

Luteal phase length, endometrial edema, and behavior differentiate post-ovulatory events in a giant panda (*Ailuropoda melanoleuca*)

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Abstract

Despite decades of reproductive research on the giant panda (*Ailuropoda melanoleuca*), the post-ovulatory phase continues to confound zoologists in conservation and breeding centers around the world, often resulting in significant investments of time and resources without reproductive success. The purpose of this project was to document and compare post-ovulatory characteristics during a non-productive and productive breeding in the same individual in consecutive years. A multidisciplinary approach was used to monitor the visiting female giant panda at the Toronto Zoo through the luteal phase of her first two full reproductive cycles in 2014 and 2015. Monitoring occurred via urine-endocrine analysis, weekly ultrasound examinations, and continuous behavioral observations. The 2014 reproductive cycle consisted of a pseudopregnancy, characterized by an extended luteal phase (241 days), the identification of endometrial edema and folding during ultrasound examinations, and a lack of strong association between behavior patterns and urinary progesterone secretion. The 2015 reproductive cycle included increased feeding time through the primary progesterone rise compared to the previous year, followed by simultaneous decrease in appetite and increases in inactivity, resting, sitting upright, and pre-partum-associated behaviors. These changes began 25 days before the birth of twins on Day 153 post-ovulation. Both fetuses were detected via ultrasound 15 days pre-partum. These results suggest that an absence of pre-partum behaviors, ultrasound evidence of endometrial edema without a fetus, and an extended luteal phase may be indicative of pseudopregnancy in giant pandas. Simultaneous monitoring of morphology, behavior, and urinary-endocrine profiles showed clear differences between successful and unsuccessful reproductive years.

KEYWORDS

delayed implantation, reproduction, ultrasound, urinary hormone analysis

1 | INTRODUCTION

As the ex situ population of giant pandas (*Ailuropoda melanoleuca*—from here on, panda) has steadily increased in size since the 1970s, so has the scientific investigation of the physiological, behavioral and endocrine correlates of their reproduction (Brambell, 1976; Czekala et al., 2003; Durrant et al., 2003; Hodges et al., 1984; Kersey et al., 2010a, 2016; Kleiman, 1983; Mainka et al., 1990). Pandas are seasonally monoestrous, and having advanced indication of a birth event greatly benefits zoological institutions, as management of the birth and subsequent neonatal care requires extensive planning (Kleiman, 1983; Schaller et al., 1985; Steinman et al., 2006). However, studies regularly report examples of female pandas displaying physiological and behavioral changes typical of pregnancy, with no subsequent parturition (Chaudhuri et al., 1988; Hodges et al., 1984; Steinman et al., 2006). Pseudopregnancy (or pseudocyesis, see Murphy [2018, pp. 376–377]) is the most commonly cited explanation for this outcome in pandas, a phenomenon that is well documented in other members of the Order Carnivora (various felids, Dehnhard et al., 2012; *Mustela putorius*, Hammond & Marshall, 1930; *Ursus americanus*, Hellgren et al., 1990; *Eumetopias jubatus*, Sattler & Polasek, 2017; *Chrysocyon brachyurus*, Velloso et al., 1998). However, panda reproduction is complicated by a highly variable period of delayed implantation (embryonic diapause; Murphy, 2018), and an unknown (though undeniably short) gestation length (Kersey et al., 2010b; Steinman et al., 2006). Identification of a developing fetus can only occur in a short window for pandas, one that is often reduced further by females becoming increasingly uncooperative pre-partum (Hildebrandt et al., 2006; Kersey et al., 2016; Sutherland-Smith et al., 2004). There are reports of both successful birth of cubs undetected by ultrasound and cubs identified on ultrasound that were never born (Kersey et al., 2010b; Sutherland-Smith et al., 2004; Willis et al., 2011). Therefore, it has been suggested that reproductive events reported as pseudopregnancies may in fact be instances of failed embryonic or fetal development (Chaudhuri et al., 1988; Steinman et al., 2006; Willis et al., 2011; G. Zhang et al., 2004). Furthermore, a high degree of individual variation in the physiological, behavioral, and endocrine signs of reproduction has been reported both among animals and within the same animal from year to year, precluding clear differentiation of reproductive status (Kersey et al., 2010b; McGeehan et al., 2002; Snyder et al., 2004). Although there are some hormone-based diagnostic markers of pregnancy in other species which experience pseudopregnancies (see Brown et al., 1994; Finkenwirth et al., 2010), these do not translate to pandas, and efforts to find a clear marker for them are ongoing (Roberts et al., 2018; Steinetz et al., 2005; Willis et al., 2011). Data across disciplines comparing pregnant and nonpregnant luteal phases in the same individual remain scarce for this species (Narushima et al., 2003; Steinman et al., 2006).

