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 anoestral 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.


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 perioestrual 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 progestosterone (P₄) 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, anoestral, 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).
using urinary versus faecal evaluations. Duration of the secondary progestagen 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 progestagen 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 progestagen concentrations were averaged across years within females, mean urinary progestagen during the secondary rise was threefold higher compared to the primary rise interval \((88.6 \pm 9.4\) vs \(27.4 \pm 1.1\) ng/mg creatinine \((\text{Cr})), whereas mean faecal progestagen was sevenfold higher during the secondary versus primary rise \((5048.3 \pm 853.3\) vs \(733.8 \pm 37.0\) ng/g). For SB452, the mean urinary progestagen concentration was sevenfold higher during the secondary than primary rise \((116.9 \pm 13.2\) vs \(16.7 \pm 0.6\) ng/mg \(\text{Cr})\). Meanwhile, there was a threefold increase in mean faecal progestagen during the secondary compared to primary rise \((1342.7 \pm 125.6\) vs \(428.5 \pm 29.7\) ng/g).

**Year-to-year faecal progestagen 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 progestagen 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 progestagen during the primary (but not secondary) rise differed \((P<0.05)\) among all 3 years. For SB452, faecal progestagen during the primary rise in 2002 was similar \((P>0.05)\) to 2003, but faecal progestagen for both years was lower \((P<0.05)\) compared to 2004 values. Faecal progestagen 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 progestagen 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 progestagen excretion pattern were quite consistent between animals and across years within a given individual.

### Table 1

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

Progestagen concentrations within reproductive state within females with different subscripts differ \((P<0.05)\).
Faecal progestagen patterns in non-parturient giant pandas

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

Faecal progestagen patterns in parturient giant pandas

Longitudinal faecal progestagen profiles for representative giant pandas that were mated and/or AI and then gave birth are provided in Fig. 4. For this cohort, faecal progestagen concentrations for the 50-day period post-E peak were 36.3–300.5 ng/g greater than during the 50-day pre-E surge interval. The duration of the primary rise averaged \(83.5 \pm 9.5\) days during which time faecal progestagen averaged \(286.3 \pm 124.4\) ng/g, whereas the secondary rise in progestagen averaged \(2574.4 \pm 568.8\) ng/g and lasted \(36.8 \pm 2.2\) days. Within this specific ‘pregnancy group’, two dams gave birth to singletons (SB446 and SB473) and two to twins (SB487 and SB414). Mean faecal progestagen concentrations in twinning dams (\(622.8 \pm 182.3\) ng/g) actually were lower \((P<0.05)\) than females producing singleton cubs (\(1331.8 \pm 208.8\) ng/g). For all giant pandas that gave birth \((n=11)\), average durations of the primary and secondary progestagen rise intervals were \(80.5 \pm 3.9\) days (range, 61–104 days) and \(38.8 \pm 3.0\) days (range, 26–55 days) respectively (total luteal phase duration, \(121.5 \pm 4.4\) 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, \(228.7 \pm 9.2\) days).

Faecal progestagens in parturient versus non-parturient individuals

Progestagen data were compared between giant pandas that did or did not give birth. Faecal progestagen 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 progestagen 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 progestagen excretion (parturient, \(119.0 \pm 7.4\) days versus non-parturient, 137.8±12.8 days).

Co-chromatographic profiles depicting faecal progestagen 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)
co-eluted in the range of $^3$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 progestagen 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 progestagen 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 progestagen production that was detectable by longitudinal analysis of hormonal metabolites excreted in freshly voided faeces. The excretion pattern was unusual in that an initial progestagen 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 progestagen 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 progestagen 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 progestagen 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
mechanisms associated with this accelerated increase in progestagen 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 progestagen excreted in faeces, a biomaterial that is much easier to recover than blood or urine.

A two-tiered, post-ovulatory progestagen 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 progestagen 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 P₄ 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 P₄ 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 progestagens 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 progestagen rise. Regardless, we speculate that conceptus presence has little impact on CL function because faecal progestagen 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 progestagen 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 foetuses 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 Koubi, 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 progestagen 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 ± 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 progestagen excretion between parturient and non-parturient females, which was not unusual. Within the order Carnivora, progestagen 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 nigriceps; 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 progestagen 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 progestagen 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 progestagen 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 progestagen 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 1974, DeMatteo et al. 2006), 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. 

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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 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 progestagen 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. 1997). A urine sample was considered too dilute and was 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%.

Progestagen 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 progestagen metabolites in the faecal extracts (Monfort et al. 1997). Pooled sample extracts that contained high progestagen 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 progestagen excretion during the anoestral 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 progestagen concentrations returned to baseline range (baseline mean ± 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 progestagen 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 ± 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.

**Funding**

This study was funded by Friends of the National Zoo.

**Acknowledgements**

We thank Susan Walker, Nicole Abbondanza, Karen Steinman, Copper Aitken-Palmer, Adrienne Crosier and Nicole Presley for their logistical support. Special thanks to Nicole Savageau, Bridgette von Holdt, Jessica Beckman, Valerie Parkman, Serena Enloe and Corrinni Bazlett for laboratory assistance. We also acknowledge the work of the keeper and curatorial staffs at the Smithsonian’s National Zoological Park, Zoo Atlanta and China Conservation and Research Centre for the Giant Panda for sample collection.

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Received 5 January 2010
First decision 2 February 2010
Revised manuscript received 15 April 2010
Accepted 20 April 2010