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Noninvasive monitoring of reproductive function by determination of faecal progestagens and sexual behaviour in a herd of Przewalski mares in a semireserve

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We examined the reproductive activity and sexual behaviour of a herd of Przewalski mares *Equus ferus przewalskii* Poljakov, 1881 that were born in zoos and lived in a semireserve since 1992 during five periods in 1995–1997 of 4–6 weeks each. Ovarian activity was detected by the analysis of faecal progestagens. In addition, behavioural detection of oestrus and continuous recording of the daily activity with a storage telemetry system were carried out and compared with the analytical data. Faecal 20α-hydroxypregnane analysis revealed ovarian activity to be 100% (April/May 1995), 25% (May/June 1996), 88% (October/November 1996), 63% (January/February 1997) and 100% (April/May 1997) of the mares sampled. Behavioural observations showed a seasonal pattern with maximal sexual interactions in April/May 1995/1997 and only few interactions in winter. Detailed activity records in individual animals revealed an oestrus related increase from 14 h/d to 15.6 h/d. Our results show a tendency of seasonality which support the view that Przewalski mares are seasonal breeders with sexual activity in spring and early summer. In May/June 1996 a dysregulation of reproductive activity associated with a persistent increase in locomotor activity occurred. We hypothesise external disturbances from a shooting yard close to the semireserve. Compared to behavioural observations, faecal progestagen analysis seem to be the most convenient method to investigate reproductive activity in free ranging Przewalski mares.

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Introduction

For nearly one hundred years, Przewalski horses *Equus ferus przewalskii* Poljakov, 1881 have been maintained in zoos far from their natural habitat. The free living population is extinct since 1969 when the last Przewalski horse, a stallion, was seen in the wild (Bouman and Bouman 1994). At the end of World War II, there were 31 horses in captivity of which only nine reproduced. All of them were

descendants of animals caught during successful, but stressful captures around the turn of the century. Since then the size of the captive population has grown to 1840 registered Przewalski horses in 1995 (Volf 1996, W. Zimmermann and L. Kolter, pers. comm.), a large enough population to regard a reintroduction as feasible. The zoo population, however, is under different selection pressures than wild populations, and the zoo environment is sufficiently different from the natural environment to change the ontogeny of behaviour patterns. In order to ensure the long-term survival of reintroduced Przewalski horses, only careful selection of individuals and cautious adaptation of suitable animals in semireserves will guarantee the success of reintroduction efforts. Important prerequisites are sufficient knowledge of ethology, habitat requirements and reproductive behaviour of the species (Van Dierendonck *et al.* 1996).

Seasonal reproductive activity is usually considered an adaptation to seasonal natural environments and is triggered by changes in day light duration (Aschoff 1955). As in many other species, the primary zeitgeber for the activation of reproduction in equids is the change in daily light length (Sharp and Ginther 1975, Fraser 1992). Such seasonal activation is meant to ensure that conception and birth occur during that period of the year when food availability in the natural habitat is sufficient to maximize survival of offsprings.

For Przewalski horses, nothing is known about seasonal breeding pattern in the wild. In the zoo population of the northern hemisphere, the birth of foals have been observed throughout the year but peaks during the period from April to August with a pronounced maximum in May (W. Zimmermann and L. Kolter, pers. comm., Monfort *et al.* 1994). In the southern hemisphere, 69% of the foals were born at almost the same time (Monfort *et al.* 1994), which reflects endogenous mechanisms and suggests that photoperiod is not the only determinant in timing of the breeding season.