In this study, we describe physiological, morphological, and behavioral changes that occurred following artificial insemination (AI) in the same female panda for two consecutive luteal phases; one of which resulted in no implantation of embryos, while the other produced twin cubs.

2 | MATERIALS AND METHODS

All animal procedures were performed in accordance with the species-specific animal care and welfare guidelines of the Association of Zoos and Aquariums, of which the Toronto Zoo is an accredited institution.

2.1 | Animal management

In March 2013, a pair of pandas intended for breeding arrived for a 5-year stay at the Toronto Zoo (43.82°N, 79.18°W); the male (SB 732, born September 2008) from Chengdu Research Base of Giant Panda Breeding, and the female (SB 676 born August 2007) from Chongqing Zoo. Throughout the observation period, both pandas received water ad libitum and a diet of 99% bamboo (*Phyllostachys aurea*, *P. glauca*, and *P. rubromarginata*), with supplemental leaf-eater biscuits, apples, and pears. Honey, sugar cane, and corn stalks were occasionally provided for enrichment purposes. Aside from occasional events of passing mucoid feces, the pandas were healthy throughout the entire observation period (Edwards et al., 2006).

Panda habitations included off-display holding pens (12.0, 12.8, and 9.3 m²), larger on-display indoor day rooms (72 and 68.3 m²), and on-display outdoor exhibits (865 and 350 m²). SB 676 was limited to climate-controlled indoor enclosures when external temperatures were below -10°C, above 30°C, and in key instances of SB 676's reproductive cycle when urine samples were essential for hormone analysis. SB 676 showed some signs of estrus in the spring of 2013, but not enough to suggest sexual maturity and receptivity.

Time of ovulation was determined by urinary hormone analysis in both study years, identifiable by a rapid decrease in urinary estrone conjugate (E1C) concentrations from peak seasonal values after a period of clear hormonal and behavioral estrus. On the day of ovulation physical introductions between SB 676 and SB 732 were attempted to encourage natural breeding, with SB 676 rejecting SB 732 in all instances. In 9 h, following these rejections paired AI procedures were performed (see Czekala et al., 2003). In 2014, frozen-thawed semen collected from two males in China were used for insemination. In 2015, fresh semen from SB 732 collected by electroejaculation was used along with the frozen-thawed semen from China for insemination (as Huang et al., 2002, 2012; Moore et al., 1984).

2.2 | Urinary hormone analysis

Urine samples were collected from January 5, 2014 through October 23, 2015. Zookeepers collected urine samples daily, immediately upon arrival, and additionally as soon as possible following deposition. Daily cleaning of floors prevented cross-contamination of samples between days. Collected samples were frozen immediately and kept at -20°C until analysis. They were analyzed by enzyme

immunoassay for estrone conjugate (E1C: R522-2) and progestagen (CL425) concentrations as described previously (Monfort et al., 1989). Hormone data were normalized to urinary concentrations of creatinine (Cr) and displayed as ng/ml Cr.

2.3 | Ultrasound evaluation

SB 676 was trained using positive reinforcement to voluntarily participate in weekly transabdominal ultrasound examinations while lying in dorsal recumbency. For these procedures, SB 676's lower ventrum was shaved and a conductive gel (EcoGel 200, Eco-Med Pharmaceutical Inc.) was applied to her skin. Ultrasound scans were performed using a portable ultrasound unit (Sonosite Edge; FUJIFILM SonoSite Canada) with a linear array transducer (Sonosite L52x Rectal Probe, 52 mm broadband [10–5 MHz frequency range], 15 cm maximum scan depth; FUJIFILM SonoSite Canada). During each of these examinations, the bladder and uterine body were first identified and then, when possible, each of the uterine horns was followed cranially. These examinations began on May 26 in 2014 and on May 27 in 2015; occurring on average once weekly to the end of her luteal phase each year, as determined by the return of urinary progesterone metabolites to baseline levels.

