

RESEARCH ARTICLE

A Comparison of Fecal Steroid Metabolite Concentrations Between Harem and Bachelor Stallions in a Free-Ranging Population of Przewalski's Horses (*Equus ferus przewalskii*)

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The aim of this study was to determine whether concentrations of reproductive steroid hormone metabolites significantly differed between harem stallions and bachelor stallions in the free ranging group of Przewalski's horses (*Equus ferus przewalskii*) at the Hortobágy National Park in Hungary. Throughout the study, fecal samples were collected from 21 harem stallions and 15 bachelor stallions and analyzed for immunoreactive estrogen and androgen metabolites. Harem stallions demonstrated significantly higher concentrations of estrogen ($P < 0.001$) and epi-androsterone ($P < 0.001$), but not testosterone ($P = 0.426$). These findings confirm that sociosexual status has a significant effect on androgen concentrations in individual Przewalski stallions. Zoo Biol. 36:127–131, 2017. © 2017 Wiley Periodicals, Inc.

Keywords: estrogen; epi-androsterone; testosterone; sociosexual hierarchy

INTRODUCTION

The endangered Przewalski's horse (*Equus ferus przewalskii*) is the last extant species of truly wild horse [Goto et al., 2011]. Due to successful breeding in captivity, reintroduction efforts in the 1990s began in Mongolia and China. There are currently over 300 free-ranging and native-born individuals surviving in Mongolia and China, and over 2000 total animals worldwide kept in semi-reserves, wild animal parks, and zoos [Ryder, 1993; Boyd and Bandi, 2002; Wakefield and Zimmerman, 2002; Xia et al., 2014].

Testosterone and estrogen both play a necessary role in maintaining stallion libido and normal semen parameters, ability to ejaculate, and affecting sexually dimorphic physical traits [Thompson et al., 1978, 1980]. In other species, testosterone is similarly correlated with sexually dimorphic appearance and behaviors, such as vocalization patterns, activity levels, territoriality and marking, and aggression [Pardridge et al., 1982; Fadem and Corbett, 1993; Lovren et al., 2001; Wallen, 2005; Yon et al., 2008;

van Kesteren et al., 2012; Mendonça-Furtado et al., 2014]. Feral domestic horses also form harem groups and bachelor stallion herds. Harem domestic stallions show increased libido and aggression, and have consistently higher serum testosterone concentrations compared to their bachelor

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counterparts [McDonnell, 1995, 2000; McDonnell and Murray, 1995]. This study aims to quantify endocrinologic information between harem and bachelor stallions in a discrete population of Przewalski's horses, which can be extrapolated for use in future analyses of the species. Our hypothesis was that harem Przewalski's stallions would exhibit higher fecal androgen metabolite concentrations than bachelor stallions.

MATERIALS AND METHODS

Study Area

The Pentezug Wild Horse Reserve is located in eastern Hungary, approximately 150 km east of Budapest in the Hortobágy National Park. The park was established in 1973 and encompasses approximately 820 km² of Puszta, or central European steppe. The climate is semi-arid, and ecologically categorized as sub-continental forest and steppe. The average annual temperature falls between 21°C and 22°C in the summer months and -2.5°C in the winter months. The average annual rainfall is approximately 500 mm, and snowfall approximately 2–10 cm, distributed throughout 40–45 days per year [Zimmermann et al., 2009]. The Pentezug Wild Horse Reserve consists of 2470 ha located in the core zone of the National Park.

The horses in the Hortobágy National Park live and breed in harem units, consisting of one mature breeding stallion, up to eight adult mares, and their offspring. There are no predators threatening this population. Home ranges are not maintained by Przewalski horse harems in the Pentezug population, but rather horses share the entire area of the reserve, moving generally together as one large herd consisting of more than 20 harem groups. Bachelor groups follow the harem groups and aggressive interactions between bachelor and harem stallions are frequent. There is no observed relationship between age of stallion and harem size in this population. Dominance is related to aggression and length of time being a harem stallion (Brabender and Zimmermann, unpublished data).

In addition to the horses, a large herd of domestic cattle (*Bos primigenius taurus*), carefully bred to phenotypically resemble reconstructed aurochs (*Bos primigenius*), use this area for grazing. The reserve is fenced off by Gallagher electric-fences (three strings, 1.40 m high) on all boundaries. These fences were chosen on the basis that they would provide a surmountable barrier to the natural inhabitants of the reserve including roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa scrofa*), and the great bustard (*Otis tarda*) but still keep the cattle and horses contained [Zimmermann et al., 2009].