To date several approaches have been used to study the physiological aspects of (seasonal) reproductive activity of Przewalski mares. New techniques to track oestrous cycles include noninvasive methods that measure faecal progestagens (Schwarzenberger *et al.* 1992) or urinary oestrogens (Monfort *et al.* 1991). Only few data exist on sexual behaviour. Data from Pony mares (*Equus caballus*) and from semi-free-ranging Przewalski mares suggested an unusually extended behavioural oestrus, lasting for 7–8 days (Asa *et al.* 1979, Monfort *et al.* 1991). From domestic animals, an increase in general locomotor activity during oestrus is well known. In cows for example, behavioural oestrus is accompanied by a distinct increase in locomotor activity of 100% to 300% compared with baseline activity levels during non-oestrous periods (Pennington *et al.* 1986). In free ranging Przewalski horses, an increase in locomotor activity could be used for oestrus detection, but information on this species was not available until now.

It was the aim of our study to investigate the reproductive activity and sexual related behaviour of a herd of Przewalski mares under seminatural conditions and to obtain information on the correlations between endocrine status, sexual related behaviour and locomotor activity in a group of Przewalski mares. For this purpose

monitoring of ovarian activity was performed by measuring progestagens in faecal samples. Behavioural observations and continuous records of daily activity with a storage telemetry system complemented the physiological investigations. The data obtained from the different methods should be compared according to their reliability for the detection of oestrus.

Material and methods

Animals

This study was carried out with a group of 7–12 Przewalski mares that were born in zoos and lived in a 36 ha semireserve 70 km north of Berlin (52°55' northern latitude) since 1992. The semireserve consisted of open pasture and a narrow edge of forest (pine *Pinus sylvestris* and oaks *Quercus robur*). The pasture contained greatly varying grasses and clover (especially *Lolium perenne* and *Dactylis glomerata*, together with *Trifolium repens*), but also perennials (*Urtica dioica*, *Artemisia* sp., *Cirsium arvense*, *Cirsium vulgare*).

The mares were 3–6 years old at the beginning of the study. They had free access to pasture and fresh water, minerals were offered as salt blocks. However, no additional feeds were provided.

Faeces collection

Faecal samples were collected twice a week during five periods of 4–6 weeks each, in April/May 1995 and May/June 1996 (12 animals), October/November 1996 and January/February 1997 (8 animals) and April/May 1997 (7 animals). They were stored at –20°C until assayed.

Sexual behaviour

On the day of faecal collection, visual observations on sexual related behaviour were conducted for 6 h by always the same experienced observer (A.S.). The following behaviours were recorded: following (maintaining extended and unusual close contact within one body lenght to another mare during walking and grazing), proximity (standing within one body lenght to another animal for extended periods), tail raising (tail held of standing in proximity to other mares), urination (in proximity to other mares), flehmen (typical ungulate facial display consisting of raising the head and curling the upper lip) and striking vocalisation (squeaking). If an animal showed at least one of these behaviours several times on a given day, it was considered to be sexually active. To compare observation periods independently from the number of animals and observation days, we calculated an index of sexual behaviour (ISB: percentage of animals with sexual related behaviour during one observation period).

The index includes the number of days on which sexual related behaviour was observed multiplied by the number of animals displaying such behaviour on these days (sexual active animal days, SAAD), the number of animals (n) present during the observation period, and the number of observation days (D), as follows:

$$\text{ISB (\%)} = \frac{\text{SAAD}}{n \times \text{D}} \times 100$$

Activity recording

Continuous recording of activity was carried out with a new storage telemetry system (ETHOSYS, producer: IMF technology, Frankfurt/O, Germany; Scheibe *et al.* 1998) for four individuals during May/June 1996 and for two individuals in April/May 1997. The system consisted of collars, a central receiving station and a software package for data transmission to a PC. The collars contain sensors, a signal processing and storage unit, a radio receiver and a transmitter. They recorded general locomotor activity and stored the results every 15 min in an internal memory. The resulting data set was transferred automatically to the central station by radio when the animals approached the salt lick.

