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Endocrine milieu of perioestrus in the giant panda (*Ailuropoda melanoleuca*), as determined by non-invasive hormone measures

David C. Kersey[A,D,E], *David E. Wildt*[A], *Janine L. Brown*[A], *Rebecca J. Snyder*[B], *Yan Huang*[C] *and Steven L. Monfort*[A]

[A]Center for Species Survival, Smithsonian Conservation Biology Institute, Front Royal, VA 22630, USA.

[B]Zoo Atlanta, Atlanta, GA 30315, USA.

[C]China Conservation and Research Center for the Giant Panda, Wolong Nature Reserve, Wenchuan, Sichuan 623006, China.

[D]Present address: Western University of Health Sciences, College of Veterinary Medicine, Pomona, CA 91766, USA.

[E]Corresponding author. Email: dkersey@westernu.edu

Abstract. The aim of the present study was to determine the efficacy of faecal hormonal measures for evaluating ovarian activity in a significant sized cohort of giant pandas during the perioestrual period. Faecal excretion of oestrogen and progestagen metabolites corresponded with urinary patterns and receptive behaviours. Longitudinal assessment of 10 females revealed that, on average, faecal oestrogen concentrations started to rise ($P < 0.05$) above baseline (baseline mean ± s.e.m.; $64.7 \pm 6.6\,ng\,g^{-1}$) 5 days before the preovulatory oestrogen peak ($484.6 \pm 126.8\,ng\,g^{-1}$), which was followed by a gradual descent over 4 days to nadir. Mean faecal progestagen metabolite concentrations increased approximately twofold above baseline (from 186.2 ± 37.7 to $347.2 \pm 75.7\,ng\,g^{-1}$; $P < 0.05$) during the 20-day interval after the preovulatory oestrogen surge. Variability within and among females precluded the use of a threshold of oestrogen or progestagen metabolites to predict reproductive status, yet faeces collected 2–3 days per week provided sufficient data to recognise that an individual was in the perioestrual period. Finally, in females that were examined for at least 3 consecutive years, there was an 18–53 day variation in the onset and an 8–13 day variation in the duration of perioestrual behaviour from year to year. In summary, these findings indicate that gonadal hormone profiles associated with the period immediately before, during and after oestrus are accurately revealed by analysis of the fibrous faeces of the giant panda. This approach has potential value for providing point-in-time information on the reproductive status of free-living individuals.

Additional keywords: bear reproduction, faecal hormones.

Introduction

A substantial amount of information has been generated over the past 25 years describing the reproductive biology of the female giant panda (for reviews, see Durrant *et al.* 2006; Howard *et al.* 2006; Steinman *et al.* 2006). Males and females reach sexual maturity at approximately 5 and 3 years of age, respectively (Kleiman *et al.* 1979; Schaller *et al.* 1985; Steinman *et al.* 2006). The species is unusual in that the adult female is reproductively receptive for <1% of the year, being mono-oestrual during a breeding season that ranges from February through to June (for a review, see Steinman *et al.* 2006). The obligate perioestrual period appears to be characterised hormonally by a gradual 10-day increase in urinary oestrogen excretion, with a rapid decline in oestrogen excretion signalling ovulation (Monfort *et al.* 1989; Lindburg *et al.* 2001; Czekala *et al.* 2003). Peak oestrual behaviours are observed over only a single, annual 1–3-day period that generally corresponds with a peak in oestrogen excretion (Monfort *et al.* 1989; Czekala *et al.* 1998, 2003; Lindburg *et al.* 2001; Steinman *et al.* 2006). This abbreviated reproductive period has historically complicated efforts to propagate giant pandas in *ex situ* collections (Zhang *et al.* 2006). For example, if a designated pair is sexually incompatible, time is short to find an alternative mate or plan for AI. Regardless, as the collective knowledge describing the basic biology of the giant panda has grown, so has managed care and breeding success (Wildt *et al.* 2003; Edwards *et al.* 2006*a*, 2006*b*; Ellis *et al.* 2006; Howard *et al.* 2006; Zhang *et al.* 2006), with >275 individuals now living in the world's *ex situ* collection (Xie and Gipps 2007).

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Although there are multiple publications addressing the reproductive endocrinology of the giant panda, all data have been generated collectively from studying only 23 individuals, and virtually all detailed descriptions about perioestrus originate from the assessment of urinary steroid metabolite excretion in just three females (for a review, see Steinman *et al.* 2006). There is a need to determine whether these earlier findings from only a few individuals typify norms for the species. Furthermore, although urinary hormone monitoring has been helpful for understanding basic ovarian function, collecting urine can be problematic, especially when giant pandas are living on a soil substrate in a large enclosure. Whereas urinary evaluations offer little opportunity for studying giant pandas living in nature, faecal samples can be readily identified and collected in bamboo-rich habitats. Faecal steroid metabolite analyses have been used to study the endocrinology of other Ursidae, including the brown bear (*Ursus arctos*; Ishikawa *et al.* 2002, 2003; von der Ohe *et al.* 2004; Dehnhard *et al.* 2006), Himalayan black bear (*Ursus thibetanus*; Young *et al.* 2004), sloth (*Melursus ursinus*; Young *et al.* 2004), spectacled bear (*Tremarctos ornatus*; Dehnhard *et al.* 2006) and sun bear (*Helarctos malayanus*; Onuma *et al.* 2002; Schwarzenberger *et al.* 2004; Hesterman *et al.* 2005). Faecal hormone monitoring was attempted previously in a single female giant panda after testing a range of extraction and assay methods (Kubokawa *et al.* 1992). However, that study relied on only a single sample per month from one animal, which prevented rigorous testing of the validity and feasibility of this approach for assessing reproductive activity.

Thus, the specific objectives of the present study were to validate immunoassays for assessing faecal metabolites of oestrogens and progestogens in the giant panda and to then determine the association between faecal steroid excretion patterns and concomitant urinary steroid measures and sexual behaviour characteristic of perioestrus. A major advantage of the present study was our comparative approach (assessing faeces *v.* urine) and the availability of faecal samples from a significant sized cohort of giant panda females. Our findings also serve as a foundation for a parallel study (D. C. Kersey, D. E. Wildt, J. L. Brown, R. J. Snyder, Y. Huang and S. L. Monfort, unpubl. data) designed to document the excretion patterns of sex steroid metabolites associated with acyclicity, delayed implantation, pseudopregnancy and pregnancy in this species.