Animals

This study was a prospective cohort study involving 36 Przewalski's stallions from the Pentezug area in Hungary. Horses included in the study were identified as harem

stallions ($n = 21$; age range 6–16 years, mean = 9.5 years, median = 10 years) or bachelor stallions ($n = 15$; age range 6–10 years, mean = 6.25 years, median = 6 years). One stallion lost his harem status midway through the study period and has appropriate timed samples included in both groups. Bachelor stallions less than 6 years of age were excluded from analysis. All stallions occupying harem status were included. To be eligible for inclusion, horses had to be repetitively identifiable from photographs and physical descriptions listed in the 2014 Przewalski's Horse International Studbook, and occupy a clear harem or bachelor status within the population. Samples were excluded from the study if they were attributable to a horse whose harem status was discernible but identity was not, or who was culled during the study period.

Collection

Sample collection was interspersed between the months of April 24 and June 12, 2014. Collections were carried out on foot, starting from an observational distance of 10–40 m from the horses. Once observed in its natural environment, the herd would be watched until a representative stallion defecated, and the location of the feces would be noted. Binoculars were utilized to visualize each individual stallion as well as the feces and notable geographic markers. Samples were collected when it was safe to do so without disturbing the herd in their natural environment, within a timespan of 10 min post-defecation. Because of association with the same mares and yearlings in the harem, and defecation marking behavior that is displayed more consistently by harem stallions, identifying individuals, and obtaining repeat samples from harem stallions was easier than for bachelor stallions. Each sample was stored in a four ounce plastic cup, labeled with the horse's name, date, and a sample number, and frozen on site after the completion of each collection period. As samples were obtained opportunistically under field conditions; 48 fecal samples were obtained from the 21 harem stallions (samples/stallion ranged from 1 to 5) and 36 fecal samples were obtained from the 15 bachelor stallions (samples/stallion also ranged from 1 to 5).

Sample Analysis

The frozen samples were transported to the University of Veterinary Medicine in Vienna at the conclusion of the study period for analysis. Fecal samples were thawed at 4°C for 12 hr and extracted using a methanol/diethylether method as described and used for several other mammalian species [Palme et al., 2013]. Wet feces was mixed and vortexed with 4.5 ml aqueous methanol (80%). After centrifugation 1 ml of the supernatant methanol was transferred into a new vial, mixed with 0.5 ml of a 5% NaHCO₃ in water solution, and re-extracted with 3.0 ml diethyl ether. The ether phase was transferred into a new vial, and evaporated. The extracts were re-dissolved and diluted

with assay buffer. Aliquots were analysed using group-specific enzyme-immunoassays (EIA) for immuno-reactive androgens (epiandrosterone and testosterone metabolites), and total estrogens. The rabbit polyclonal antibodies used in the EIAs were against 5 α -androstane-3 β -ol,17-one 3-HS:BSA; (epiandrosterone); 4-androstene-17 β -ol, 3-one 3CMO:BSA (testosterone), and estradiol-17 β -OH 17-HS:BSA (total estrogens). The intra- and interassay variation in these assays was <10% and 15%, respectively. All samples were assayed in duplicate. Samples were repeated if the duplicates differed by 10% or more. Dilutions of fecal extracts were parallel to the standard curves of the assays used. Quality control included intra- and interassay variability, coefficient of variation, and fecal extracts that were diluted parallel to the standard curve.

Comparisons were drawn between estrogen, epiandrosterone, and testosterone concentration of each sample within each cohort. Concentrations were determined as ng/ml androgens, and then divided by the dry weight of feces extracted to give the results as ng/g feces. Due to the right skewedness in the data, it was log transformed for the purposes of analysis. The replicate measurements taken on each horse were accounted for using mixed-effect linear regression, resulting in each harem, and bachelor stallion representing a "random effect." Each cohort remained a "fixed effect" throughout the analysis. Model-predicted marginal means and standard errors from each cohort are reported. Significance was set at $P < 0.05$.

RESULTS

Estrogen concentrations ranged from 0.05 to 22.71 ng/g. Epi-androsterone concentrations ranged in value from ≤ 0.01 to 53.92 ng/g. Testosterone concentrations ranged in value from ≤ 0.01 to 4.70 ng/g.

The mean concentration for estrogen and epi-androsterone were higher in harem stallions than bachelor stallions (estrogen: 6.30 ± 0.67 ng/g vs. 3.41 ± 0.76 ng/g, $P < 0.05$; epi-androsterone: 5.56 ± 1.69 ng/g vs. 1.76 ± 1.90 ng/g, $P < 0.05$). Values for testosterone were higher in harem stallions, but were not significantly different from those in bachelor stallions (0.32 ± 0.15 ng/g, vs. 0.24 ± 0.16 ng/g, $P = 0.426$). Values are compared between groups in Figure 1.