The resulting time series were analysed for 24 h activity time budget and periodicities. For rhythmical analysis, the original time series were subdivided into partially overlapping periods of seven days, with a time lag of one day in between. Periodicities were identified by power spectral analysis and appropriate tests for statistical significance (Sollberger 1965, Sinz 1978, Andel 1984, Doberenz 1985, Diggle 1990). The different power spectra were compared by a value called "Degree of Functional Coupling (DFC)" (Sinz and Scheibe 1976, Scheibe *et al.* 1999). It expresses the sum of power of the 24h-period and all ultradian harmonic rhythms (SI_{harm}) as a percentage of the total power of all significant periods (SI_{total}). Harmonic ultradian periods were periods of 12, 8, 6, 4.8, 4, 3.3, and 3 h period length, following this definition:

$$DFC = \frac{SIharm}{SItotal} \times 100$$

A degree of 100% would mean, that an animal acts in the same way at the same time of the day on consecutive days. The resulting DFCs were mapped continuously for each individual.

Measurement of faecal 20α-hydroxypregnanes

Faecal samples (0.5 g) were extracted for 30 min with 9 volumes of 90% methanol. After centrifugation (15 min at 1200 × g) the supernatant was diluted 1 : 1 with water and aliquot portions of 10 µl were subjected to the assay. All hormone measurements were carried out in duplicate using microtitre plate enzyme immunoassay procedures. 20α-hydroxypregnanes were measured using an antiserum raised in rabbits against 5β-pregnane-3,20α-diol-3-gluc-BSA (pregnanediol, E. Möstl, Vienna, Austria), the corresponding 3-glucuronid-peroxidase conjugate as label, and pregnanediol (5β-pregnane-3α,20α-diol) as standard. The antiserum showed the following cross-reactivities relativ to 5β-pregnane-3α,20α-diol (100%): 20α-dihydroprogesterone, 211%; 5α-pregnane-20α-ol-3-one, 60%; 5α-pregnane-3β,20α-diol, 36%; 5α-pregnane-3α,20α-diol, 34%. Progesterone, 5α-pregnane-20-one, 5β-pregnane-20-one, 5α-pregnane-3α-ol-20-one and 5α-pregnane-3,20-dion were below 0.1%.

The assays were carried out using the second antibody technique (Meyer *et al.* 1990). The sensitivity of the assay was defined as 2 standard deviations from the signal given by the zero blank and was 13 ng/g. Serial dilutions of faecal extracts from follicular and luteal phase samples were parallel to the standard curve. The mean intra- and interassay coefficients of variation, calculated from a pool of faecal samples, were 8.7% and 10.5%, respectively.

HPLC

For progestagen separation, individual sample extracts were concentrated fivefold and 500 µl were loaded onto a Ultrasep ES100/RP-18/6 mm (Sepserv, Berlin) HPLC column (4 × 250 mm). Progestins were separated by reverse-phase chromatography using a methanol:buffer (20 mM Tris, pH 7.2) mixture (78 : 22) at a flow rate of 1 ml/min. Fractions were collected at 20 sec intervals and diluted with 1 volume of water before 20 µl of the samples were directly introduced into the assay system.

Comparison of analytical and behavioural data

The measurement of progestagen concentrations in faeces were assumed to indicate cyclic ovarian activity and to estimate the days of oestrus. Values below 0.4 µg/g and values above 1.0 µg/g were considered to be indicative of the follicular phase or of the presence of a functional corpus luteum respectively. We had to consider that behavioural oestrus in Pony and Przewalski mares lasted over a 7-day period from preovulatory day 5 until postovulatory day 2 (Asa *et al.* 1979, Monfort *et al.* 1994), and a time lag for steroids from blood plasma until their appearance as metabolites in faeces of 1 day (Palme *et al.* 1996). Thus behaviours during the time period from 4 days before to 3 days after the faecal progestagen minimum were defined as oestrus-specific, those outside this interval as dioestrus-related.

