Unique biphasic progestagen profile in parturient and non-parturient giant pandas (*Ailuropoda melanoleuca*) as determined by faecal hormone monitoring

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Abstract

The luteal phase of the giant panda has been exclusively assessed by studying urinary hormone patterns in a very few individuals. To better understand hormonal dynamics of protracted progestagen excretion in this endangered species, we monitored hormonal metabolites in the fibrous faeces of multiple females in the USA and China. Giant pandas that were anoestrous during the breeding season excreted baseline progestagen throughout the year. In contrast, there were two distinctive periods when progestagen excretion increased in females that experienced behavioural oestrus, the first being modest, lasting for 61–122 days, and likely reflecting presumptive ovulation. This increase was far surpassed by a secondary rise in progestagen excretion associated with a rejuvenated luteal capacity or hormone production from an extra-gonadal source. The duration of this 'secondary' rise in progestagen excretion averaged ~45 days and terminated in a decline to baseline coincident with parturition or the end of a non-parturient luteal interval. Data revealed that, even with a complex, biphasic progestagen profile, the longitudinal patterns produced by giant pandas were relatively consistent among animals and across years within individuals. However, progestagen excretion patterns throughout this period could not be used to discriminate among non-pregnant, pregnant or pseudopregnant states.

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Introduction

The reproductive biology of the female giant panda has received considerable attention over the past 30 years (Snyder *et al.* 2004, Howard *et al.* 2006, Steinman *et al.* 2006). Aside from the species' natural charisma, the reproductive biology of the giant panda is intriguing because females are seasonally monoestrous, having the opportunity to conceive during only one 24–72 h period annually. Because the *ex situ* population is important as 'insurance' for wild counterparts and as a potential source of animals for future reintroductions (Ellis *et al.* 2006), there is urgency in ensuring that every animal reproduces, despite the narrow window of fertility. Therefore, individual females are typically monitored intensively during the annual single oestrus to allow accurately timed matings and/or artificial inseminations (AIs).

Until recently, endocrine data on the giant panda were largely derived from evaluating hormonal metabolites in urine (Steinman *et al.* 2006). We recently demonstrated the ability to detect changes in oestrogen and

progestagen metabolites in bamboo-laden, fibrous faeces during this species' dynamic perioestrous interval (Kersey *et al.* 2010). Most previous endocrine studies in giant pandas have focused on behavioural oestrus and ovulation, when there appears to be a modest post-ovulatory rise in urinary progestagen (usually termed the primary rise; Steinman *et al.* 2006). There has been little attention directed at understanding the prolonged luteal phase in pregnant versus non-pregnant individuals. Of ten reports on this topic, only three giant pandas in total have been monitored for urinary progestagen profiles from ovulation to parturition or through a non-parturient phase (sometimes referred to as 'pseudo-pregnancy'; Bonney *et al.* 1982, Hodges *et al.* 1984, Chaudhuri *et al.* 1988, Masui *et al.* 1989, Monfort *et al.* 1989, McGeehan *et al.* 2002, Narushima *et al.* 2003, Dehnhard *et al.* 2006, Steinman *et al.* 2006, Spady *et al.* 2007). In these cases, a secondary urinary progestagen rise has occurred 74–122 days after the end of oestrus (Steinman *et al.* 2006) that has suggested a shift in

hormonal source, perhaps related to nidation. The giant panda has long been considered to experience delayed implantation (Hodges *et al.* 1984, Monfort *et al.* 1989, Zhang *et al.* 2009), a strictly mammalian strategy prominent in carnivores, especially ursids (Hamlett 1935, Conaway 1971, Weir & Rowlands 1973, Renfree & Calaby 1981, Mead 1989, Sandell 1990, Lindenfors *et al.* 2003, Spady *et al.* 2007). After fertilization, the embryo is sustained as a blastocyst until an unidentified trigger stimulates implantation and resumption of development (Renfree & Calaby 1981, Mead 1989, Sandell 1990). Although peak in urinary progestagens coincides with ultrasonographic foetus detection in giant pandas (Zhang *et al.* 2009), it is unknown whether nidation occurs with the onset of a secondary progesterone (P_4) rise, as has been demonstrated in the mink (*Mustela vison*; Allais & Martinet 1978), European badger (*Meles meles*; Canivenc & Bonnin 1981) and spotted skunk (*Spilogale putorius*; Mead 1981). The phenomenon of pseudopregnancy (i.e. when a female experiences the same physiological and behavioural changes as a pregnant counterpart; Erskine 1998) is recognized in other ursids, including a prolonged and elevated progestagen phase with profiles that are indistinguishable from pregnant conspecifics (e.g. American black bear, *Ursus americanus*; Schulz *et al.* 2003; Asiatic black bear, *Ursus thibetanus*; Sato *et al.* 2001; brown bear, *Ursus arctos*; Tsubota *et al.* 1992; sun bear, *Helarctos malayanus*; Schwarzenberger *et al.* 2004). A similar prolongation of progestagen excretion occurs in the giant panda, although this method is uninformative in diagnosing pregnancy (Monfort *et al.* 1989, Steinman *et al.* 2006).