DISCUSSION

The purpose of this study was to compare the androgen and estrogen hormone concentrations between harem and bachelor stallions in a free-ranging population of Przewalski's horses.

Harem stallions demonstrated higher values than bachelor stallions in both estrogen and epi-androsterone, both of which are testosterone metabolites. The lack of significance in differences in testosterone between the two groups is likely due to the metabolism of testosterone causing

hard-to-detect fecal metabolite concentrations. Although the group-specific assay used for the analysis of testosterone metabolites is a "broad spectrum" antibody against "17 β -ol androgens," these types of androgen metabolites are not really excreted in stallion fecal samples and therefore this assay is not well suited for its use in stallion fecal samples. Comparably low fecal testosterone metabolite values were also reported in free ranging stallions of the Misaki breed in Japan [Khalil et al., 2009], as well as in other species [Ganswindt et al., 2003; Mendonça-Furtado et al., 2014]. The much higher concentrations of estrogen and epiandrosterone metabolites suggest these assays are better suited for studying fecal steroid hormone metabolites in stallions. The differences detected in estrogen and epiandrosterone likely reflect serum testosterone concentrations and demonstrate a physiological correlation between social structure of the Przewalski's horse population and reproductive hormone concentrations; similar trends have been documented in both feral and domesticated equids [McDonnell, 1995, 2000; McDonnell and Murray, 1995; Khalil et al., 1998, 2009]. Support for this interpretation would be validated with comparisons between serum and fecal hormone concentrations in Przewalski stallions.

The basic social unit in equids is the family group, usually consisting of one stallion, one or more non-related mares, their foals, and subadult offspring. Generally one of two distinct mating systems is employed in equid groups: one based on family groups and bachelor groups co-existing in home ranges (e.g., Plain's Zebras, Przewalski's Horses), and one based on highly territorial family groups excluding roaming bachelor groups (e.g., Grevy's Zebras, Asiatic Wild Asses; [Klingel, 1975; Klingel, 1982]). The significantly higher concentrations of reproductive hormones in harem stallions in Przewalski's horses likely play an important role in behaviors exhibited by harem stallions, and not by bachelor stallions. Domestic harem stallions spend significantly more time than bachelor stallions in guarding, surveillance, and aggressive behaviors [McDonnell and Murray, 1995]. Przewalski stallions exhibit similar behaviors to domestic stallions, with even higher incidences of aggressive behaviors [Feh, 1988]. Although this has yet to be documented, higher testosterone concentrations in some individuals may offer insight into aggressive, aberrant behaviors. One specific example, which has been recognized in multiple observational reports of reintroduced Przewalski's horses, is adult male horses committing infanticide [Ryder and Massena, 1988; Feh and Munkhtuya, 2008]. Observational studies of Przewalski stallion infanticide do not support the explanation of sexual selection. Because it is observed in both harem and bachelor stallions, committing infanticide does not confer any reproductive advantage on the stallion. It is not known if stallions that commit infanticide have higher serum concentrations of testosterone than other stallions. With regard to this study, infanticide was not observed in this population of Przewalski's horses during the study period. As such, we were unable to correlate potential hormone variations with aberrant,