Results

Characterization of immunoreactive progestagens in faecal samples

The elution positions (Fig. 1) of 20α-dihydroprogesterone (1; 8 ml), 5α-pregnane--20α-ol-3-one (2; 10.7 ml), 5β-pregnane-3β,20α-diol (3; 11.7 ml), 5β-pregnane--3,20α-diol (4; 13.3 ml) had been determined previously in seperate HPLC runs after injection of 6 ng of each standard and analysis of the HPLC fractions in the pregnanediol EIA. After calibration 8 selected faecal samples containing different amounts of steroids from mares in the luteal phase were extracted, separated by the HPLC-system, subsequently analysed with the EIA and compared with the different steroid standards. From these samples 3 examples are shown in Fig. 1. The results demonstrate individual differences in the composition of immunoreactive metabolites. In all extracts, however, two immunoreactive progestagens corresponding to the elution positions of 20α-dihydroprogesterone and 5α-pregnane--20α-ol-3-one were confirmed as main metabolites. In addition, two minor peaks, one coeluting with 5β-pregnane-3β,20α-diol and a second more polar substance were detected. Thus, the pregnanediol EIA used seems to be appropriate to analyse progestagens in faecal samples. In addition, measurements of extracts and the corresponding HPLC fractions with an assay system using a specific antibody raised against 20α-dihydroprogesterone (Schwarzenberger *et al.* 1992) with low

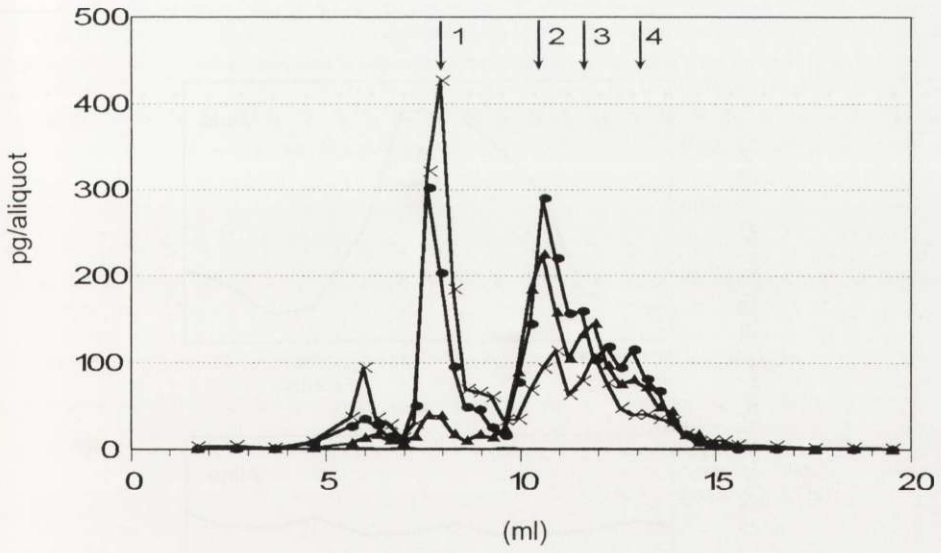

Fig. 1. Characterization of gestagen metabolites in faecal samples collected during the corpus luteum phase of three Przewalski mares (×, ●, ▲). The elution positions of the 4 corresponding gestagens had been determined after injection of 6ng of each steroid and analysis of the HPLC fractions in the EIA (1: 20α-dihydroprogesterone, 8 ml; 2: 5α-pregnane-20α-ol-3-one, 10.7 ml; 3: 5β-pregnane-3β,20α-diol, 11.7 ml; 4: 5β-pregnane-3α,20α-diol, 13.3 ml).

crossreactivities against other gestagens revealed clearly lower levels in HPLC fractions and extracts (data not shown). Therefore, all subsequent analyses were performed with the pregnanediol assay.