Materials and methods

Study animals and facilities

North America

During the study (2001–2005), the Smithsonian National Zoological Park (SNZP; 39°N, 77°W) and Zoo Atlanta (ZA; 33°N, 84°W) each maintained one adult female and one adult male giant panda. The female housed at SNZP (Studbook (SB) 473) was born on 22 July 1998 and exhibited pubertal oestrus in April 2002 (aged 3.7 years). The ZA female (SB452) was born on 25 August 1997 and experienced first oestrus in April 2001 (3.5 years of age). Fresh water was available at all times and giant pandas were fed primarily a bamboo diet (~75%) supplemented with fruit and a high-fibre biscuit that included amino acids, minerals and vitamins. All bamboo was harvested locally and kept fresh under sprinklers to prevent desiccation before feeding. Animals had access to outdoor (100–300 m^2) areas during daylight and were housed indoors (50–100 m^2) at night. Both areas allowed continuous inter-pair olfactory and auditory contact and frequent visual communication. Direct physical interaction between male and female giant pandas was allowed, and recorded, during behavioural oestrus, as determined from visual observations of proceptive and receptive behaviours (McGeehan *et al.* 2002) by keepers and curatorial staff, with intermittent contact throughout the remainder of the year (but only for the SNZP pair). Behavioural observations occurred during work hours (0700–1700 hours), except during periods of receptive behaviours (e.g. lordosis, tail-up; McGeehan *et al.* 2002), when females were monitored 24 h per day. Dates of attempted or confirmed copulations and/or AI were recorded and used to establish physiological relationships with hormonal profiles.

China

The China Conservation and Research Center for the Giant Panda at the Wolong Nature Reserve (Wolong; 31°N, 103°E) has consistently maintained one of the world's largest *ex situ* giant panda populations, currently with approximately 40 adult females and 25 adult males. For the present study (2001, 2002, 2004), faecal samples were collected from 10 healthy females (mean age 8.8 years; range 3–16 years) that displayed overt signs of proceptive and receptive oestrual behaviour (McGeehan *et al.* 2002). Water was available *ad libitum* and, in general, the diet consisted primarily of bamboo (~80–90% of total diet) supplemented with a high-fibre biscuit produced on site. Females were housed individually in enclosures that included indoor (30–60 m^2) and outdoor (100–300 m^2) areas, with outdoor access limited to daylight hours. Physical contact between the sexes was limited to periods of behavioural oestrus, as determined by behavioural observations, although all females were in auditory, visual and olfactory proximity to at least one male conspecific throughout the year.

Sample collection and processing

Urine

Freshly voided urine was aspirated from the enclosure floor 3–7 days per week with a clean plastic syringe and then transferred to a 12 × 75-mm plastic tube, labelled with animal identification number and date, and stored frozen (−20°C) until analysis. Care was taken to avoid contamination of urine with faeces or water (to avoid a dilution effect). If a fresh urine specimen could not be secured in the morning, samples were collected as excreted throughout the day. Samples excreted during the night were labelled as 'overnight' specimens and assigned a time of 0000 hours (midnight).

Creatinine

All urine samples were indexed for creatinine (Cr) to account for variations in water content (Taussky 1954). The Cr concentrations in urine (0.05 mL; diluted 1 : 20 in bovine serum albumin (BSA)-free phosphate buffer) were determined as described previously (Monfort *et al.* 1990). Samples with Cr concentrations <0.1 mg mL^{-1} were considered too dilute (probably from water

contamination), a criterion that excluded approximately 7% of all samples. The hormone concentration in a given urine sample ($ng\,mL^{-1}$) was divided by Cr concentration ($mg\,mL^{-1}$) to derive a final hormone concentration that was expressed as the concentration of the hormone per mg Cr excreted ($ng\,mg^{-1}$ Cr).

Faeces

Generally, faecal samples were collected within 1 h of excretion, 5–7 days per week in North America or 2–5 days per week in China. Due to the variable fibre content of giant panda faeces (Edwards *et al.* 2006*b*), keepers preferentially selected faecal boluses (1–2 boluses per defecation, depending on size) that contained a greater amount of digested bamboo (score >50 as per Edwards *et al.* 2006*b*). Generally, faecal boluses ranged in consistency from formed but soft, slightly moist (e.g. score 50) to formed, very hard, dry crumbly (e.g. score 100); however, most faecal boluses (~70%) conformed to a score of 75 as per Edwards *et al.* (2006*b*). Faecal samples were collected in resealable plastic bags, labelled with the animal number and date and stored frozen (−20°C) until processing.

Faecal samples were freeze-dried in a lyophiliser (Labconco, Kansas City, MO, USA) and crushed in plastic bags to separate powdered faeces from larger pieces (>0.5 cm) of undigested bamboo, including whole or parts of leaves or culm. Fine powdered faeces were stored frozen (−20°C) in labelled and capped 12 × 75-mm plastic tubes and later thawed to allow 0.1 g of each sample to be extracted (in 90% ethanol) for the analysis of hormonal metabolites using the procedures of Wasser *et al.* (1994). Extracts were then dried under air and resuspended in 1 mL BSA-free phosphate buffer before being stored frozen. The mean (± s.e.m.) hormone recovery rate for extraction from faecal powder was 84.9 ± 1.3% (based on a preliminary trial involving the addition of ^{3}H-oestradiol).

Endocrine analyses

High-pressure liquid chromatography

High-pressure liquid chromatography (HPLC) analyses (Varian ProStar; Varian Analytical Instruments, Walnut Creek, CA, USA) were conducted to identify faecal oestrogen (E) and progestagen (P) metabolites, as described previously (Monfort *et al.* 1991, 1997). Pooled faecal extracts with high E and P immunoreactivity were concentrated 20-fold and spiked with respective radioactive hormone tracers (E run, ~14 000 c.p.m. mL^{-1} each ^{3}H-oestrone sulfate (E1S) and ^{3}H-oestradiol; P run, ~14 000 c.p.m. mL^{-1} ^{3}H-progesterone (P4)) to act as cochromatographic markers. Radioactivity was assessed in eluted fractions to identify the retention times for the radioactive markers. Eluates were evaporated to dryness, resuspended in 0.13 mL buffer and hormone concentrations were quantified by an oestrone glucuronide and P enzyme immunoassay (EIA; see below). Fractional immunoreactivity was compared with retention times for known radioactive markers to establish the presumptive identity of excreted steroidal metabolites.