Given the success of studying the dynamics of perioestrus via faecal monitoring in this species (Kersey *et al.* 2010), we used the same approach to examine the extended (and largely ignored) post-ovulatory interval. Because faecal assessments in mammalian species generally 'dampen' the hormonal fluctuations usually measured in blood (Monfort 2003, Schwartz & Monfort 2008), we hypothesized that monitoring progestagens in faeces would provide a more accurate representation of the magnitude and temporal patterns of the primary versus secondary progestagen rise. In this context, we took advantage of giant pandas housed in two zoological collections in North America as well as one of the largest captive breeding centres in China. This unique accessibility allowed the first ever, long-term, comparative assessment of giant pandas that were cycling, anoestrous, pregnant or non-parturient.

Results

We examined a total of 30 reproductive seasons (range, 1–4 per female) for the 14 giant pandas. Ten were acyclic with no expression of behavioural oestrus or change in baseline faecal progestagen (see below).

On 19 occasions, signs of oestrus were evident, and natural breeding and/or AI occurred. One female demonstrated oestrus, but was not mated or inseminated. Of the giant pandas that were mated and/or AI, 11 became pregnant and gave birth to cubs, whereas the remaining 9 did not give birth and were classified as non-parturient.

Mean luteal faecal progestagen profile

Figure 1 depicts the mean progestagen pattern from pre-ovulation through the entire luteal phase for all individuals in which a combined primary and secondary hormonal rise was evident. The existence of a primary progestagen elevation was apparent where faecal progestagen concentrations were higher ($P<0.05$) for the 50-day period after, compared to the 50-day interval before, the urinary E peak (283.7 ± 13.5 vs 215.4 ± 15.8 ng/g). The secondary rise (2289.5 ± 236.9 ng/g) occurred when progestagen concentrations increased 6.3-fold ($P<0.05$) over primary rise concentrations (360.8 ± 13.9 ng/g). The initial rise began the day after the urinary E peak and, on average, lasted 88.8 days (± 6.6 days; range 61–122 days), whereas the shorter ($P<0.05$) secondary rise lasted 44.7 days (± 3.5 days; range 30–64 days).

Urinary versus faecal progestagen patterns

The correlations between matched urine and faecal progestagen patterns for SB473 and SB452 (one cycle each; Fig. 2) were strong ($r=0.80$ and $r=0.62$ respectively; $P<0.05$). According to our criteria, the total luteal durations for SB473 and SB452 were 13 days longer (149 days) and 7 days shorter (141 days) respectively, using urinary as opposed to faecal evaluations. Duration of the primary rise was 14 days shorter (88 days) in SB473 but not different in SB452 (88 days)

Figure 1 Mean ($\pm$ S.E.M.) progestagen profile of ten females during the pre-luteal period and primary and secondary rise intervals. Data are aligned to day of peak faecal oestrogen excretion. Scale of inset graph illustrates change in faecal progestagen excretion from preluteal to primary rise concentrations.

Figure 2 Matched urinary (closed circles, solid lines) and faecal (crosses, dashed lines) progesterone metabolite concentrations from females (A) SB473 and (B) SB452. Data are aligned to day of peak urinary oestrogen excretion.

using urinary versus faecal evaluations. Duration of the secondary progesterone rise was nearly twice the length in SB473 (61 days) but not different in SB 452 (56 days) using urinary versus faecal evaluations.