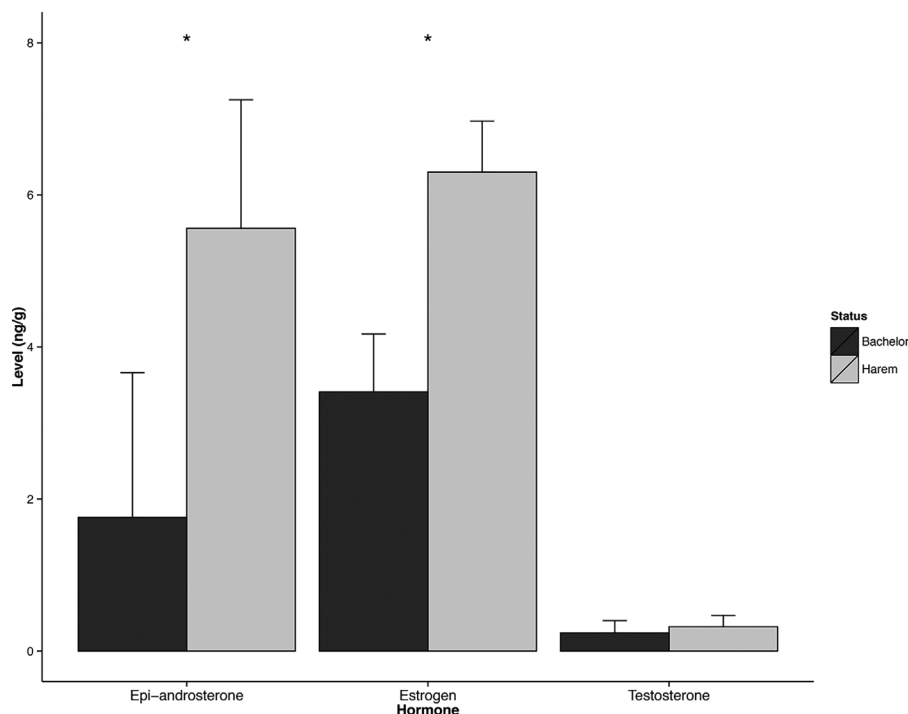


Fig. 1. Comparison of androgen metabolites in feces of free-ranging harem and bachelor Przewalski stallions collected during the summer. Statistical difference at $P < 0.05$ indicated by *.

aggressive behaviors. Aggressive behavior in other species, however, has been correlated with elevated testosterone concentrations [Pardridge et al., 1982; Wallen, 2005; Yon et al., 2008; van Kesteren et al., 2012; Mendonça-Furtado et al., 2014]. In the future, if possible, it would be interesting to determine the relative androgen concentrations from males exhibiting infanticide compared to “normal” bachelor and harem stallions.

Endocrinological differences between individual stallions have been documented in both feral and domesticated equids [McDonnell, 1995, 2000; McDonnell and Murray, 1995]. This information has been used to successfully manipulate sociosexual conditions of stallions to produce desirable changes in reproductive physiology. For example, stallions with low libido placed in a pasture with solely mares (or even just in close proximity with mares) for an extended period of time will exhibit higher testosterone levels and more frequent displays of sexual behavior than if they are housed in isolation or only in proximity to other stallions [Boyd, 1986; McDonnell and Murray, 1995; Khalil et al., 2009]. Similar manipulations, when possible, in managing wild species with a harem social structure have been practiced in some zoos with anecdotal success, stimulating reproductive behaviors in males that previously had not displayed strong interest in mating. This social arrangement of housing an intended breeding male Przewalski stallion with multiple mares is the common management strategy currently employed in most zoos. The findings of this study provide evidence for why these manipulations may work in Przewalski horse captive breeding management programs.

When faced with the challenge of breeding two individuals in captivity that are not, for whatever reason, breeding, the tendency may be to consider assisted reproductive technologies. Although technology available today (electroejaculation, hormonal manipulation of cycles, artificial insemination, etc.) makes assisted captive breeding, in theory, increasingly attainable [Collins et al., 2014; Deng et al., 2014], it is not without serious challenges. This study provides defensible data that Przewalski's stallion reproductive physiology could be manipulated by social interactions to possibly increase endogenous reproductive steroid production with potential, consequent increase in sperm production and in secondary male behaviors. Artificially elevating the perceived sociosexual status of a domestic stallion by surrounding the stallion with mares has been shown to effect an increase in endogenous testosterone and, in some cases, consequent increase in exhibiting reproductive behaviors and sperm production [McDonnell, 1995, 2000; McDonnell and Murray, 1995; Burger et al., 2012].

These sociosexual observations are not limited to equids. Increased serum testosterone concentrations have been positively correlated in captive white and black male rhinoceros with respect to the number of female rhinoceros housed with the males [Christensen et al., 2009]. Male rhinoceros of both species were grouped into those housed alone, those housed only with other males, those housed with one female, two females, or greater than two females. Serum samples were collected from males on at least a monthly basis for a year. Males housed with two or more females had higher testosterone concentrations than males housed

without females or males housed with only one female. Similar findings have been reported in primates and canids [van Kesteren et al., 2012; Mendonça-Furtado et al., 2014].

This study was additionally limited to characterizing reproductive steroid hormone concentrations. Recent investigations into the physiology of some Przewalski's populations have demonstrated that there are significant elevations in glucocorticoid concentrations of harem stallions in response to environmental triggers, and less significant fluctuations in bachelor stallion glucocorticoid concentrations [Wolter et al., 2014]. Thus, documentation of baseline values of glucocorticoid concentrations in bachelor and harem Przewalski's stallions should also be a worthwhile endeavor.

CONCLUSIONS

This paper presents baseline concentrations of reproductive steroid hormones in male Przewalski's horses. Consistently, harem stallions exhibited higher fecal reproductive androgen metabolite concentrations than bachelor stallions.

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