This inhibits the release of follicle stimulating hormone and luteinizing hormone, which prevents estrus cyclicity [21]. GnRH vaccination is an effective contraceptive for up to 3 years and causes infertility in an average of 63% vaccinated horses [22]. Administration of exogenous steroids are another reversible contraceptive that has been utilized in free-roaming mares. Various progesterone and estradiol-based injections and implants have exhibited unreliable results with respect to estrus suppression and have not proven to be effective contraceptives [23]. Transcervically-administered intrauterine devices (IUDs) have been tested for contraceptive use in free-roaming mares. IUDs containing copper have resulted in 80% of mares being infertile the first year but then dropped to 29% and 14% in subsequent second and third years, respectively [24].

I. D. Hypothesis

The purpose of the present study is to analyze two contraceptive techniques, one that is irreversible and targets free-roaming stallions and one that is reversible and targets domestic mares. We hypothesized that these methods would be safe and effective to assist BLM managers in controlling horse populations without resulting in unwanted behaviors.

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II. Effects of long-acting injectable progesterone given two days after luteolysis on estrus suppression in mares

II.A. Introduction

The mare is a seasonally polyestrous long-day breeder with a 21-day estrous cycle [1]. In the northern hemisphere mares typically cycle from mid-February to mid-November, with spring and fall transition periods between February and May and September and October respectively [1]. The length of behavioral receptivity (estrus) during the estrous cycle can range from 4-7 days, with ovulation occurring 24-48 hours prior to the end of the estrous period [1]. Typical estrous behaviors when presented to a stallion or a gelding include squatting with the tail raised, urinating, and everting the clitoris [1]. The mare will cease to show estrous behavior following ovulation, due to the increasing serum concentration of progesterone from the corpus luteum. This phase of the estrous cycle is known as diestrus and usually lasts 15-19 days [1]. Temperament and performance of mares can vary with the ovarian cycle with some mares becoming distracted and unmanageable during estrus [2]. These behaviors are undesirable in performance horses, particularly in the height of the competitive season.

Administration of exogenous progesterone or progestin (synthetic form of progesterone) can suppress estrous behavior in mares. Intramuscularly-administered progesterone suspended in sesame oil can be effective for suppressing estrous behavior but it must be given daily (0.3-0.6 mg/kg of body weight) to maintain effective concentrations of progesterone for blocking estrous behavior [3]. Daily oral administration of altrenogest (Regu-Mate®; Intervet, Millsboro, Delaware, USA), a synthetic progestin, at a dosage of 0.044 mg/kg of body weight is also

effective at suppressing estrus within three days of initiating treatment with a return to estrous behavior four days following cessation [4]. Though effective, it is sometimes difficult to administer a consistent dosage of altrenogest due to poor palatability. In cattle, medroxyprogesterone acetate (Depo-Provera®; Upjohn, Kalamazoo, Michigan, USA), has been effective in suppressing estrus and maintaining pregnancy [3]. However, this product is unable to suppress estrus [5] or maintain pregnancy [6] in mares when administered intramuscularly at 250 mg or 1000 mg per mare, respectively. A single subcutaneous implant containing norgestomet (Synchromate-B®; Searle Agri Inc., Elburn, Illinois, USA; 6 mg/mare) [7] or up to 80 subcutaneous implants containing progesterone (totaling 2 g/mare) with estradiol benzoate (totaling 200 mg/mare) (Synovex-S®; Syntex Animal Health, West Des Moines, Iowa, USA) [8] were also unable to suppress estrus in mares.

A long-acting proprietary formulation of progesterone in a low viscosity non-aqueous liquid is compounded for use in mares at 200 mg/mL (BioRelease® P_4 LA200; BET Pharm, Lexington, Kentucky, USA) [9]. It was determined that serum progesterone concentrations of 2-6 ng/mL could be achieved for about 10 days following injection of 1.4 g of this long-acting progesterone formulation [10]. In a study to determine its efficacy for pregnancy maintenance, Ball and coworkers compared dosages of 0.75 g, 1.5 g and 2.25 g of long-acting progesterone and concluded that the 2.25 g/mare dosage was most effective for pregnancy maintenance [11].

The specific objective for this investigation was to determine if serum progesterone concentrations, achieved from a 2 g injection of long-acting progesterone (200 mg/mL; as recommended by the manufacturer) would inhibit behavioral estrus in mares for a prolonged period. This study will expand the current literature by reporting on the efficacy of a compounded long-acting progesterone formulation for estrus suppression in mares. Based on the

results of previous studies demonstrating prolonged elevation of progesterone concentrations (>2 ng/mL) for at least 10 days [10,11], we hypothesized that this dosage would result in estrus suppression during this period.

II.B. Materials and Methods

Between the months of June and August, five mares of various breeds (Thoroughbred, Appaloosa, Anglo Arab) ranging in age from 13-19 years (mean = 16.2 years) were group-housed and fed a diet of mixed-grass hay and water ad libitum. Subjects were randomly selected from the teaching horse herd at the Oregon State University Carlson College of Veterinary Medicine, located in Corvallis, Oregon, USA. All of the experimental procedures described in this study were approved by the Institutional Animal Care and Use Committee at Oregon State University prior to the research. Timing of ovulation was determined by daily transrectal palpation and ultrasonography, using a 5.0-MHz, B-mode, linear array ultrasound scanner (Sonovet SV600, Universal Medical Systems Inc., Bedford Hills, New York, USA). The mares were monitored for three estrous cycles before treatment and one estrous cycle after treatment to ensure that all mares were cycling normally. Estrous cycling was determined by rectal palpation and ultrasonography. Endogenous progesterone was removed through lysis of the corpus luteum seven days post-ovulation with prostaglandin F2alpha (PGF2α; Lutalyse®; Upjohn Pharmacia, Kalamazoo, Michigan, USA; 10 mg intramuscularly). It was necessary to remove endogenous progesterone so that the serum concentration of progesterone measured and the suppression of estrous behavior recorded was only the result of the exogenous progesterone administered. Two days following PGF2α administration, each mare received a 2 g dose of long-acting progesterone

(BioRelease® P_4 LA200; BET Pharm, Lexington, Kentucky, USA), 10 mL intramuscularly in the neck. All mares received progesterone from the same bottle.