Repeated annual assessments (each for 3 or more years) of the Smithsonian National Zoological Park (SNZP) and Zoo Atlanta (ZA) females revealed that progesterone concentrations were always lower ($P < 0.05$) during the primary compared to secondary rise interval (faecal measurements are presented in Table 1). However, when urinary versus faecal patterns were compared, there was no consistency in the magnitude of the increase between the primary and secondary rise. For example, when progesterone concentrations were averaged across years within females, mean urinary progesterone during the secondary rise was

threefold higher compared to the primary rise interval (88.6 ± 9.4 vs 27.4 ± 1.1 ng/mg creatinine (Cr)), whereas mean faecal progesterone was sevenfold higher during the secondary versus primary rise (5048.3 ± 853.3 vs 733.8 ± 37.0 ng/g). For SB452, the mean urinary progesterone concentration was sevenfold higher during the secondary than primary rise (116.9 ± 13.2 vs 16.7 ± 0.6 ng/mg Cr). Meanwhile, there was a threefold increase in mean faecal progesterone during the secondary compared to primary rise (1342.7 ± 125.6 vs 428.5 ± 29.7 ng/g).

Year-to-year faecal progesterone patterns

When annual reproductive cycle data were averaged for each of SB473 and SB452, there was no difference ($P > 0.05$) between these individuals in durations of the primary or secondary progesterone rise or total length of the luteal phase (Table 1). The correlation between date of luteal phase onset (i.e. day after the E peak) and the duration of the luteal phase was also non-significant ($P > 0.05$) for both females (SB473, $r = 0.08$; SB452, $r = 0.10$). Among years, the average fold increase between the primary and secondary rise was similar within females ($P > 0.05$). For SB473, mean faecal progesterone during the primary (but not secondary) rise differed ($P < 0.05$) among all 3 years. For SB452, faecal progesterone during the primary rise in 2002 was similar ($P > 0.05$) to 2003, but faecal progesterone for both years was lower ($P < 0.05$) compared to 2004 values. Faecal progesterone during the secondary rise was similar ($P > 0.05$) in 2002 and 2003, but concentrations for both of these years were higher ($P < 0.05$) than in 2004. Overall, average progesterone concentrations did not differ ($P > 0.05$) between the two females during the primary or secondary phases (Table 1). Collectively, data indicated that the magnitude and duration of the biphasic progesterone excretion pattern were quite consistent between animals and across years within a given individual.

Table 1 Year-to-year evaluations of mean faecal progesterone concentrations ($\pm$ S.E.M. ng/g) and durations ($\pm$ S.E.M. d) during the luteal phases of two adult female giant pandas.

Female	Year	Day of year of oestrogen peak	Primary rise		Secondary rise		Fold increase from primary to secondary rise	Total luteal duration
			Duration	Progesterone (ng/g)	Duration	Progesterone (ng/g)		
SB473	2002	117	109	424.6 ± 16.7^a	52	1338.2 ± 154.8	3.2	161
	2003	94	103	648.7 ± 37.7^b	40	2996.8 ± 545.5	4.6	143
	2004	123	98	777.9 ± 42.0^c	39	4536.3 ± 821.2	5.8	137
	Mean	111.3 ± 8.8	103.3 ± 3.1	617.1 ± 103.2	43.7 ± 4.2	2957.0 ± 923.5	4.5	147.0 ± 7.2
SB452	2002	92	105	238.7 ± 23.8^d	42	2583.3 ± 662.7^f	10.8	147
	2003	84	61	369.4 ± 38.8^d	64	2548.2 ± 382.9^f	6.9	125
	2004	74	95	427.3 ± 30.4^e	53	1342.7 ± 125.6^g	3.1	148
	Mean	83.8 ± 5.2	87.0 ± 13.3	345.1 ± 55.8	53.0 ± 6.4	2158.1 ± 407.8	7.0	140.0 ± 7.5

Progesterone concentrations within reproductive state within females with different subscripts differ ($P < 0.05$).

Faecal progesterone patterns in non-parturient giant pandas

Faecal E and progesterone excretion profiles for four representative, non-parturient females are presented in Fig. 3. For these individuals, mean progesterone concentrations for the 50-day period after the E surge were $89.7\text{--}318.5$ ng/g greater than progesterone concentrations during the 50 days before oestrus. The primary rise in faecal progesterone averaged 416.6 ± 122.0 ng/g and lasted 85.8 ± 15.4 days, whereas the secondary rise in progesterone averaged 3173.8 ± 650.3 ng/g and lasted 42.3 ± 5.4 days.