Oestrone glucuronide EIA

Oestrogen values were assessed in unprocessed urine and faecal extracts using a single antibody oestrone glucuronide EIA (Stabenfeldt *et al.* 1991), with minor modification. Oestrone glucuronide antibody (R583) was obtained from Coralie Munro (University of California, Davis, CA, USA) and coated to microtitre plates (96-well; Nunc-Immuno, Maxisorp; Fisher Scientific, Pittsburgh, PA, USA). Faecal extracts and urine were diluted before analysis (unprocessed urine, equivalent to 0.2–2.5 µL; faecal extract, equivalent to 0.5–5.0 µL). Plates were read on a microplate reader (Dynex MRX; Dynex Technologies, Chantilly, VA, USA) when optimal optical density (OD; reading filter, 405 nm; reference filter, 540 nm) of the maximum binding wells was reached (i.e. 1.00 OD). The interassay coefficients of variation (CV) for two internal controls ($n = 87$ assays) were 13.8% (mean binding, 41.8%) and 11.4% (mean binding, 74.5%), whereas the intraassay CV was <10%. Both urine ($r^2 = 0.99$; $P < 0.05$) and faeces ($r^2 = 0.98$; $P < 0.05$) demonstrated displacement curves parallel to those of standard hormone preparations. Significant ($P < 0.05$) recovery was achieved following the addition of standards to both urine ($y = 0.76x + 4.38$; $r^2 = 0.98$) and faecal ($y = 1.24x + 19.54$; $r^2 = 0.98$) extracts.

Progesterone EIA

Faecal and urinary P metabolite immunoreactivity was determined using a single-antibody P4 EIA (CL425; C. Munro; Graham *et al.* 2001). Samples of unprocessed urine and faecal extract were diluted (equivalent to 0.04–2.5 µL) before P4 EIA assessment. The interassay CV for the two internal controls ($n = 54$ assays) was 12.2% (mean binding, 37.0%) and 13.2% (mean binding, 67.8%), whereas the intraassay CV was <10%. Serially diluted, unprocessed urine ($r^2 = 0.99$; $P < 0.05$) and faecal ($r^2 = 0.96$; $P < 0.05$) extracts both demonstrated displacement curves that were parallel to those of standard hormone preparations. Significant ($P < 0.05$) recovery was achieved following the addition of P4 to both urine ($y = 0.88x - 1.13$; $r^2 = 0.98$) and faecal ($y = 0.67x - 2.03$; $r^2 = 0.98$) extracts.

Statistical analyses

Baseline concentrations of urinary and faecal E and P were determined through an iterative process described by Moreira *et al.* (2001) with minor modification. Briefly, baseline values were assessed yearly for each female for each hormone. Values in excess of two standard deviations (s.d.) of baseline were removed from the dataset until no values exceeded 2 s.d. of the baseline mean. Mean baseline hormone concentrations are expressed as the mean ± s.e.m. The duration of hormonal perioestrus was considered the day from an initial increase in oestrogens above baseline (mean + 2 s.d.) to the day oestrogen values returned to within baseline range. Receptive oestrual behaviours were defined as tail-up, walking backwards and lordosis (McGeehan *et al.* 2002).

To standardise profiles for the perioestrual period, urinary and faecal hormone metabolite concentrations were aligned to the day of peak E (Day 0). To examine the association between urinary and faecal measures, data were aligned to the day of peak urinary E. The day of the year (based on the Gregorian calendar) was used to assess differences in the time of onset of perioestrus within and among females. For EIA validations, parallelism was

assessed via non-linear regression, whereas hormone recovery was assessed using linear regression. Datasets were tested for normality (Kolmogorov–Smirnov test) before the significance of differences were assessed. Relationships between urinary and faecal hormone data were determined by calculating a Pearson product moment correlation. Statistical significance for independent datasets was assessed using a Mann–Whitney test, and dependent datasets were tested using a Wilcoxon signed rank test. All analyses were conducted with SigmaStat 3.1 (Systat Software, Point Richmond, CA, USA).

Results

High-pressure liquid chromatography

Faecal E immunoreactivity quantified after HPLC separation (Fig. 1*a*) coeluted with ^{3}H-E1S (Fraction 17) and ^{3}H-oestradiol (Fraction 64), which constituted 11.9 and 21.7% of total immunoreactivity, respectively. The HPLC analysis of faecal P immunoreactivity (Fig. 1*b*) revealed seven prominent peaks. The largest peak (Fraction 10) constituted 8.7% of total immunoreactivity and exhibited the same retention time as pregnanediol-3-glucuronide. A small proportion of the total immunoreactivity (2.6%) was associated with P4 (Fraction 66). The presumptive identities of the six other major immunoreactive peaks (12.4% of total immunoreactivity) detected by the P4 EIA were not established.

Urinary and faecal oestrogen and progestagen comparisons

Concurrent urinary and faecal E and P profiles from a single perioestrual event from two female giant pandas housed at SNZP (SB473) and ZA (SB452) with hormone excretion