Beginning two days before injection of long-acting progesterone, estrous behavior was determined by individually exposing mares to a stallion every other day until ovulation occurred. A numerical scale for quantifying estrous behavior developed by Gorecka and colleagues [12] (Table II.1) was used to determine the level of behavioral estrus displayed by each mare. The scale used was a one-to-eight scale ranging between completely non-receptive with attempts to attack the stallion to completely receptive to the stallion showing no non-receptive behavior. Blood samples were collected from the jugular vein every other day until the subsequent ovulation. Serum was separated and stored at -20°C until assayed for progesterone. Progesterone concentrations were determined by a commercial solid-phase radioimmunoassay (Coat-A-Count; Diagnostic Products Corp, Los Angeles, California, USA) previously validated for use in mares [13]. The assay sensitivity was 0.02 ng/mL and the intra-assay coefficient of variation was <5%..

Data were expressed as a mean ± standard deviation. Mean serum progesterone concentrations and estrous behavior were compared to pretreatment values over time using an analysis of variance (ANOVA) where treatment was given on day 0. Significance was defined as $p<0.05$.

II.C. Results

All of the mares were cycling normally prior to and following treatment cycle (data not shown). In all mares, serum progesterone concentrations were less than 1 ng/mL on the day of treatment with long-acting progesterone (indicating complete luteolysis). Mean serum

progesterone concentrations were found to be significantly higher on days 1 and 2 compared to day -1 and on days 1, 2, 4, and 6 compared to day 0, confirming the removal of endogenous progesterone concentration (Figure II.1). In addition, serum progesterone concentrations were significantly higher on days 1 and 2 compared to days 8 and 10, demonstrating the clearance of the exogenous progesterone from circulation. None of the mares displayed non-receptive behavior at the time of long-acting progesterone administration (Figure II.2). Estrus suppression was not observed in mares following progesterone treatment in spite of elevated serum progesterone concentrations (>2 ng/mL) ($p>0.50$; Table II.2). Unexpectedly, all of the mares developed diffuse swelling and pain around the injection site that persisted for up to 8 days (Figure II.3). In addition to the local tissue reaction, 50% of mares developed an increased core body temperature greater than 38°C (101°F) beginning one day after the injection and lasting up to four days.

II.D. Discussion

Loy and Swan first demonstrated the estrus suppressive effects of exogenous progesterone in mares [14]. Munro and colleagues [15] found that with few exceptions, estrous behavior was correlated with progesterone concentrations below 2 ng/mL. In the current study, none of the mares demonstrated behavioral estrus suppression following administration of long-acting progesterone (2 g/mare), despite elevated serum progesterone concentrations ranging from 1.07-18.53 ng/mL (Table II.3). It is not known if pharmacologic removal of endogenous progesterone with PGF2α could have affected the behavioral responses to exogenous progesterone. Additional research administering exogenous progesterone to mares toward the

end of diestrus but without artificially shortening the estrous cycle would be needed to answer this question.

In addition to progesterone, estrous behavior is also influenced by serum estradiol 17-β concentrations [16]. Overlapping increasing endogenous estradiol 17-β concentrations with decreasing exogenous progesterone concentrations may explain the failure to suppress estrus (receptive behavior) in the current study. Receptive behavior is variable between mares as can be seen in the current study. Some mares showed receptive behavior only when they were in physiological estrus, but others showed no non-receptive behavior when they were in physiologic diestrus. Decreased uterine and cervical tone, endometrial edema and a growing dominant follicle were observed in all mares following treatment, which culminated in ovulation an average of 13.7 ± 2.9 days following exogenous progesterone treatment. The interval to ovulation following PGF2α administration was similar when compared to a previous report by Loy and colleagues of an average of 10-12days [17]. Although the administration of long-acting injectable progesterone to estrous mares (2 g/mare) was ineffective at suppressing behavior towards the stallion, this study shows that this treatment may serve as a means for ovulation postponement during routine breeding management.

Adverse local reactions similar to those reported here have been observed following subcutaneous injections of 500 mg of crystalline progesterone [14]. However, in the present study, half of the mares also became febrile following the injection. A sample of the progesterone formulation was cultured and found to be negative for the presence of aerobic and anaerobic bacteria. Since no local injection site reactions were seen following the administration of intramuscular PGF2α two days prior to administering the long-acting progesterone, it can be concluded that the adverse reactions observed were direct results of administration of this long-

acting compounded progesterone formulation (200 mg/mL; 10 mL). This is in contrast to a study that found no significant injection site reactions using 300-750 mg (2-5 mL) of a less concentrated progesterone formulation (150 mg/mL) by the same manufacturer (150 mg/mL) for equine pregnancy maintenance where a 10 mL dose was split into two injection sites on the same side of the neck [18]. Further studies are needed to determine if the source of the inflammatory reactions observed in the present study were a result of the concentration of the long-acting compounded progesterone formulation (200 mg/mL) or the volume administered (10 mL).

II.E. Conclusion

In conclusion, we found that administration of long-acting compounded progesterone (2 g/mare) resulted in elevated serum progesterone concentrations for 10 days but did not significantly suppress estrus. Based on the possibility of local and systemic adverse reactions, the use long-acting injectable progesterone formulations are not recommended for estrus suppression in performance horses.