Faecal progesterone patterns in parturient giant pandas

Longitudinal faecal progesterone profiles for representative giant pandas that were mated and/or AI and then gave birth are provided in Fig. 4. For this cohort, faecal progesterone concentrations for the 50-day period post-E peak were $36.3\text{--}300.5$ ng/g greater than during the 50-day pre-E surge interval. The duration of the primary rise averaged 83.5 ± 9.5 days during which time faecal progesterone averaged 286.3 ± 124.4 ng/g, whereas the secondary rise in progesterone averaged 2574.4 ± 568.8 ng/g and lasted 36.8 ± 2.2 days. Within this specific 'pregnancy group', two dams gave birth to singletons (SB446 and SB473) and two to twins (SB487 and SB414). Mean faecal progesterone concentrations in twinning dams (622.8 ± 182.3 ng/g) actually were lower ($P < 0.05$) than females producing singleton cubs (1331.8 ± 208.8 ng/g). For all giant pandas that gave birth ($n = 11$), average durations of the primary and secondary progesterone rise intervals were 80.5 ± 3.9 days (range, 61–104 days) and 38.8 ± 3.0 days (range, 26–55 days) respectively (total luteal phase

duration, 121.5 ± 4.4 days). Length of gestation (calculated on the basis of date of first breeding or AI event to parturition) ranged from days 191 to 273 (mean, 228.7 ± 9.2 days).

Faecal progesterone in parturient versus non-parturient individuals

Progesterone data were compared between giant pandas that did or did not give birth. Faecal progesterone concentrations were higher ($P < 0.05$) in non-parturient females compared to parturient females during both primary (460.9 ± 28.0 vs 291.0 ± 20.9 ng/g respectively) and secondary (3125.5 ± 339.4 vs 3096.5 ± 455.3 ng/g respectively) rise intervals. The magnitude of the average increase in faecal progesterone from the primary to the secondary rise was similar ($P > 0.05$) for non-parturient (6.0 ± 2.5 -fold) and parturient (11.7 ± 6.5 -fold) females. Likewise, the durations of the primary (parturient, 82.3 ± 8.4 days versus non-parturient, 95.5 ± 10.0 days) and secondary (parturient, 36.8 ± 2.2 days versus non-parturient, 42.3 ± 5.4 days) rise intervals were similar ($P > 0.05$). As a result, there were no differences ($P > 0.05$) in the overall length of the biphasic interval of elevated progesterone excretion (parturient, 119.0 ± 7.4 days versus non-parturient, 137.8 ± 12.8 days).

Co-chromatographic profiles depicting faecal progesterone immunoreactivity after high pressure liquid chromatography (HPLC) separation from two parturient (SB414 and SB446) and two non-parturient (SB473, 2002; SB452, 2003) females are presented in Fig. 5. Correlation between the two data sets was significant ($r = 0.84$; $P < 0.05$). Broad immunoreactive peaks (fractions 4–13) that co-eluted with pregnanediol glucuronide were detected in all individuals. Additionally, a broad band of immunoreactivity (fractions 63–80)

Figure 3 Representative profiles of faecal oestrogen (closed circles, solid lines) and progesterone (open circles, dashed lines) metabolites of non-parturient females (A) SB414, (B) SB544, (C) SB490 and (D) SB473. Open triangles denote days of natural breeding, and the closed diamonds represent days of artificial insemination. All data are aligned day of year.

Figure 4 Representative profiles of faecal oestrogen (closed circles, solid lines) and progesterone (open circles, dashed lines) metabolites of parturient females (A) SB446, (B) SB487, (C) SB414 and (D) SB473. Open triangles denote days of natural breeding, closed diamonds indicate days of artificial insemination and closed stars mark the day of parturition. All data are aligned to the day of year. Scale of inset graph (profile B) illustrates the variability in peak progesterone excretion among females.

co-eluted in the range of $^3\text{H-P}_4$ (fraction 66). At least, six additional unidentified immunoreactive peaks (fractions 20–60) were detected in both parturient and non-parturient females. Overall, the spectrum of immunoreactive progestagens was indistinguishable in both parturient and non-parturient females.