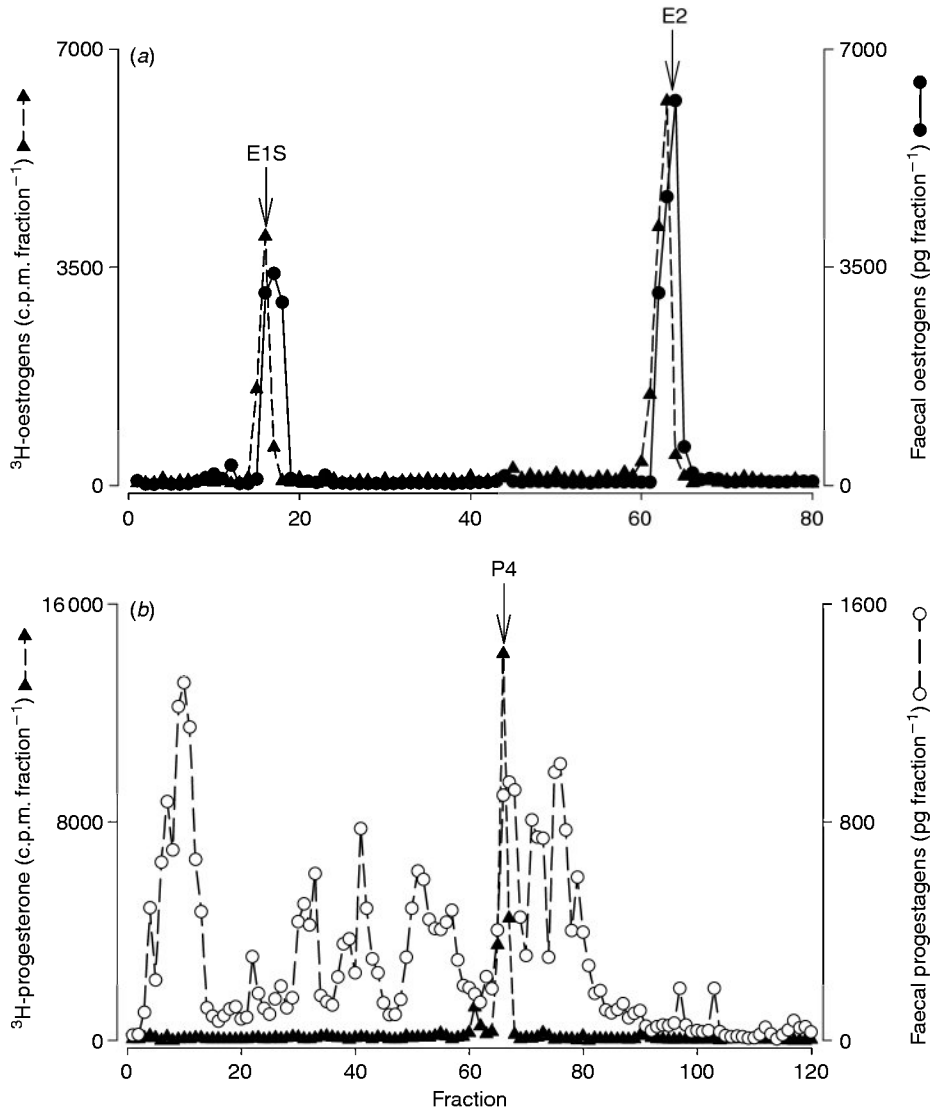

Fig. 1. High-pressure liquid chromatography cochromatographic profiles of (*a*) ^{3}H-oestrogens and (*b*) ^{3}H-progesterone, with corresponding faecal oestrogen and progestagen metabolites, respectively. Arrows mark elution of oestrone sulfate (E1S), oestradiol (E2) and progesterone (P4).

aligned to the day of urinary E peak (Day 0) are shown in Fig. 2. Profiles were generated from E and P data of paired (i.e. collected on the same day) urine and faecal samples. The correlation between urinary and faecal E excretion was significant for SB473 ($r = 0.79$; $P < 0.05$) and SB452 ($r = 0.69$; $P < 0.05$), as was the relationship between urinary and faecal P ($r = 0.49$ for SB473 and $r = 0.67$ for SB452; $P < 0.05$).

For SB473, the initial increase over baseline (+2 s.d.) in urinary E occurred on Day −6 (from 10.1 ± 0.5 to 19.1 ng mg^{-1} Cr), whereas faecal E exceeded baseline (+2 s.d.) concentrations on Day −3 (from 98.6 ± 4.8 to 324.1 ng g^{-1}; Fig. 2*a*). Peak faecal E concentrations (744.4 ng g^{-1}) were observed 1 day before peak urinary E concentrations (150.4 ng mg^{-1} Cr). Urinary E excretion subsequently declined to baseline levels by Day 4, whereas faecal E concentrations reached baseline 1 day later (Day 5). There was a significant increase in both urinary and faecal P during the 20-day period after maximal excretion of E (12.8 ± 1.5 ng mg^{-1} Cr and 710.7 ± 98.8 ng g^{-1}, respectively) compared with the 20-day interval that preceded the E peak (4.7 ± 1.2 ng mg^{-1} Cr and 235.6 ± 22.0 ng g^{-1}, respectively; $P < 0.05$; Fig. 2*c*).

Urinary E concentrations in female SB452 first increased above baseline (+2 s.d.) on Day −9 (from 9.7 ± 0.5 to 14.3 ng mg^{-1} Cr), whereas faecal E increased above baseline by Day −5 (from 72.5 ± 4.8 to 120.9 ng g^{-1}; Fig. 2*b*). In female SB452, peak urinary and faecal E concentrations were observed on Days 0 and 1, respectively (266.8 ng mg^{-1} Cr and 600.9 ng g^{-1}, respectively). Urinary E excretion declined to baseline by Day 7, whereas faecal E excretion had reached baseline levels 5 days earlier (i.e. on Day 2). Similar to what was observed for female SB473, urinary and faecal P excretion was significantly greater for female SB452 during the 20-day period after the peak in urinary E excretion (13.3 ± 1.0 ng mg^{-1} Cr and 551.9 ± 91.9 ng g^{-1}, respectively) compared with the 20-day period prior to peak E excretion (3.6 ± 0.4 ng mg^{-1} Cr and 194.3 ± 21.0 ng g^{-1}, respectively; $P < 0.05$; Fig. 2*d*).

Mean perioestrual profiles

Mean perioestrual faecal E and P profiles for female SB473 (in 2002, 2003, 2004 and 2005) and female SB452 (in 2002, 2003 and 2004) are shown in Fig. 3. For female SB473, faecal E excretion increased above baseline by Day −6 (from 153.0 ± 2.4 to 167.2 ng g^{-1}, respectively), peaked at 687.9 ± 56.0 ng g^{-1} (representing an approximate 4.5-fold increase ($P < 0.05$) above nadir) and then decreased precipitously to baseline by Day 1 (110.7 ng g^{-1}; Fig. 3*a*). Faecal P content during the 20 days before and after peak E excretion differed significantly (297.1 ± 24.0 *v.* 730.7 ± 45.0 ng g^{-1}, respectively; $P < 0.05$). For female SB452, the onset of perioestrus coincided with a rise in mean E excretion from 36.7 ± 1.3 to 79.0 ng g^{-1} on Day −9, a peak on Day 0 (452.5 ± 106.0 ng g^{-1}; an approximate 11.5-fold increase over baseline; $P < 0.05$) and then a decrease to baseline by Day 5 (37.9 ng g^{-1}; Fig. 3*b*). Faecal P excretion 20 days before and after the E peak differed significantly (146.7 ± 14.0 *v.* 381.7 ± 48.0 ng g^{-1}, respectively; $P < 0.05$). Mean peak faecal E concentrations for females SB473 and SB452 were not significantly different (687.9 ± 56.0 *v.* 452.5 ± 106.0 ng g^{-1}, respectively; $P > 0.05$).