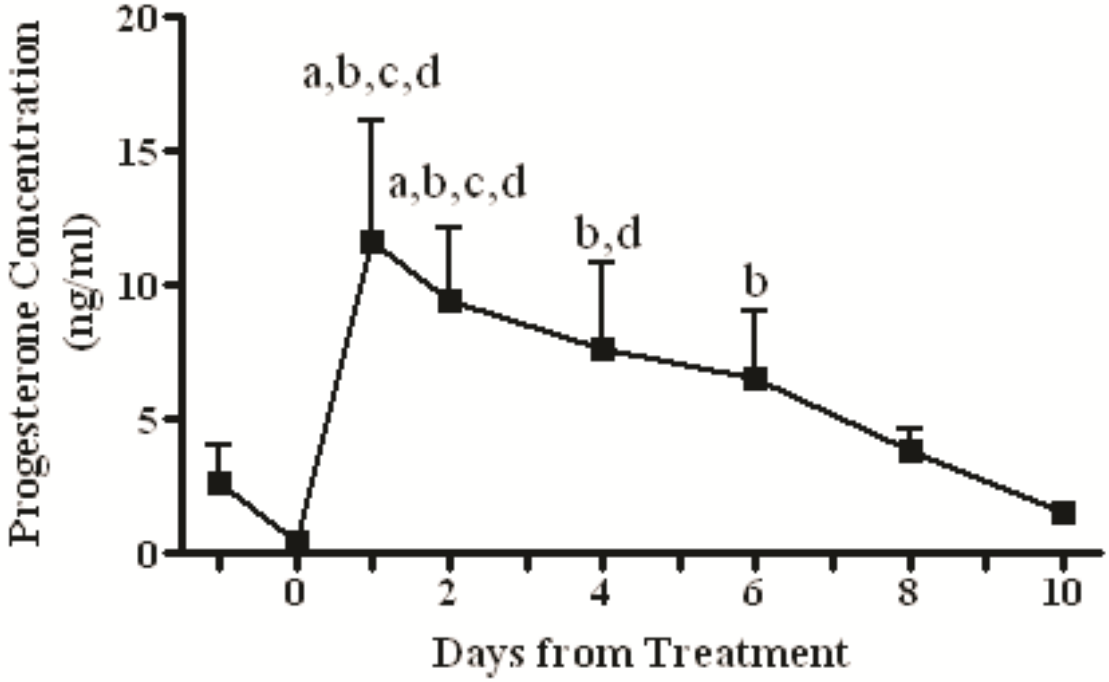

Figure II.1. Mean serum progesterone concentrations one day prior until ten days following intramuscular administration of long-acting progesterone formulation (2 g/mare). a- serum progesterone concentration is significantly different from day -1, b- serum progesterone concentration is significantly different from day 0, c- serum progesterone concentration is significantly different from day 8, d- serum progesterone concentration is significantly different from day 10.

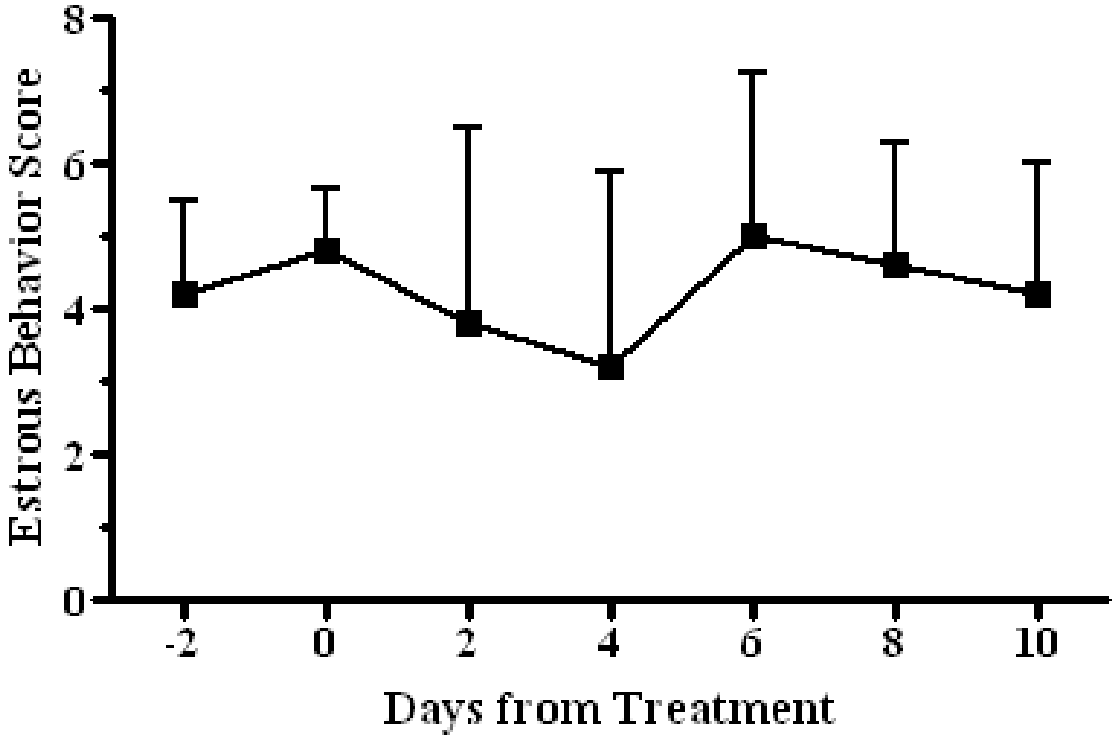

Figure II.2. Mean estrous behavior scores two days prior until ten days following intramuscular administration of long-acting progesterone formulation (2 g/mare). Day of treatment is indicated as day 0. Estrous behavior did not significantly change over time ($p>0.50$).