Monitoring of corpus luteum function

Faecal progestagen analysis revealed cyclic ovarian activity to be 100% (April/May 1995), 25% (May/June 1996), 88% (October/November 1996), 63% (January/February 1997) and 100% (April/May 1997) of the mares sampled (Table 1). A depression of cyclic ovarian activity occurred from October/November 1996 to January/February 97 during the seasonal anoestrus period in winter until the onset

Table 1. Investigation of cyclic ovarian activity in Przewalski mares by determination of faecal progestagens and sexual behaviour.

Month	Number of observed mares	Percentage with ovarian activity monitored by faecal progestagens	Index of sexual related behaviour (ISB)
April/May 1995	12	100	20.9
May/June 1996	12	25	13.0
October/November 1996	8	88	13.5
January/February 1997	8	63	3.1
April/May 1997	7	100	18.2

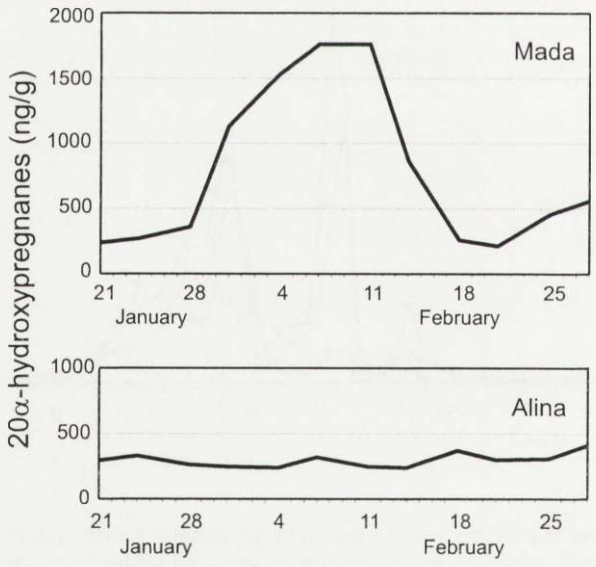

Fig. 2. Concentrations of faecal 20α-hydroxypregnanes of an acyclic mare (Alina) and an animal with cyclic ovarian activity (Mada) during January/February 1997.

of cyclicity between February and April (Monfort *et al.* 1994). One faecal profile for each reproductive status during January/February 1997 is shown in Fig. 2. In the acyclic animal (Alina) pregnanediol levels remained at constantly low values (298 ± 55 ng/g; mean ± SD). In contrast, the second animal (Mada) revealed a cyclic pattern that increased from 300 ng/g to 1750 ng/g during the luteal phase, thereafter decreasing again to initial values after a cycle length of about 24 days.

The same individuals that showed seasonal cyclicity of their reproductive activity in 1995 and 1997 were dysregulated in 1996. A typical example of one individual (Lulu) is shown in Fig. 3. Ovarian activity with distinct follicular and corpus luteum phases were detected in spring 1995 and 1997. In contrast to these