Faecal progestagens in acyclic giant pandas

Ten acyclic profiles in our study were characterized by the absence of a faecal E surge, sexual behaviours and a sustained increase in faecal progesterone excretion (representative individuals in Fig. 6). All of these females were of reproductive age (5–12 years), and five of ten had given birth earlier (including SB382, Fig. 6C), although none were nursing cubs during the year of assessment. Faecal progesterone in acyclic giant pandas (101.9 ± 4.5 ng/g) was lower ($P < 0.05$) than baseline concentrations in non-parturient (295.1 ± 21.2 ng/g) or parturient (142.9 ± 10.5 ng/g) females.

Discussion

This study demonstrated that ovulating giant pandas experienced a protracted, biphasic increase in progesterone production that was detectable by longitudinal analysis of hormonal metabolites excreted in freshly voided faeces. The excretion pattern was unusual in that an initial progesterone rise early after ovulation was sustained for a variable interval that lasted 61–122 days. This interval was then followed by a distinctive 3- to 20-fold elevation that lasted another 28–63 days before declining to signal birth or the end of a non-parturient phase. A general biphasic progesterone pattern has been reported from earlier urinary monitoring studies of this species, but only in a few individuals

(Hodges *et al.* 1984, Chaudhuri *et al.* 1988, Masui *et al.* 1989, Monfort *et al.* 1989, Mainka *et al.* 1990, McGeehan *et al.* 2002, Narushima *et al.* 2003). The present study was important because of the increased confidence generated from simultaneously examining a large cohort of giant pandas, some of which were observed year to year. Doing so permitted careful examination of the relatively modest, yet significant primary progesterone rise that characterized the immediate post-oestral interval, even in unmated females. This finding confirmed previous assertions by others (Monfort *et al.* 1989, Mainka *et al.* 1990, Steinman *et al.* 2006, Spady *et al.* 2007) that luteal formation was spontaneous and obligate in this species. Whereas the duration of the initial progesterone rise varied highly among individuals and across years, we found great consistency in the magnitude and duration of the shorter, hyper-elevated secondary hormonal rise. Understanding the

Figure 5 Co-chromatographic profiles depicting faecal progesterone immunoreactivity after HPLC separation from two parturient (open circles, solid lines) and two non-parturient (closed circles, dashed lines) females. Triangle indicates peak elution of $^3\text{H-P}_4$ (fraction 66) that was added as a co-chromatographic marker to both faecal pools before HPLC analysis.

Figure 6 Representative profiles of faecal oestrogen (closed circles, solid lines) and progesterone (open circles, dashed lines) metabolites of acyclic females (A) SB439, (B) SB474, (C) SB382 and (D) SB495. All data are aligned to day of year. Scales for faecal oestrogen and progesterone concentrations and day of year are the same as presented in Figs 3 and 4.

mechanisms associated with this accelerated increase in progesterone excretion and its potential association with implantation offers rich opportunities for further research. This was especially evident given that it was possible to track both subtle and marked fluctuations in progesterone excreted in faeces, a biomaterial that is much easier to recover than blood or urine.

A two-tiered, post-ovulatory progesterone rise has been noted in previous studies of the giant panda (reviewed in Steinman *et al.* (2006)). However, ours was the first effort to differentially quantify the duration, amplitude and temporal excretion patterns between the two phases and across a large cohort of pregnant versus non-pregnant individuals of this rare species. The post-ovulatory biphasic progesterone profiles for the giant panda generally were highly consistent with findings in other ursids (American black bear; Foresman & Daniel 1983, Palmer *et al.* 1988, Hellgren *et al.* 1991, Tsubota *et al.* 1998, Schulz *et al.* 2003; brown bear; Tsubota *et al.* 1992; spectacled bear, *Tremarctos ornatus*; Dehnhard *et al.* 2006; Asiatic black bear; Sato *et al.* 2001) as well as mustelids (mink; Murphy & Moger 1977, Allais & Martinet 1978, Papke *et al.* 1980; European badger; Canivenc & Bonnin 1981; western spotted skunk; Mead 1981). This includes earlier suggestions that the duration of the secondary phase is less variable than that of the primary phase (Enders 1952, Murphy & Moger 1977, Allais & Martinet 1978, Papke *et al.* 1980, Canivenc & Bonnin 1981, Mead 1981, Foresman & Daniel 1983). Enders (1952) first postulated that the second, enhanced P_4 rise was associated with implantation of a previously free-floating embryo, a concept later proven for the mink (Allais & Martinet 1978, Murphy *et al.* 1993, Douglas *et al.* 1998), European badger (Canivenc & Bonnin 1981, Yamaguchi *et al.* 2006) and spotted skunk (Mead 1981). What is much less well understood is the mechanism(s)