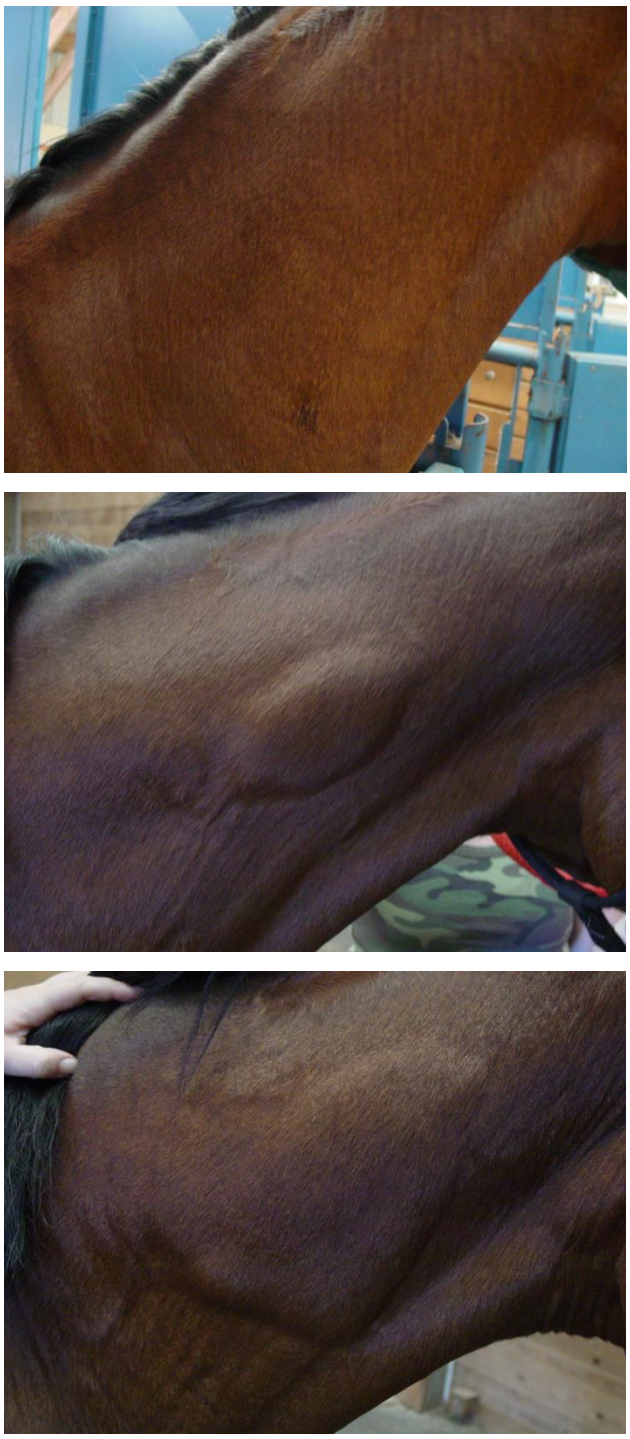

Figure II.3. Typical local reaction that developed following intramuscular administration of long-acting progesterone formulation (2 g/mare) before the treatment (day 0, top), one day after treatment (day 1, middle) and two days after treatment (day 2, bottom).

Score	Description
1	Non-receptive behavior (tail switching, moving around, squealing, holding ears back, attempts to kick); mare attacks or kicks the teasing stallion
2	Non-receptive behavior (tail switching, moving around, squealing, holding ears back, attempts to kick); no severe attack towards the stallion
3	Non-receptive behavior (tail switching, squealing, holding ears back attempts to kick); mare stands still
4	Mare stands still indifferently; neither receptive nor non-receptive behavior
5	Mare shows estrous behavior: stands still, raises tail or winks, accompanied by some non-receptive behavior (tail switching, squealing, holding ears back, attempts to kick)
6	Mare shows estrous behavior: stands still, raises tail or winks: no non-receptive behaviors
7	Mares shows full estrous behavior: stands still, raises tail or winks, pass fluids and lowers pelvis (postures), accompanies by some non-receptive behavior (tail switching, holding ears back, attempts to kick)
8	Mare shows full estrous behavior: stands still, raises tail, winks, pass fluids and lowers pelvis (postures); no non-receptive behaviors

Table II.1. Behavioral estrus scoring rubric. Behavioral estrus was quantified using a one-to-eight scale ranging between completely non-receptive with attempts to attack the stallion to completely receptive to the stallion showing no non-receptive behavior [11].

Mare ID	day -2	day 0	day 1	day 2	day 4	day 6	day 8	day 10
Christian	4	4	4	2	2	2	5	6
Ruby	5	6	2	2	2	6	6	6
Piper	2	4	2	2	2	4	4	2
Rosie	5	5	5	5	2	5	2	4
Tilly	5	5	5	8	8	8	6	3

Table II.2. Individual estrous behavior scores from two days prior until ten days following intramuscular administration of a long-acting injectable progesterone formulation (2 g/mare).

Mare ID	day -1	day 0	day 1	day 2	day 4	day 6	day 8	day 10
Christian	4.44	0.47	13.95	7.50	6.28	7.94	4.54	1.74
Ruby	3.35	0.90	7.18	6.91	5.26	5.83	4.44	1.72
Piper	1.12	0.18	18.53	10.04	4.68	3.01	ov	
Rosie	2.75	0.55	8.86	13.61	9.84	5.87	2.92	1.07
Tilly	1.32	0.00	10.24	9.20	12.16	9.79	3.39	1.56

Table II.3. Serum progesterone concentrations (ng/mL) from one day prior to ten days following intramuscular administration of a long-acting injectable progesterone formulation (2 g/mare). Ovulation (ov) occurred an average of 13.7 ± 2.9 days following exogenous progesterone treatment with one mare having ovulated at day 8.

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III. Comparison of Serum and Hair Testosterone Concentrations in Free-roaming Stallions

III.A. Introduction

In many species, such as rock hyrax (*Procavia capensis*) and white-browed sparrow-weavers (*Plocepasser mahali*), male social rank is correlated with testosterone concentrations ([TEST]) such that dominant males have higher circulating [TEST] [1]. Glucocorticoid stress hormones, such as cortisol ([CORT]) and corticosterone, increase in primates and rodents that belong to lower social ranks [2]. Although [TEST] have been measured in free-roaming stallions previously, it has never been correlated to social hierarchy [3,4,5].