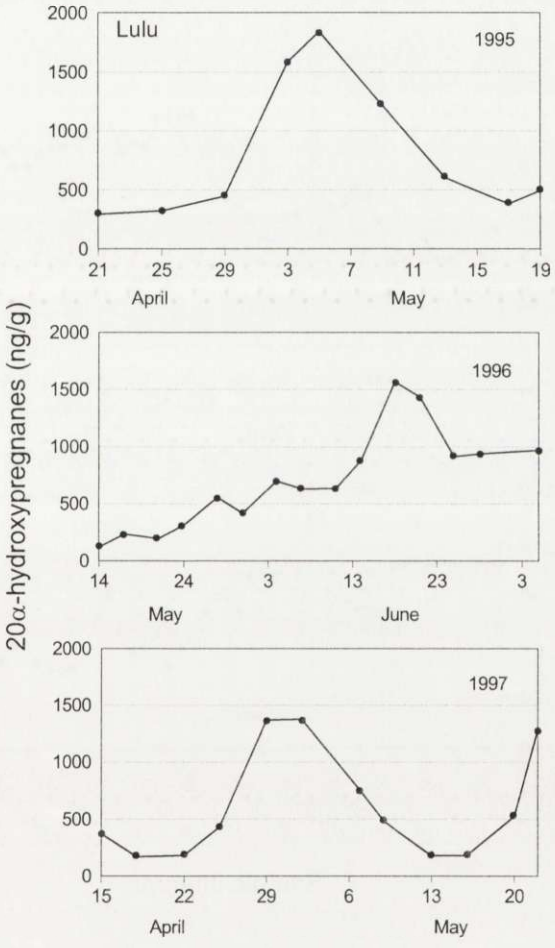

Fig. 3. Concentrations of faecal 20α-hydroxypregnanes in faeces of mare Lulu with cyclic ovarian activity in April/May 1995 (above) and in April/May 1997 (below), as well as an unexplained period of dysregulation (persistent corpus luteum activity) during May/June 1996 (centre).

episodes, no detectable cyclicity occurred in May/June 96. During this period values increased steadily from the onset of sampling over a period of 30 days, reaching luteal phase levels around the middle of June and remained high until the end of the sampling period due to a persistent CL.

Investigation of sexual related behaviour

If an animal showed sexual related behaviour on a given day, it was considered sexually active. The calculated index of sexual behaviour (ISB) for each observation period during the entire study is shown in Table 1. It demonstrates clear differences between the periods with maximal sexual related behaviour in April/May 1995 (20.9%) and 1997 (18.2%), a depression in May/June 1996 (13%) and autumn (13.5%, October/November 1996) and a low level of sexual related behaviour in

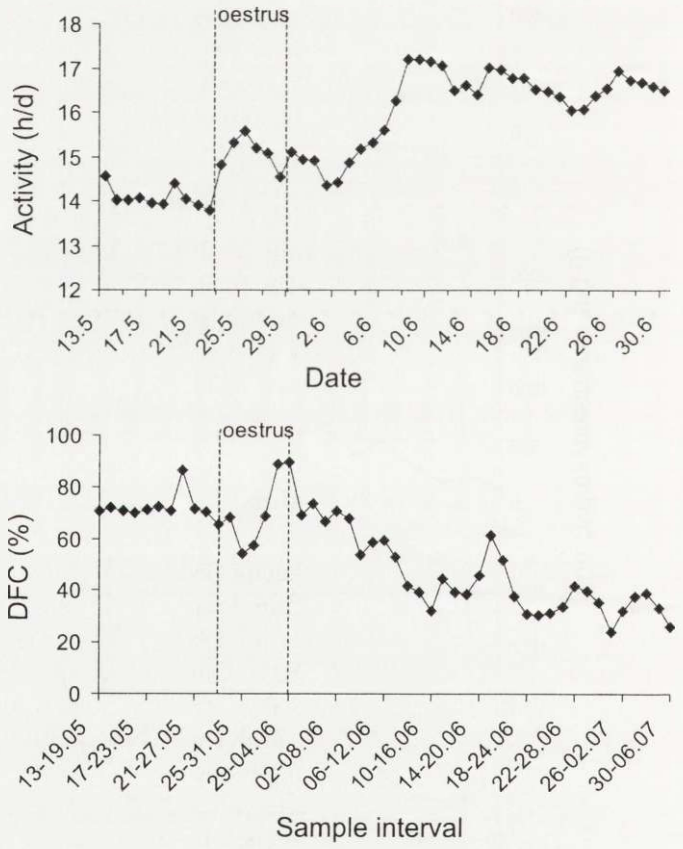

Fig. 4. Variation of daily activity time as percentage of the mean from one mare during May/June 1996. The degrees of functional couplings (DFC) were calculated from the activity data obtained from the same mare (for explanation see text). The day of oestrus was identified by progestagen analysis and is indicated.

winter (3.1%, January/February 1997). A comparison of all behavioural observations with the two phases of the ovarian cycle showed that 69% of all sexual behaviours occurred during the follicular phase, whereas 31% were distributed throughout the luteal phase. However, there were individual differences between the mares. Some animals showed sexual behaviour during every oestrus period, whereas in other animals sexual behaviour was registered only occasionally.