Faecal data collected throughout the year were examined for variations within and between animals with respect to the onset of perioestrus and the duration of oestrus. For female SB473, increased receptive behaviours commenced 24 April 2002, 2 April 2003, 1 May 2004 and 11 March 2005, giving a range

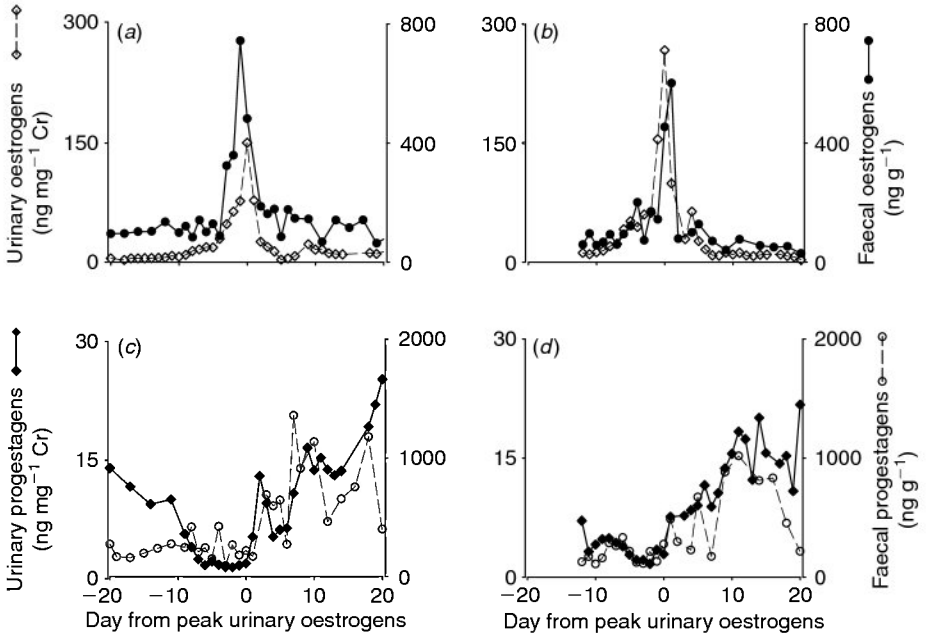

Fig. 2. (*a*, *b*) Oestrogen (◇, urinary; ●, faecal) and (*c*, *d*) progestagen (♦, urinary; ○, faecal) concentrations during perioestrual periods from females SB473 (*a*, *c*) and SB452 (*b*, *d*). Data were aligned as day from peak urinary oestrogen excretion.

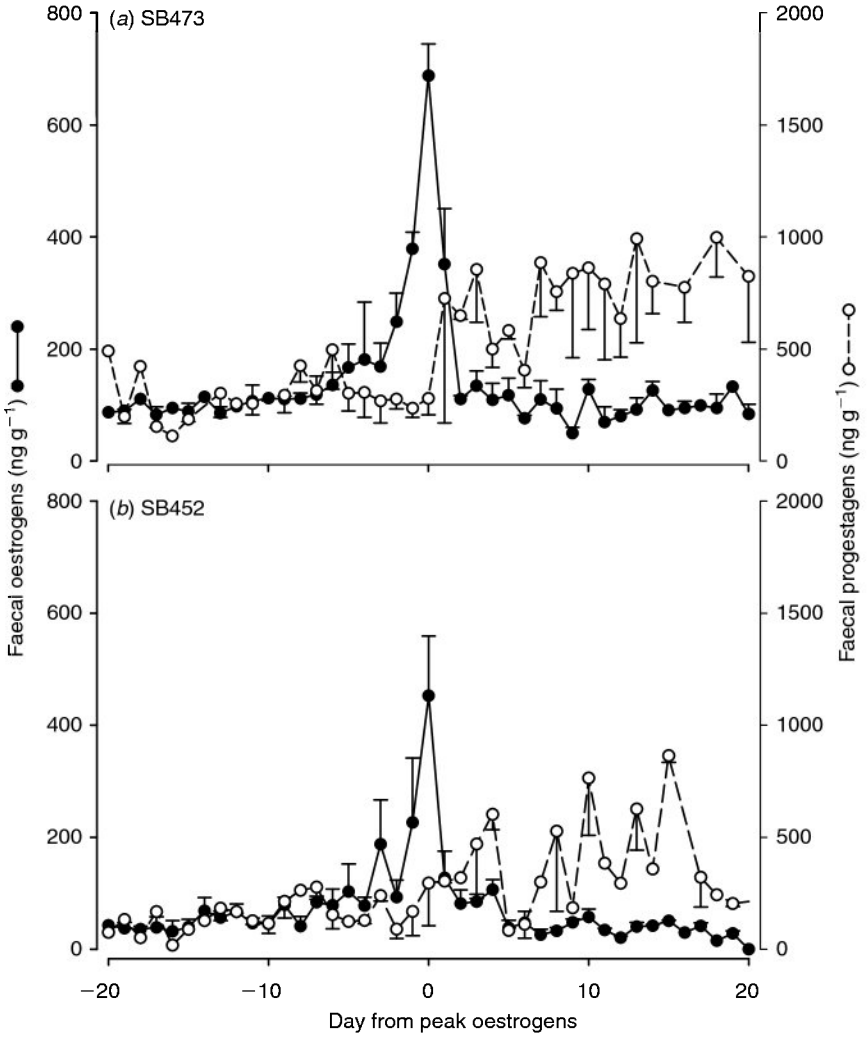

Fig. 3. Mean (± s.e.m.) faecal oestrogen (●) and progestagen (○) metabolite immunoreactivity during the perioestrual period for (*a*) female SB473 (in 2002, 2003, 2004 and 2005) and (*b*) female SB452 (in 2002, 2003 and 2004).

in the onset of perioestrus of 53 days. This female exhibited overt perioestrual behaviours (e.g. tail-up, walking backwards and bleating) that lasted 10 days (2002), 8 days (2003), 9 days (2004) and 8 days (2005), giving a within-animal variation in the duration of oestrus of only 2 days over the 4-year study period. For female SB452, receptive behaviours commenced 2 April 2002, 25 March 2003 and 14 March 2004, giving a range in the onset of perioestrus of 18 days. Female SB452 exhibited overt oestrus behaviour for 9 days (2002), 8 days (2003) and 13 days (2004), giving a within-animal variation in the duration of oestrus of 5 days over the 3-year study period.