When analyzing hormone concentrations in wild animals, a variety of different samples can be tested, such as blood, saliva, feces, or urine [6]. Unlike the other types of samples, hair is stable at room temperature for long periods and represents average hormone concentrations over time [7].

The current study could benefit behavioral research in wildlife by showing that noninvasive sample collection can be done to evaluate behavior correlations with physiological changes. Also, current managers of the Bureau of Land Management (BLM) wild horse and burro department as well as other range managers working with free-roaming horse populations could benefit from this study when making informed decisions concerning animal welfare and population control.

The purpose of this study was to compare [TEST] and [CORT] in serum and hair from stallions of different social ranks. We hypothesized that dominant stallions would have higher

[TEST] and submissive stallions would have higher [CORT]. We also hypothesized that these differences would be observed in hair samples but not in blood samples do to the minute-by-minute changes occurring in the latter.

III.B. Materials and Methods

Stallions used in this study were part of the free-roaming horse herds (n=15) managed on the Confederated Tribes of Warm Springs Reservation, located in Central Oregon, who oversaw and approved the animal care procedures throughout sample collection. The herds were gathered mid-May 2015 for the purpose of castration. The stallions to be castrated were sorted from the herd and put into holding pens before being individually moved to a chute for castration. Behavioral evaluations were conducted over a two-day observation period while horses were in various holding pens prior to castration. A 5-point behavior score was created with 1 being most submissive and 5 being most dominant. A horse that received a score of 1 exhibited tense behaviors with a high flight response. While a horse that received a score of 5 exhibited behaviors such as fighting, rearing, and lunging toward other stallions.

Samples were collected when each stallion was anesthetized for castration. Blood samples were collected from the jugular vein using a Vacutainer® tube without an anticoagulant in the serum tubes and a 19-gauge needle. Blood samples were allowed to clot and then centrifuged at 3000 rpm for 15 minutes. Serum was pipetted off and stored at -20°C. A mane hair sample was pulled out by the root. The [TEST] and [CORT] were extracted from the hair using a previously described method [1]. Briefly, 100 ± 20 mg of hair was weighed, minced into 3-4 mm pieces, and sonicated in methanol (2 mL) at 20ºC for 30 minutes. The samples were then

incubated overnight at 50°C in a water bath with gentle shaking. The methanol was pipetted off into a new glass vial and evaporated to dryness under nitrogen. The samples were then reconstituted with 125 µL of assay buffer (#80-0170, Assay Designs, Inc., Ann Arbor, MI).

Chemiluminescence (Immulite 1000, Siemens Healthcare Diagnostics, Tarrytown, NY) was used to measure [TEST] and [CORT] from serum and extracted hair samples according to the manufacturer's protocol. For [TEST], the Total Testosterone Kit (#LKTW1) by Immulite was used with an assay sensitivity of 0.15 ng/mL. The serum intra-assay CV was at 12.8% and the hair intra-assay CV was at 10.3%. For [CORT], the Cortisol Kit (#LKCO1) by Immulite was used with an assay sensitivity of 1 ng/mL. The serum intra-assay CV was 5.1% and the hair intra-assay CV was 9.4%.

Behavior scores for each stallion over two days were averaged and then stallions were categorized based upon their average behavior score as either submissive (with a score of <2.5), neutral (2.5-3.5), or dominant (>3.5). Serum and hair [TEST] and [CORT] were compared to the average behavior scores using an analysis of variance (ANOVA). Significance was defined as $p<0.05$.

III.C. Results

Of the stallions sampled, 20% exhibited behaviors that were classified as submissive, 53% were neutral, and 27% were dominant. Contrary to our first hypothesis, serum [TEST] was not significantly higher in dominant stallions and serum [CORT] was not significantly higher in submissive stallions (Table III.1). Hair [TEST] was higher than serum [TEST] in all stallions regardless of social rank. On the other hand, serum [CORT] was higher than hair [CORT], with

the latter measuring just at the assays lower limit of detection. Although the magnitude of these results is modest, they lay the foundation for additional research to be carried out after castration that could have significant implications on animal welfare in regard to herd social hierarchy.

III.D. Discussion

Free-roaming horse management is a complex issue incorporating social, economic, emotional, political, and environmental factors. On federally owned lands free-roaming horses are covered under the provisions of the Wild Free-Roaming Horses and Burro Act of 1971 [8]. However, on Native American owned lands, each tribe can have a different management policy based on its sovereignty. The horse herd reduction program for the Confederated Tribes of Warm Springs includes castration and either the release of geldings back to the range or the selling of geldings to be trained as ranch and rodeo horses.

This is the first study to report on the use of hair samples to measure [TEST] or [CORT] in horses. Hair hormone concentrations differed significantly from serum hormone concentrations. Hormone concentrations deposit into hair over weeks or months and are not subject to fluctuations seen in serum hormone concentrations [1]. In free-roaming stallions, serum [TEST] and [CORT] have been found to be highest at 0800 h, lowest at 2300 h, with oscillations in between [3]. In addition, when stallions are housed with other stallions (as the stallions were in the current study prior to castration) serum [TEST] significantly decreases compared to when stallions are housed with mares [9]. Either may explain the difference observed between hair and serum [TEST].

Cortisol concentrations were low in both serum and hair as well as numerically similar between all three behavior categories. Because stallions were separated from their individual harems and housed together in pens for two days, it is likely that their social hierarchy was disrupted. Literature reveals that correlations between [CORT] and social rank becomes nonexistent when the social hierarchy is disrupted [10]. This may explain why no difference in serum [CORT] were observed across the various social ranks in the present study. In other species, such as the mongoose, [CORT] were similarly found to have no correlation between various males with different social ranks [11].

Our research was limited to a group of fifteen stallions from a single location. Also, there was no follow-up behavioral evaluations after the geldings were released onto the land. Future research should evaluate the long-term effects of castration on [CORT] in free-roaming geldings.

While it was unexpected that [TEST] and [CORT] did not differ between social ranks, these results are important in understanding the social hierarchy of free-roaming stallions before castration occurs and lays the foundation for further studies into the effect of castration after release back onto rangelands.