Continuous activity recording

Of the four animals for which detailed activity records were available for the period of May/June 1996, only one (Mada) showed physiological signs of oestrus according to the progestagen analysis. This animal (Fig. 4) increased its level of daily activity from 14 h/day during prooestrus to a value of 15.6 h/d around the time of oestrus (25th May). This was followed by a temporary decrease. Between 8th and 12th of June an increase to a level of 17.2 h/d occurred, which was found also in the three other animals recorded. A short oestrus dependent increase in activity was also observed in cycling mares in May 1997, however, no continuous increase was detected for these animals until the end of the sampling period (data not shown).

The calculation of DFCs revealed that in May/June 1996 the daily activity patterns became variable and unstable. This results in an uncoupling of the rhythmic pattern of daily activity (Fig. 4). Starting from a coupling degree of 70% a decrease to 40% and less at the beginning of June was calculated for Mada, remaining at this low level until the end of the recording period. Simultaneously, a decrease to a DFC of around 40% was obtained also from the three other animals during this period. The DFC of the activity records of the two animals studied in May 1997 showed no such decline (68% resp. 71%).

Discussion

The analysis of faecal ovarian progestagens provides a powerful noninvasive method to detect ovulatory cycles, gestation length and seasonal reproductive activity in wildlife species. (Schwartz *et al.* 1995, Heistermann *et al.* 1996, Schwarzenberger *et al.* 1996, Brown *et al.* 1997). Our progestagen measurements in Przevalski horses used faecal samples collected twice weekly with an antiserum against 5β-pregnan-3,20α-diol-3-gluc-BSA and the corresponding 3-glucuronid--peroxidase conjugate as label. The faecal progestagen concentrations revealed distinct differences between samples from the follicular (< 0.4 μg/g) and the corpus luteum phase (> 1.0 μg/g). After HPLC separation of faecal samples two main immunoreactive substances were detected eluting in the same way as 20α-dihydroprogesterone and 5α-pregnane-20α-ol-3-one. However, identity cannot be confirmed, because coelution with standards in different chromatographic systems has to be demonstrated. In addition, Schwarzenberger *et al.* (1992) did not confirm the

presence of 20α-dihydroprogesterone in faeces of Przewalski mares. Therefore, the concentrations reported should be considered as a measurement of a group of metabolites recognised by the antibody (20α-hydroxypregnanes). No immunoreactive substance at the position of progesterone was demonstrable.

Our monitoring of faecal progestagens further support the view that Przewalski horses are seasonal breeders, however, this cannot be concluded definitely from this first study. The high proportion of cycling mares both in April/May 1995 and 1997 is consistent with the mating season of Przewalski horses. A more detailed study on seasonality with even numbers of intervals across the seasons should follow.

In domestic horses (Aschoff 1955, Ortavant *et al.* 1985) and in well adapted free ranging populations, as the Askania-Nova Przewalski horse population, a clear seasonal reproduction pattern occurred with sexual activity in summer followed by spring-summer foaling periods. This strategy is a prerequisite for successful colt development and their early sexual and physiological maturation (Steklenev 1995). In zoo populations of Przewalski horses, however, reproduction out of season occurs occasionally (Monfort *et al.* 1994). Similarly, 5 of 8 mares in our study showed ovarian activity already in January/February 1997. In the domestic horse a small proportion of mares exhibit oestrous cycles during the nonbreeding season (Fitzgerald and Schmidt 1995), even if they were kept in total darkness (Colquhoun *et al.* 1987). The mechanism responsible for this phenomenon is not known. One reason for the absence of a clear seasonality in our study might be the lack of stallions compared to the natural harem (polygynous) social organization of Przewalski horses (Houpt and Boyd 1994). Oestrus synchronization by male cues, oestrus detection or copulation followed by pregnancy were not possible in our purely female study group.