Average and individual perioestrual profiles from animals in China

Faecal E data from the 10 females housed at the China Conservation and Research Center for the Giant Panda (Wolong Nature Reserve) revealed that, on average, peak E concentrations were 7.4-fold times greater than baseline values (484.6 ± 126.8 *v.* 64.7 ± 6.6 ng g^{-1}, respectively; $P < 0.05$; Table 1). Additionally, faecal P concentrations were, on average, 1.9-fold greater in the 20-day period after the periovulatory E peak than in the 20-day period before the peak (347.2 ± 75.7 *v.* 186.2 ± 37.7 ng g^{-1}, respectively; $P < 0.05$).

Representative individual perioestrual profiles of four of these 10 females are shown in Fig. 4. The temporal excretory dynamics of hormone excretion in this cohort were similar to what was observed in the more intensively sampled giant pandas at SNZP and ZA (Figs 2, 3). The between-animal range in the onset of receptive perioestrual behaviours was 42 days (ranging from Day 109 for female SB544 to Day 151 for female SB414). Owing to limited sampling for female SB446 during the perioestrual period, only a single E point was recorded showing an increase in faecal E fourfold above baseline on Day 130 (581.2 ng g^{-1}), which occurred 7 days before the onset of receptive behavioural oestrus and copulation (Fig. 4*a*). Faecal P excretion in female SB446 20 days before and after

Table 1. Date of peak faecal oestrogen and peak concentrations of females housed at the China Conservation and Research Center for the Giant Panda at the Wolong Nature Reserve

Studbook no.	Date of peak faecal oestrogen	Peak oestrogen concentration (ng g^{-1})
374	23 February 2002	432.2
382	7 February 2002	250.6
397	3 March 2002	194.8
404	17 March 2002	328.8
414	19 May 2001	278.1
414	4 June 2002	465.4
418	15 May 2001	480.1
439	18 April 2004	1546.7
446	10 May 2001	581.2
487	2 May 2004	126.9
544	24 April 2004	534.8

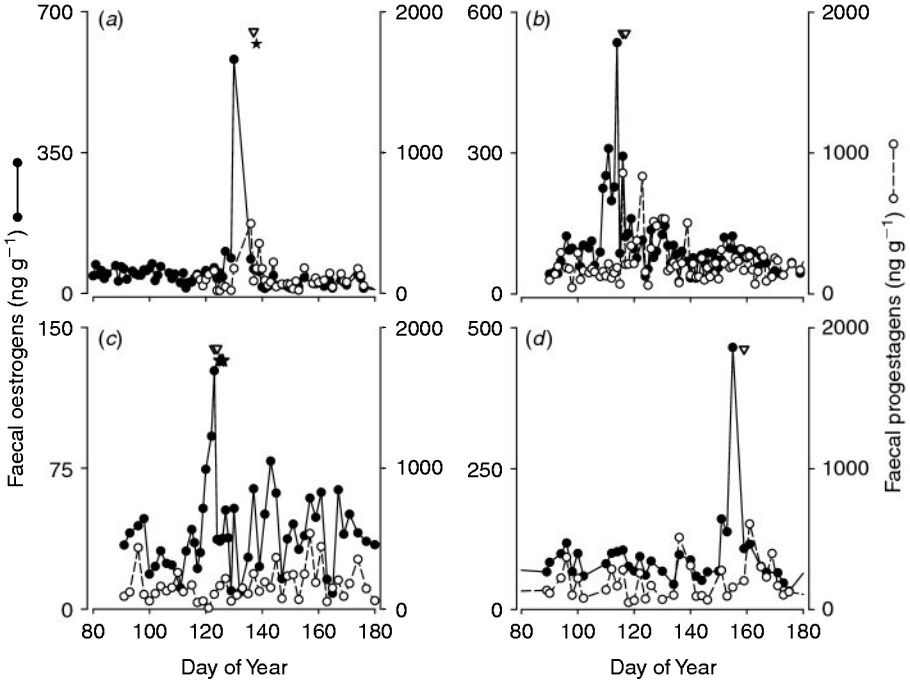

Fig. 4. Representative faecal oestrogen (●) and progestagen (○) metabolite excretion profiles for females (*a*) SB446, (*b*) SB544, (*c*) SB487 and (*d*) SB414. (▽), day(s) of natural breeding; (★), timing of AI. Data are aligned to the Day of Year. Note differences in the faecal oestrogen scales among individuals.

mating did not differ significantly (114.8 ± 35.0 *v.* 112.2 ± 21.8 ng g^{-1}, respectively; $P > 0.05$). Female SB544 mated on Day 116, 5 days after faecal E increased above baseline and 1 day after the faecal E peaked (534.8 ng g^{-1}) sevenfold above baseline (Fig. 4*b*). Mean faecal P excretion for this individual 20 days before and after mating differed significantly (151.2 ± 13.6 *v.* 355.5 ± 53.8 ng g^{-1}, respectively; $P < 0.05$). For female SB487, the faecal E peak coincided with receptive behavioural oestrus and copulation (Day 123), but peak E concentrations (126.9 ng g^{-1}) were reduced compared with those in other females and exceeded baseline by only fourfold (Fig. 4*c*). Faecal P concentrations for female SB487 20 days before and after mating were not significantly different (121.3 ± 22.8 *v.* 146.9 ± 18.9 ng g^{-1}, respectively; $P > 0.05$). Peak faecal E concentrations in female SB414 increased sixfold above baseline on Day 155 (465.4 ng g^{-1}), with copulation on Day 159 (no faecal samples were collected from Day 156 to 159; Fig. 4*d*). There were no significant differences in faecal P concentrations for this female for the 20 days before and after mating (202.1 ± 54.6 *v.* 267.7 ± 59.9 ng g^{-1}, respectively; $P > 0.05$). Of the four individuals for which data are presented in Fig. 3, females SB446 and SB487 produced singleton and twin cubs, respectively, after a gestation of 128 and 122 days, respectively. The other two giant pandas were presumed to have experienced a pseudopregnancy, a non-pregnant state with hormones and behaviours mimicking pregnancy (Monfort *et al.* 1989; Mainka *et al.* 1990; Narushima *et al.* 2003; Steinman *et al.* 2006).