Our study plays a part in the on-going challenge of finding the right contraceptive strategy to combat the growing free-roaming horse populations that have posed a problem for many fields. Many groups managing free-roaming horse populations have moved to various other contraceptive strategies that are administered to mares over the stallions. This is done mainly due to the chance that male-oriented contraception may disrupt normal seasonal breeding and foaling patterns [12]. Vasectomies were considered for reproductive control of the dominant stallions, but shown to be unsuccessful due to an increase in bachelor stallion reproductive success [13]. Castration has not been widely studied as a contraceptive tool for free-roaming

horses. If castrated stallions are returned to the range, it is important to consider the effect of castration on herd hierarchy and animal welfare.

III.E. Conclusion

It has been concluded from this study that [TEST] and [CORT] did not differ significantly between social ranks among free-roaming stallions. However, hormone concentrations obtained from hair samples were significantly different from serum samples. While it was expected that hair and serum concentrations would differ, the incidence of variance between [TEST] and [CORT] and social rank, needs to be studied further to better understand the social hierarchy of free-roaming horses and the affects population control methodology impact that structure.

Behavior	Serum [TEST] (ng/mL)	Hair [TEST] (ng/100mg)	Serum [CORT] (ng/mL)	Hair [CORT] (ng/100mg)
Submissive	35.3 ± 26.5	188.6 ± 89.6	10.2 ± 5.4	1.0 ± 0.0
Neutral	35.9 ± 26.0	173.4 ± 43.0	9.4 ± 3.0	1.0 ± 0.0
Dominant	71.0 ± 54.2	183.5 ± 42.6	10.9 ± 1.4	1.3 ± 0.6

Table III.1. Mean ± standard deviation in serum and hair testosterone ([TEST]) and cortisol concentrations ([CORT]) taken from free-roaming stallions managed by the Confederated Tribes of Warm Springs Reservation in May 2015. There were no significant differences between the behavior groups.

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IV. Conclusion & Future Directions

While reproductive management has been extensively studied in other wildlife species, techniques for managing free-roaming horses are relatively new. Even reproductive management methods with domestic horses is constantly being evaluated and improved to find the most effective and safe techniques that regard animal welfare.

The present study identified the inability of a long-acting compounded progesterone to suppress estrus in mares despite elevated serum progesterone concentrations for 10 days. In contrast to previous studies, local and systemic adverse reactions occurred following administration of this product.

The present study was the first to use hair samples from horses to analyze the endocrine profiles. This method could be used in future studies where physiological information about long term endocrine status is desired. Testosterone and cortisol concentrations from serum and hair samples did not correlate to observed behavior in free-roaming stallions suggesting potential co-dependencies between bachelor bands that may eliminate the need to reassert hierarchy.

The effects of castrating and releasing stallions back on to the range, as in the case for some of the horses managed by the Confederated Tribes of the Warms Springs Reservation, needs to be observed and studied in the future to evaluated how castration affects harem distribution and the potential success of bachelor stallions. Also, due to samples being opportunistically collected at time of castration, the long-term endocrine concentrations retrieved from hair from gathered free-roaming stallions needs to been assessed further to understand if the concentrations are representative of observed behavior of social hierarchy while on the range.

The current state of the free-roaming horse and burro population affects many fields. In order to best control the population size, more research into reproductive management needs to be conducted to identify the best method to safely and effectively decrease the repopulation rates to preserve the integrity of natural resources on public lands.

Appendix A. Experimental Protocol for the Extraction of Testosterone and Cortisol from Hair

Experiment performed on November 20, 2016

1. Weigh out hair sample
   Label and zero out dry vial on analytical scale. Place the root portions of the hair sample in its corresponding vial until 100 ± 20 mg of hair is in the vial. Discard the rest of the sample.

2. Mince
   Using fine scissors, mince the hair sample into 3-4 mm pieces while within the vial.

3. Incubate
   Add 2 mL of methanol to each vial containing the hair samples.
   Sonicate samples at 20°C for 30 minutes.
   Incubate samples overnight in a shaking incubator at 50°C.

4. Evaporate
   Pipette methanol supernatant into new dry vials and discard residual hair vials.
   Place the methanol vials under a stream of nitrogen overnight.

5. Reconstitute
   Add 125 mL assay buffer to each vial.
   Vortex each vial for 1 minute to remove residual sample from the sides of the vials.
   Incubate vials at 50°C for 30 minutes.
   Vortex each vial for 2 minutes.
   Incubate in shaking incubator at 50°C for 2 hours.
   Vortex vials for 2 minutes.

6. Chemiluminescence
   Run Testosterone kit (#LKTW1) and Cortisol kit (#LKCO1) with samples according the manufacturer's instructions on Immulite 1000 (Siemens Healthcare Diagnostics, Tarrytown, NY).

Appendix B. Abstract Presented at the Society for Theriogenology Annual Conference in Fort Collins, CO 2017

Serum and hair testosterone concentrations do not differ in stallions between social ranks

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In many species, male social rank has been found to correlate with testosterone concentration ([T2]), with more dominant males having higher circulating [T2]. However, the influence of [T2] on social ranking has not been evaluated yet in feral horse herds. The purpose of this study was to compare [T2] and cortisol concentration ([CORT]) in serum and hair in stallions from a variety of different social ranks. We hypothesized that dominant stallions would have higher [T2] and submissive stallions would have higher [CORT]. We also hypothesized that there would be a correlation between serum and hair [T2] and [CORT].