The behavioural detection of oestrus is confounded by the occurrence of oestrus-like behaviour independent of gonadal stimulation. This is supported by Munro *et al.* (1979), who found no consistent relationship between net behavioural scores and the circulating concentrations of oestrogens or androgens in Pony mares. Our observation schedule (twice weekly) led to the detection of only 50% of all oestrus periods. This was probably possible because horses have an unusually long oestrus (Asa *et al.* 1979). A higher observation frequency might lead to a higher degree of oestrus detection. Sexual behaviours were distributed throughout the entire ovulatory cycle, but were more intense in the periovulatory period (69%) than on days outside the periovulatory period (31%). This is compatible with data from Pony mares living together with stallions in harem groups. Sexual interactions between males and females and copulatory behaviour were observed in ovariectomized and seasonally anovulatory domestic horse mares (Asa *et al.* 1980, Asa 1986). This led to the suggestion, that the ovary plays a major role in actively suppressing oestrus responses during the luteal phase of the cycle (Asa *et al.* 1980). This indicates that exclusive behavioural observations seemed to be insufficient to monitor reproductive activity in Przewalski mares.

Similar to the progestagen analysis, the behavioural data revealed different intensities of reproductive activity throughout the observation periods. The maximum frequency of sexual behaviour occurred during spring, followed by a slight depression in summer and autumn, whereas only few behaviours were observed in winter. In all mares investigated we obtained an oestrus-dependent increase in locomotor activity. This is in agreement with data from domestic animals like cows, where locomotor activity increased by 100 to 300% during the time of standing oestrus (Pennington *et al.* 1986).

At the beginning of June 1996, however, an unexpected and persistent increase in locomotor activity occurred accompanied by lowered DFC for all animals recorded, implying that behavioural rhythms in the diurnal and ultradian frequency range were highly unstable. In previous analyses, we found a more unstable pattern of behavioural rhythms in Przewalski horses than in sheep or cattle (Berger and Scheibe 1994), but stable rhythmic patterns were characteristic for undisturbed conditions. The distinct and parallel reduction of the DFC in our four animals can only be interpreted as an indication for disturbances from the environment. This assumption could be substantiated from the two animals recorded in May 1997. From these data no persisting drop in DFC had been calculated (mean DFC: 71 and 68%, respectively). Therefore, we had to assume, that the environmental conditions in 1996 differed substantially from those in 1997. This can also be supported by the patterns of progestagens in the faeces, which revealed a desynchronisation of ovrian activity in 6 of 8 mares when reproductive activity was likely.

Our investigations revealed, that in the second half of May 1996 a shooting yard had been set up at a distance of only 2 km from the semireserve producing a noise level of 75 dB. Visual observation showed a strong influence of shooting on horse behaviour. This suggests that the shooting resulted in unstable activity patterns and desynchronisation of ovarian activity. We suppose, that shooting noises may have caused high levels of stress and that this kind of stress could be the reason for the unespected results. This is supported by studies on the effects of aircraft noises on reproduction, behaviour and cortisol secretion in farm livestock (Stephan and Heuwieser 1982, Zoldag *et al.* 1983).

Our results demonstrate that the analysis of faecal pregnanediol is a reliable method to investigate the reproductive activity of mares in the field. Behavioral observations may be used additionally but are neither specific nor sensitive enough to monitor reproductive activity in free ranging mares. Such investigations are an essential prerequisite for the reintroduction of this and other species into the wild. In addition, the development of analytical methods to monitor stress in captive and free ranging wildlife using the advantages of non-invasive methods of sampling should be intensified (Palme and Möstl 1997, Hofer and East 1998).

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