Discussion

The present study validated a non-invasive alternative to urinary hormone monitoring for assessing the single, annual perioestrual period in the endangered giant panda. This is important for understanding the hormonal factors that trigger sexual receptivity, ovarian follicle development, behavioural oestrus and ovulation in this rare species. The findings demonstrated that it was possible to extract and then measure key steroidal metabolites in the fibrous faeces of the giant panda, including identification of the oestrogen surge associated with presumed maximal follicle activity. Overall, faecal hormone monitoring permitted tracking general patterns of reproductive activity in faeces collected at 2–3 days periods.

Despite giant panda faeces being extremely fibrous, it was possible to adapt established methods (Wasser *et al.* 1994) to achieve excellent metabolite extraction efficiency (~85%) comparable to that reported earlier in the Malayan sun bear (*U. malayanus*; 81%; Hesterman *et al.* 2005) and other diverse mammals, including the maned wolf (*Chrysocyon brachyurus*; >90%; Velloso *et al.* 1998), black-footed ferret (*Mustela nigripes*; >90%; Brown 1997), cheetah (*Acinonyx jubatus*; >90%; Brown *et al.* 1996), moose (*Alces alces*; ~100%; Monfort *et al.* 1993) and African elephant (*Loxodonta africana*; >75%; Wasser *et al.* 1996). Achieving uniformly high extraction efficiency is important for generating datasets that can be compared within and among animals over long time intervals. This is especially relevant in the case of giant panda faeces, which can vary substantially (even within the same individual) in quality and consistency, ranging from hard, compact bamboo boluses to loose mucoid stools (Edwards *et al.* 2006*b*). Regardless, we determined that, once dried, faeces of variable quality and consistency could be readily pulverised to produce a uniform-appearing powder that, upon analysis, yielded hormonal patterns that met expectations based on previous urinary evaluations and concurrent observations of reproductive behaviours, especially oestrus.

Due to a hydrophobic nature, steroids often are hepatically conjugated to water-soluble moieties (i.e. sulfate or glucuronic acid) to facilitate excretion in the water-soluble environment of the gut (Whitten *et al.* 1998). Bacterial enzymes hydrolyse steroidal conjugates in the large intestine, which results in excretion of 'free', unconjugated steroids (Monfort 2003; Schwartz and Monfort 2008). However, HPLC analysis in the present study demonstrated that a substantial proportion of oestrogen and progestagen metabolites in giant panda faeces were excreted as steroidal conjugates. This observation can no doubt be explained, in part, by the rapid gut transit time in this species (mean 7.1 ± 0.4 h; range 6.0–8.8 h; Edwards *et al.* 2006*b*), which could result in reduced enzymatic hydrolysis and an increased proportion of excreted steroidal conjugates. This would also be consistent with our findings that faecal oestrogens and progestogens were accurately assessable in the giant panda using immunoassays and antisera directed at steroidal conjugates. The unusually short clearance rate through the gut also likely decreased the time from hormone hepatic metabolisation and conjugation to excretion of the faecal bolus. Comparatively, this lag time is substantially shorter than what has been observed in felids (8–50 h; Brown *et al.* 1994), ruminants (12–24 h; Morrow and Monfort 1998), non-human primates (24–48 h; Wasser *et al.* 1994) and hind-gut fermenters (24–48 h; Wasser *et al.* 1996). Furthermore, a propensity for the giant panda to defecate up to 20 times per day also suggests that excreta data represent discrete, point-in-time endogenous events, more so than with urine, which embodies more of a 'pooling effect' (Monfort 2003). This probably explains why excreted E concentrations remain elevated for longer in the urine than in the faeces in this species.

For more than 25 years, urinary oestrogens have been measured in the giant panda to track reproductive changes associated with the short, annual perioestrual period (Bonney *et al.* 1982; Hodges *et al.* 1984; Chaudhuri *et al.* 1988; Monfort *et al.* 1989; Mainka *et al.* 1990; Lindburg *et al.* 2001; McGeehan *et al.* 2002; Czekala *et al.* 2003; Steinman *et al.* 2006). Receptive oestrual behaviours, including tail-up and walking backwards, are most often correlated with peak and declining urinary oestrogen concentrations. Perioestrus is characterised hormonally by oestrogen values that rise gradually from baseline to peak excretion over the course of 1–2 weeks, then decline precipitously at the presumptive time of ovulation (for a review, see Steinman *et al.* 2006). The urinary oestrogen profiles during perioestrus from the intensively monitored females SB473 and SB452 (Fig. 2) conformed to previously published data, including finding receptive oestrual behaviours at and after the oestrogen peak. However, we also observed the same basic trend in faecal oestrogen, although the temporal dynamics between urinary and faecal measures differed slightly. For both females, the duration of elevated perioestrual oestrogen was longer in the urine than in the faeces (by 2–9 days). Furthermore, the initial significant rise in oestrogen during perioestrus occurred in the urine 3–4 days before being discovered in the faeces. Both measures revealed that oestrogen was metabolised rapidly because both urinary and faecal oestrogens decreased sharply after the periovulatory peak, returning to nadir within 7 days. Yet, because the excretory fate of urinary oestrogens is likely more rapid than that of the faecal oestrogens, steroidal changes may be detected sooner in the urine than in the faeces. The marked post-surge decrease in oestrogen excretion no doubt reflected a rapid changeover in follicular function at ovulation, which could help managers to predict the impending end to sexual receptivity and, thus, opportunities for mating or AI (McGeehan *et al.* 2002; Snyder *et al.* 2004; Steinman *et al.* 2006). In addition, although the duration of elevated oestrogen excretion is comparatively short, even the periodic collection of faecal samples may reveal a single point-in-time indication of a surge that is predictive of impending oestrus (e.g. the 15-fold increase in faecal oestrogens above baseline observed from only one sample in SB446; Fig. 4*a*).