responsible for provoking this significant P_4 surge. Early morphological studies in several carnivore species have revealed that corpora lutea (CL) form rapidly post-ovulation and remain unchanged until lutein cells increase in size, change in cellular ultrastructure and secrete more progesterone coincident with implantation (Wimsatt 1963, Mead 1986, Douglas *et al.* 1998). An intriguing area for future research is determining how CL rejuvenation or enhancement is mediated and whether an extra-gonadal source of steroid contributes to the hormonal milieu associated with the secondary progesterone rise. Regardless, we speculate that conceptus presence has little impact on CL function because faecal progesterone profiles were unremarkable between parturient and non-parturient giant pandas.

Seasonality is a dominant characteristic of the giant panda's ovarian cycle (Steinman *et al.* 2006) and is likely one of the primary cues responsible for modulating embryonic diapause in the species (Sandell 1990, Zhang *et al.* 2009). Although the breeding season is distinct (February to June), we learned in the present study that there are year-to-year variations in seasonal oestrus onset within the same animal. One might expect early seasonal oestrus to be followed by a prolonged luteal phase (i.e. to optimize the timing of nidation, parturition and offspring survival), yet we found no evidence for such a mechanism. In fact, for two females monitored sequentially over multiple years, the duration and magnitude of the luteal phase were remarkably consistent, regardless of when oestrus occurred. However, our sample size was small, and more work is needed to determine whether the duration of the luteal phase adjusts relative to the seasonal timing of ovulation.

Our findings were also relevant to the topic of pseudopregnancy. We intentionally avoided using the term pseudopregnancy because it became apparent that

giant pandas can exhibit a protracted biphasic progesterone profile after ovulation and in the absence of copulation and any direct male contact. Secondly, and perhaps more importantly, giant pandas may be experiencing significant embryonic loss, with many females previously classified as 'pseudopregnant' actually undergoing failed pregnancies (Steinman *et al.* 2006). For example, Sutherland-Smith *et al.* (2004) documented two fetuses during late gestation (day 134) via ultrasound in a female that later gave birth to a single cub (day 150). Anecdotal reports of suspicious foetal loss via ultrasonographic examinations have also been noted at the Smithsonian's National Zoological Park (S Murray, personal communications) and Memphis Zoo (A J Kouba, personal communications). Post-nidation foetal loss has also been well documented in the European badger, another delayed implanting species (Cresswell *et al.* 1992, Yamaguchi *et al.* 2006). Giant pandas clearly experience true pseudopregnancy in the absence of mating or conception, including displaying strong maternal behaviours (e.g. decreased appetite, nest building and cradling inanimate objects; Steinman *et al.* 2006) during the waning days of the secondary progesterone phase. However, because it is impossible to discriminate pregnant from non-pregnant individuals using excreted steroids (either urine or, as we have discovered here, faeces), we contend that it is more accurate to retrospectively classify giant panda reproductive cycles as 'parturient' versus 'non-parturient'.

The gestation length derived from this study (121.5 $\pm$ 4.4 days; range, 99–145 days) was within the 85–185 days previously reported for this species (Zhu *et al.* 2001). Our systematic efforts to document the excretory dynamics of the luteal phase revealed an invariant pattern in progesterone excretion between parturient and non-parturient females, which was not unusual. Within the order Carnivora, progesterone profiles are indistinguishable in the pregnant versus non-pregnant domestic dog (*Canis familiaris*; Smith & McDonald 1974, Concannon *et al.* 1975), red wolf (*Canis rufus*; Walker *et al.* 2002), maned wolf (*Chrysocyon brachyurus*; Velloso *et al.* 1998, Songsasen *et al.* 2006), bush dog (*Speothos venaticus*; DeMatteo *et al.* 2006), wolverine (*Gulo gulo*; Mead *et al.* 1993), black-footed ferret (*Mustela nigripes*; Brown 1997) and dwarf mongoose (*Helogale parvula*; Creel *et al.* 1991). In the case of the giant panda, this similarity extended to a homologous milieu of immunoreactive progesterone metabolites (detected by co-chromatographic HPLC analysis) that were indistinguishable between parturient and non-parturient females.