Stallions used in this study were part of the feral horse herd managed on the Confederated Tribes of Warm Springs Reservation (n=15). After gathering stallions for the purpose of castration, behavioral evaluations were conducted on each horse in a small and large pen to determine how they interact with other stallions. A 5-point behavior score was created with 1 = highly submissive and 5 = highly dominant. Samples were collected when each stallion was anesthetized. A jugular venous blood sample was collected, allowed to clot, and serum was stored at -20°C. A mane hair sample was pulled out by the root. [T2] and [CORT] were extracted from the hair as previously described.[1] Briefly, 100 ± 20 mg of hair was weighed, minced into 3-4 mm pieces, sonicated in methanol (2 mL) at 20ºC for 30 minutes, and incubated overnight at 50°C in a water bath with gentle shaking. The methanol was pipetted off into a new glass vial and evaporated to dryness under nitrogen. The samples were then reconstituted with 125 µL of assay buffer (#80-0170, Assay Designs, Inc., Ann Arbor, MI). Chemiluminescence (Immulite 1000, Siemens Healthcare Diagnostics, Tarrytown, NY) was used to measure [T2] and [CORT] from serum and extracted hair samples. The behavior score collected from both pens was averaged and then categorized as either submissive (<2.5), neutral (2.5-3.5), dominant (>3.5). The behavior score category was compared using an ANOVA for serum and hair [T2] and [CORT]. In addition, a Pearson correlation analyses was performed to determine if there was an association between serum and hair [T2] or [CORT]. Significance was defined as $p<0.05$.

There were no significant differences between hair (ng/100mg) and serum (ng/mL) [T2] or [CORT] in submissive, neutral, or dominant stallions (mean ± standard deviation reported in the table below). There was also no correlation between serum and hair [T2] or [CORT] (R^2= 0.0257, and 0.0025, respectively).

Behavior	Serum [T2]	Hair [T2]	Serum [CORT]	Hair [CORT]
Submissive	35.3 ± 26.5	188.6 ± 89.6	10.2 ± 5.4	1.0 ± 0.0
Neutral	35.9 ± 26.0	173.4 ± 43.0	9.4 ± 3.0	1.0 ± 0.0
Dominant	71.0 ± 54.2	183.5 ± 42.6	10.9 ± 1.4	1.3 ± 0.6

While it was unexpected that [T2] and [CORT] did not differ between social ranks, this may reflect co-dependencies that exist within bachelor bands and warrant further research.

Keywords Behavior, chemiluminescence, cortisol, dominant, submissive.

Reference

1. Koren L, Mokady O, Karaskovi, et al: A novel method using hair for determining hormonal levels in wildlife. Anim. Behav. 2002;63;403-406.

Appendix C. Oregon State University Celebrating Undergraduate Excellence Symposium

Poster, 2017

Serum and hair testosterone concentrations do not differ in stallions between social ranks

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INTRODUCTION

- Male social rank has been found to correlate with testosterone concentration with more dominant males with higher circulating levels (Figure 1).[1]
- A correlation between stress hormones, such as cortisol, has been found to be associated with hierarchy in social mammals.[1]
- Hair has been found to be a reliable means for evaluating average hormone concentrations over time.[1]
- Influence of testosterone and cortisol on social ranks in feral stallions has not been evaluated.

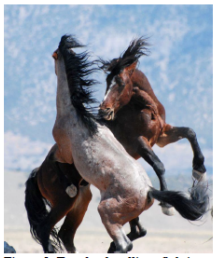

Figure 1. Two feral stallions fighting over dominance.

OBJECTIVES & HYPOTHESES

- We hypothesized that dominant stallions would have higher testosterone concentrations and submissive stallions would have higher cortisol concentrations.
- We also hypothesized that there would be a correlation between serum and hair testosterone and cortisol concentration levels.
- The objective of this study was to compare testosterone concentrations and cortisol concentrations in serum and hair in a variety of different social ranks.

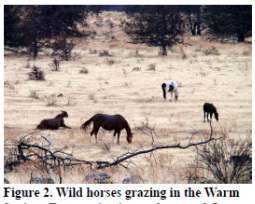

Figure 2. Wild horses grazing in the Warm Springs Reservation in north-central Oregon.

MATERIALS & METHODS

Animals

- 15 stallions were sampled from the herd managed by the Confederated Tribes of Warm Springs Reservation (Figure 2).
- Stallions were gathered for the purpose of castration.

Behavior Scoring

- Behavior evaluations were conducted on each horse when they were in a large and small pen.
- Behavior scale:
  - <2.5 = Submissive
  - 2.5-3.5 = Neutral
  - >3.5 = Dominate

Hair

- Mane hair sample pulled out by root while stallions were anesthetized.
- 100 ± 20 mg hair was weighed and cut into 3-4 mm pieces.
- Sonicated in 2 mL of methanol at 20°C for 30 minutes.
- Incubated at 50°C overnight with gentle shaking.
- Methanol was pipetted off into a new glass vial and evaporated to dryness under nitrogen.
- Reconstituted with 125 µL of assay buffer (#80-0170, Assay Designs, Inc., Ann Arbor, MI).

Serum

- Jugular venous blood samples were collected while stallions were anesthetized with a 19-gauge needle.
- Samples were allowed to clot.
- Centrifuged at 3000 rpm for 15 minutes.
- Serum was pipetted off and stored at -20°C.

Hormone Analysis

- Chemiluminescence measured testosterone and cortisol levels
- For testosterone, the sensitivity level was set to 15 ng/dL.
- For cortisol, the sensitivity level was set to 0.2 µg/dL.
- Intraassay coefficient of variation for serum cortisol and testosterone was 9.42% and 10.34% respectively.
- Intraassay coefficient of variation for hair cortisol and testosterone was 5.11% and 12.85% respectively.

Statistics

- A Pearson correlation analyses was performed to determine association between serum and hair testosterone and cortisol concentrations.
- Behavior score categories were compared using an ANOVA for serum and hair testosterone and cortisol concentrations.

RESULTS

- There were no significant differences between hair and serum testosterone and cortisol concentrations in submissive, neutral, or dominate stallions (Table 1).
- There was also no correlation between serum and hair testosterone or cortisol levels (R^2= 0.0257, and 0.0025, respectively).