All females in the present study experienced a single oestrus during the interval from February through to May, which was similar to what has been reported previously for this species (Kleiman *et al.* 1979; Bonney *et al.* 1982; Chaudhuri *et al.* 1988; Czekala *et al.* 1998; Lindburg *et al.* 2001; McGeehan *et al.* 2002; Durrant *et al.* 2002; Steinman *et al.* 2006), and for other Ursidae (Ferguson and McLoughlin 2000; Spady *et al.* 2007). For the two most intensively monitored giant pandas, the data revealed

that there was some variation in the onset of oestrus over time, and sexual receptivity appeared to occur over a 1.9–7.5-week interval from year-to-year in a given female. However, the general patterns of faecal oestrogen excretion were qualitatively similar within and between females, yet there was no evidence for a quantitative threshold in oestrogen excretion that elicited specific oestrous behaviours. For the 17 perioestrual periods presented, peak faecal oestrogen excretion represented a minimum 3.6-fold increase above baseline, but peak concentrations varied markedly among animals and even within the same female evaluated in successive years. For example, maximal faecal oestrogen values in the giant panda cohort in China varied from 126.9 to 581.2 ng g^{-1}, which was similar to the variation observed within the two intensively monitored females in the US (SB473 range 214.3–744.4 ng g^{-1}; SB452 range 245.9–600.8 ng g^{-1}). Yet, these data do not indicate that lower peak faecal oestrogen concentrations reflect inadequate ovarian function. Female SB487 (Fig. 4*c*) gave birth to twins after natural mating and AI, yet had a peak oestrogen value (126.9 ng g^{-1}) that was approximately 4.8-fold lower than the mean (614.1 ± 240.4 ng g^{-1}) of six other females, including SB544 and SB414 (Fig. 3*b*, *d*), at the same facility that did not give birth. Similarly, although the average duration of elevated faecal oestrogen during perioestrus was consistent (~8 days) with earlier studies that relied on urinary assessments (for a review, see Steinman *et al.* 2006), this range varied by 9 days (range 4–13 days) and was not an indicator of fertility. The variations in hormonal content in the faeces were not surprising because it has been known for decades that it is the temporal pattern over time, not the absolute concentrations of hormones in the blood, urine or faeces, that is most valuable as a research and management tool (for a review, see Monfort 2003). Metabolite concentrations in the faeces can also be naturally influenced by the diet (Wasser *et al.* 1993), season (Bales *et al.* 2006) and photoperiod (Brown 1997; Brown *et al.* 2002). Because the giant pandas in the present study hailed from different facilities across different countries and cultures, and even though housing and husbandry differences appeared subtle, it was impossible to explore the specifics of external factors, such as diet and light cycle, in any depth. Regardless, the data are clear that, with stricter controls and frequent sampling, it would likely be possible to use faecal hormone monitoring to examine the regulators of reproductive onset in this species.

Previous assessments of urinary progestagen metabolites have clearly documented an initial threefold primary rise above baseline that confirms ovulation and corpus luteum (CL) formation and a secondary threefold rise in progestagen metabolite excretion 74–122 days later that signals the onset of presumptive implantation (or pseudopregnancy; Hodges *et al.* 1984; Monfort *et al.* 1989; Mainka *et al.* 1990; McGeehan *et al.* 2002; Czekala *et al.* 2003; Steinman *et al.* 2006). Although overall mean faecal progestagen concentrations were increased during the 20 days after the ovulatory period in the present study, a clear primary rise in faecal progestogens was detected in only eight of 17 profiles analysed. The lack of an increase in progestagen immediately after presumed ovulation occurred despite every one of these females (pregnant or pseudopregnant) exhibiting a secondary rise (>30-fold above baseline) 63–122 days after the preovulatory faecal oestrogen peak (D. C. Kersey, D. E. Wildt, J. L. Brown, R. J. Snyder, Y. Huang and S. L. Monfort, unpubl. data). Although more investigation is needed, an inability to routinely detect the primary faecal progestagen rise suggests that one or more immunoreactive metabolites of this steroid excreted in the urine may be superior for tracking early, post-ovulatory CL function.

In conclusion, we determined that it is possible to use faecal steroid measures to identify and study the single, annual perioestrual period in the giant panda. As a result, we were able to expand information on this critical reproductive period with the inclusion of 15 giant pandas from three facilities in the US and China. Although there were clearly advantages with analysing samples collected daily or every other day, it was possible to generally track ovarian steroid metabolite patterns in faeces collected as infrequently as two to three times per week. The required protracted laboratory processing precludes using faecal measures as a quick turnaround tool for timing a natural mating or AI. However, there are at least two significant practical applications. For example, the large breeding facilities in China contain significant numbers of giant pandas, so many in fact that multiple females can be in oestrus simultaneously, making it difficult to collect urine samples, especially because most specimens are maintained on grass and dirt substrates. Faeces can be recovered any time during the day from these animals and then later analysed to examine the normality of the gonadal profiles. Also intriguing is the potential of using this approach for understanding reproductive patterns in free-living giant pandas. Monfort (2003) and Schwartz and Monfort (2008) have summarised the value of assessing steroid metabolites in the excreta of a host of wildlife species in nature. Particularly, faecal hormones have been used to understand the influence of dominance on reproductive success in African wild dogs (*Lycaon pictus*; Creel *et al.* 1997), the relationship of moose (*Alces alces*) ecological carrying capacity and carnivore colonisation (Berger *et al.* 1999) and the causes of reproductive suppression in meerkats (*Suricata suricatta*; Young *et al.* 2008). Although giant pandas are observationally elusive in the wild, their faecal samples are not. Frequent defecation (up to 20 times per day) by the giant panda increases the likelihood of finding samples without observing individuals. The use of scat detection dogs (Wasser *et al.* 2004) and radiotelemetry further increases the feasibility of collecting faecal samples under field conditions and, when combined with DNA analysis, one could attribute hormonal measures to specific, known individuals. Although it would likely be impractical to collect serial samples at a sufficient frequency to plot a perioestrual profile, the faecal approach could allow determination of whether a free-ranging female was 'reproductively active' (on the basis of a few oestrogen or progesterone values). However, the faecal method that was proven effective in the present study would have to be adapted to suit field conditions, as has been done with the baboon (*Papio papio*; Beehner and Whitten 2004) and white-tailed deer (*Odocoileus virginianus*; Washburn and Millspaugh 2002) to account for DNA and steroid conjugate and metabolite degradation. In addition, there is a growing database demonstrating the potential of measuring adrenal glucocorticosteroid (stress-related) hormones in mammals (von der Ohe and

Servheen 2002; Monfort 2003; Millspaugh and Washburn 2004; Young *et al.* 2004). We predict that it will be possible to extend these methods to permit accurate assessments of adrenal status by quantifying glucocorticosteroid metabolites excreted in the faeces, which would be a powerful means for assessing the impact of various perturbations, including human disturbance, on the well-being of wild giant pandas.

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