The topic of acyclicity in the giant panda has not been previously addressed. Some females have been classified by animal managers as experiencing 'weak oestrus' because of the lack of behavioural signs of sexual receptivity and failed ability to breed or conceive to copulation or AI (Shuling *et al.* 1997). Some cattle are

known to experience 'silent ovulation', another form of weak oestrus (Allrich 1994). Of the 30 reproductive events evaluated in giant pandas of our study, 10 (31.3%) were accompanied by the absence of or diminished sexual behaviour. Additionally, all of these individuals failed to produce an oestrogen surge or rise in progesterone throughout the year. Therefore, rather than finding data to support the concept for 'weak oestrus', it was apparent that certain females were completely acyclic and non-ovulatory. In nature, giant pandas generally produce cubs every other year because of lactational suppression related to a maternal care interval lasting up to 18 months (Zhu *et al.* 2001). In China, cubs born in captivity usually are weaned at 6 months of age, thereby allowing the female to recycle, mate and produce additional offspring the next year. Because six of ten giant panda females experiencing acyclicity in our study had given birth the previous year, we speculate that some period of anoestrus may be an obligate feature of the giant panda's life history that helps to maximize reproductive fitness.

Aside from providing new scholarly insight, our findings have potential management implications. For years, a compliment of non-invasive tools has been used to approximate the reproductive status of female giant pandas in *ex situ* collection, including through behavioural observations (Czekala *et al.* 1998, McGeehan *et al.* 2002), urinary hormone quantification (Steinman *et al.* 2006), visual changes of the vulva (McGeehan *et al.* 2002), vaginal cytology (Durrant *et al.* 2006) and/or ultrasonography in cooperative individuals (Sutherland-Smith *et al.* 2004, Zhang *et al.* 2009). Urinary progesterone monitoring has been especially useful for tracking pregnancy (or pseudopregnancy) of captive mated and/or artificially inseminated females. Because it is currently not feasible to diagnose pregnancy, a return to baseline progesterone concentrations has been used to signal either an impending birth or the ending of a 'pregnancy watch', both of which influence the use of zoological resources (Steinman *et al.* 2006). Similarly, faecal steroid measures may prove useful for assessing reproductive status in free-living giant pandas. Although giant pandas are rarely observed in the wild, females generally sustain a given territory (Schaller *et al.* 1985), and encountering giant panda faeces in the field can be common. If two to three relatively fresh faecal samples were collected over a 1- to 2-week interval, it would be possible to estimate reproductive status of a free-living female, including determining whether a given female was post-ovulatory or potentially pregnant.

Materials and Methods

Study animals and facilities

Two adult giant pandas were studied in separate North American zoos. An additional 12 adult females from a captive breeding centre in China were also monitored.

In several animals over the course of 2 and 3 years, 10 acyclic, 11 parturient and 9 non-parturient phases were analysed. All biomaterials (urine and faeces) were collected non-invasively during routine animal care of unrestrained animals. Research and management procedures (including AI) were reviewed and approved by the respective Institutional Animal Care and Use Committees of SNZP and ZA.

North America

The giant pandas living in the two North American institutions were intensively monitored on a year-to-year basis. Female SB473 (3.5 year old at study onset) was maintained at the SNZP (39°N, 77°W) and monitored in 2002, 2003, 2004 and 2005, whereas SB452 (4.5 years) was evaluated in 2002, 2003 and 2004 at ZA (33°N, 84°W). Females were managed as previously described (Kersey *et al.* 2010), and records were maintained on sexual behaviours (McGeehan *et al.* 2002), dates of attempted or confirmed copulations and/or AIs and parturition.

China

Study animals (12 females; age range, 4–16 years) were from the China Conservation and Research Centre for the Giant Panda at the Wolong Nature Reserve (31°N, 103°E). Enclosures for the females and management have been previously described (Kersey *et al.* 2010).

Sample collection and processing

Both urine and faecal samples were collected during years 2002, 2003 and 2004 from the adult female giant pandas maintained at SNZP and ZA to validate the consistency of progesterone pattern changes over time in these two types of excreta. Only faecal samples were collected from the animals in China.

Urine

Fresh urine was collected (3–7 days/week) after being aspirated, usually from the concrete floor of an enclosure, and then stored frozen (–20 °C) in labelled and sealed 12×75 mm plastic tubes until analysis. Urine was collected in the morning, but later in the day if unavailable earlier. Urine samples excreted during evening/night hours were marked as ‘overnight’ specimens and assigned an excretion time of 0000 h.