Behavior	Serum [T2] (ng/mL)	Hair [T2] (ng/100mL)	Serum [CORT] (ng/mL)	Hair [CORT] (ng/100mL)
Submissive	35.3 ± 26.5	188.6 ± 89.6	10.2 ± 5.4	1.0 ± 0.0
Neutral	35.9 ± 26.0	173.4 ± 43.0	9.4 ± 3.0	1.0 ± 0.0
Dominant	71.0 ± 54.2	183.5 ± 42.6	10.9 ± 1.4	1.3 ± 0.6

Table 1. Mean ± standard deviation in serum and hair testosterone concentrations ([T2]) and cortisol concentrations([CORT]).

DISCUSSION

- While previous studies show a correlation in higher social rank to high concentrations of testosterone, within the observed feral horse herd, it was found that this is not the case (Figure 3).[1,2]
- Cortisol levels were also found to have no correlation to social hierarchy as described in previous studies.[1,3]
- Because testosterone and cortisol concentrations did not differ between social ranks, this may reflect co-dependencies between bachelor bands.
- Hair and serum testosterone and cortisol concentrations had no correlation which confirms that fluctuations in serum hormone concentrations (collected under acutely stressful conditions) are not reflective of long term hormone concentrations. If physiologic information about long term endocrine status are desired, hormonal analysis from hair samples is likely to be more reliable.

Figure 3. Feral stallion chasing after another stallion in order to establish dominance.

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Special thanks to Dr. Tim Hazzard for running all of the endocrine samples and Dr. Jennifer Grossman for developing the hair analysis protocol used in this project.

Appendix D. Presentation given at the Society for Theriogenology Annual Conference Presentation in Fort Collins, CO 2017

Serum and hair testosterone concentrations do not differ in stallions between social ranks

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Introduction

- Dominant male mammals found to have higher circulating testosterone.
- Cortisol found to be associated with hierarchy in social mammals.
- Hair reliable means for evaluating hormone concentrations over time.

Koren et al, 2002

Hypothesis

- Purpose:
  - Compare cortisol and testosterone in hair and serum from different social ranks
- We hypothesized:
  - Dominant stallions have higher testosterone
  - Submissive stallions have higher cortisol
  - Correlation between serum and hair testosterone and cortisol concentrations

Study area

- Confederated Tribes of Warm Springs Reservation
- 15 Stallions sampled

Study area

- Behavioral evaluations:
  - Large pen
  - Small pen

Behavior scoring

- 5-point Behavior Scale
  - 1-Most submissive
  - 2-Submissive
  - 3-Neutral
  - 4-Moderately Dominant
  - 5-Very Dominant

Blood sample collection and processing

- Stallion was anesthetized
- Blood collected from jugular vein
  - Vacutainer® tube
  - 19-gauge needle
- Blood samples were allowed to clot
- Centrifuged at 3000 rpm for 15 minutes
- Serum was pipetted off
- Stored at -20°C

Hair sample collection and processing

- Mane hair sample pulled out by the root
- 100 ± 20 mg of hair was weighed
- Minced into 3-4 mm pieces

Hormone extraction from hair

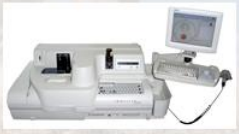
- Hormones extracted using a previously described method
  - Koren et al, 2002
- Hair sonicated in 2 mL of methanol at 20°C for 30 minutes
- Incubated overnight at 50°C in a water bath with gentle shaking

Hormone extraction from hair

- Methanol was pipetted off into new vial
- Evaporated to dryness under nitrogen
- Reconstituted with 125 µL of assay buffer (#80-0170, Assay Designs, Inc., Ann Arbor, MI)

Hormone assays

- Chemiluminescence (Immulite 1000, Siemens Healthcare Diagnostics, Tarrytown, NY)
  - Testosterone (#LKTW1)
    - Assay sensitivity: 15 ng/dL
    - Serum intra-assay CV: 12.8%
    - Hair intra-assay CV: 10.3%
  - Cortisol (#LKCO1)
    - Assay sensitivity: 0.20 µg/dL
    - Serum intra-assay CV: 5.1%
    - Hair intra-assay CV: 9.4%



Data analysis

- Behavior scores averaged
  - Submissive<2.5 (n=3)
  - Neutral 2.5-3.5 (n=8)
  - Dominant >3.5 (n=4)
- Behavior score compared using ANOVA
- Pearson correlation
  - Significant p<0.05

Results

- No significant hormone difference between behaviors

Behavior	Serum [T2] (ng/dL)	Hair [T2] (ng/dL)	Serum [CORT] (µg/dL)	Hair [CORT] (µg/dL)
Submissive	35.3 ± 26.5	188.6 ± 89.6	10.2 ± 5.4	1.0 ± 0.0
Neutral	35.9 ± 26.0	173.4 ± 43.0	9.4 ± 3.0	1.0 ± 0.0
Dominant	71.0 ± 54.2	183.5 ± 42.6	10.9 ± 1.4	1.3 ± 0.6

Mean ± standard deviation in serum and hair testosterone concentrations ([T2]) and cortisol concentrations([CORT]).

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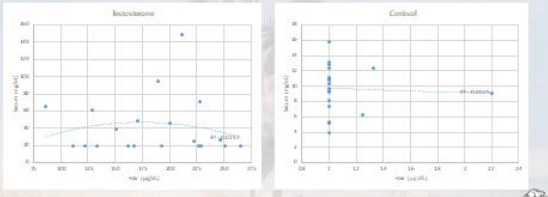
Results



- No correlation serum and hair

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Discussion

- Hypothesis 1 was not supported:
  - Dominant stallions did not have higher testosterone & submissive stallions did not have higher cortisol.
  - May reflect co-dependencies within bachelor bands that eliminate need to reassert hierarchy.
- Hypothesis 2 was not supported:
  - Serum and hair testosterone and cortisol concentrations were not correlated.
  - Likely reflects fluctuations in serum hormone concentrations not reflective of long term hormone concentrations found in hair.

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Questions?