To account for variations in water content, each urine sample was indexed for Cr (Taussky 1954, Monfort *et al.* 1989). Urine samples with a Cr mass <0.1 mg Cr/ml (~7% of all samples) were considered too dilute and were excluded from hormonal metabolite analyses. Hormone masses (ng/ml) were divided by Cr concentration (mg/ml), and final hormone values were expressed as mass of hormone per mg of excreted Cr (ng/mg Cr).

Faeces

An aliquot of freshly voided (within 1 h) faeces that visibly contained the least amount of undigested bamboo was collected 3–7 days/week from all adult females. Annual sampling began on 1 February and ended by 4 weeks after parturition or the non-parturient interval. Each sample was placed in a labelled, resealable plastic bag, and stored at –20 °C until shipped frozen by air transport to our laboratory for processing and extraction, as previously described (Kersey *et al.* 2010).

Steroid enzyme immunoassays

Faecal E metabolites were quantified using a single antibody oestrone glucuronide enzyme immunoassay (EIA; Stabenfeldt *et al.* 1991, Kersey *et al.* 2010). Inter-assay coefficients of variation (CV) for two internal controls ($n=83$ assays) were 14.5% (mean binding, 40.0%) and 14.9% (mean binding, 74.2%), and intra-assay CV was <10%.

Progesterone concentrations for urine and faeces were determined with a single antibody P₄ EIA (Graham *et al.* 2001, Kersey *et al.* 2010). Inter-assay CV for two internal controls ($n=104$ assays) were 14.3% (mean binding, 39.0%) and 14.6% (mean binding, 71.2%), and intra-assay CV was <10%.

High pressure liquid chromatography

Reverse phase HPLC (Varian ProStar; Varian Analytical Instruments, Walnut Creek, CA, USA) was used to identify progesterone metabolites in the faecal extracts (Monfort *et al.* 1997). Pooled sample extracts that contained high progesterone concentrations from parturient ($n=2$; 1 ml of three samples from each female) and non-parturient females ($n=2$; 1 ml of three samples from each female) were concentrated 20-fold and spiked with ³H-P₄ (~14 000 c.p.m./ml) to act as a co-chromatographic marker. After separation using a reverse phase C18 HPLC column (Varian Analytical Instruments), collected fractions were quantified for radioactivity to identify marker elution times. Fractions then were dried, resuspended in 0.3 ml of buffer and assayed via the P₄ EIA to detect fraction immunoreactivity alongside radioactive marker elution times to identify predominate steroidal metabolites.

Statistical analysis

Baseline faecal progesterone excretion during the anoestrous phase of the reproductive cycle was determined using an iterative process (Moreira *et al.* 2001). Luteal phase onset was considered the day after the preovulatory E peak. The end of the luteal phase was designated as the first day that progesterone concentrations returned to baseline range (baseline mean $\pm$ double s.d.) for two or more consecutive days. The subset of samples constituting the luteal phase was evaluated using an iterative process to eliminate faecal hormone concentration outliers that exceeded 2 s.d. of the mean. Duration of the primary progesterone rise was defined as the time from the initial discernible increase to an elevation 2 s.d. above the mean for at least two consecutive days. Length of the

secondary rise was considered as the interval from the end of the primary rise to the termination of the luteal phase. All hormonal metabolite concentrations and interval durations were expressed as mean $\pm$ S.E.M.

For statistical comparisons, urinary and faecal progestagen was aligned to the urinary E peak. For all other faecal hormone analyses, data were adjusted to day of year (i.e. 1 January = day 1). Relatedness between concomitant urinary and faecal progestagen concentrations as well as between HPLC analyses of parturient versus non-parturient profiles was determined using a Pearson product moment correlation. Relation between luteal phase onset and duration was determined by linear regression analysis. All data were evaluated for normality (Kolmogorov–Smirnov test) before subsequent testing. Comparisons between dependent data were made via Student's paired *t*-test (normal) or Wilcoxon signed rank test (not normal), and independent analyses were conducted with a Mann–Whitney test (not normal). Three data sets that failed normality were examined further using the Tukey–Friedman repeated-measures ANOVA on ranks (dependent data) or with the Kruskal–Wallis ANOVA (independent data). Significance was considered at a *P* value of <0.05 . Iterations were evaluated in Microsoft Excel 2007 (Microsoft Inc.), and all other statistical analyses were conducted in SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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