2.4 | Behavioral observation

SB 676 was observed live over streaming video footage taken by 19 security cameras (Pelco; Spectra D5220 (×4), Spectra D5118 (×2), Sarix IE10DN (×8), Sarix IEE20DN (×1), Sarix IMSODN10-1V (×3), Sarix D5230 (×1)) positioned around the animals' enclosures, manipulated by zoo staff via DS ControlPoint 7.7.309 (Pelco, Intel® Core™; 7-4785T CPU @ 2.20 GHz). Observations occurred on weekdays from April 28 to December 22, 2014 (excluding the weeks from June 9 to 20) and from May 14 to October 12, 2015, with additional weekend observations occurring approaching potential parturition. The basal (Daily) observation period was 9 h, from 0830 to 1730 (corresponding to a typical keeper work shift) with two extended observation days each week including the entire overnight period from 1730 to 0830. Behavior data were grouped into the base set, 12-h data sets from 0700 to 1900, and 24-h sets from 0700 to 0700 the next day. Study days where a panda was out of view for more than 1/3 of the observation period were excluded from the data set. This left a data set of 357 days, of which 355 included the "Daily" 9-h observation period, 312 Days the 12-h observation period, and 148 Days the 24-h period. The same researcher collected all observations, except on May 2, 2014, when two trained substitutes performed these duties.

Observation data were input directly into a spreadsheet-ethogram designed and operated in Microsoft Excel. This ethogram was constructed using behavior categories adapted from a 2013 study (Magnus, unpublished), and from the seminal works of Kleiman (1974), and Schaller et al. (1985).

Panda behavior observations were collected via Focal-Animal Sampling in 1-min time durations modified from Altmann (1974). At the end of each minute, the recorded behavior was enacted by the animal for the longest portion of that minute, with the first behavior engaged chosen in case of a tie. All behaviors observed were included in one of five categories: non-stereotypic behavior, stereotypic behavior, enrichment engagement, bamboo feeding, or inactivity. A more specific description characterizing the recorded behavior was also included each minute to allow for greater detail in subsequent analysis. Special care was taken to note pre-partum behaviors as described in the literature (Kersey et al., 2016; Owen et al., 2005; Zhu et al., 2001). "Event behaviors," behaviors that rarely occupied 30 s of time (e.g., scratching and urination), were also recorded as a count per minute of observation. Novel behaviors not included in the base ethogram were grouped either into one of the five main behavior categories, if duration was longer than any other behavior for that minute, or counted as an event behavior.

2.5 | Statistical analysis

Because observations were only taken from a single animal, statistical independence did not exist within our data set. However, as Owen et al. (2005) noted, effective and humane management of zoo animals regularly requires addressing the needs of an individual, rather than focusing solely upon large population trends. This is especially relevant for pandas as many facilities outside of China house two or fewer individuals long-term.

Over the course of this investigation, a total of 3740 h of behavioral observation and 567 urine samples for progestagen analysis were collected from SB 676. Data on urinary E1C concentrations were used solely to determine the date of ovulation.

Data were separated into two distinct reproductive periods based on Kersey et al. (2010a, 2011). Kersey's team used analysis of relevant reproductive steroid concentrations in the subject's urine to isolate periods of sustained elevation greater than two standard deviations from the baseline mean, calculated by repetitive elimination of outlying data points for progestagen profiles, to demarcate biologically relevant reproductive periods each year. For purposes of this study, the luteal phase (consisting of the primary rise and secondary rise) was considered to begin on the day of ovulation and AI, which was designated Day 0 on the constructed annual reproductive timelines, with all study days being assigned a number relative to this day for each reproductive year. The end of the primary rise and beginning of the secondary rise was categorized as the day when urinary progestagen concentrations increased to more than three standard deviations above the baseline average and remained at this level for multiple consecutive days. The luteal phase was considered over when urinary progestagen decreased to the baseline average. Behavioral data were averaged on the basis of these reproductive periods, as well as in 2-week divisions for graphical presentation. The five main behavior categories were transformed into a percent value of total daily observed time. Specific behavioral sub-categories

(scratching, sitting with front feet up, and rest) showed clear variation across the observation period and were also subjected to statistical analysis. Certain reproductively associated behaviors were performed too infrequently to be analyzed statistically but their patterns of occurrence were compared against the significant dates of the reproductive cycle.

For graphical representations of daily urinary hormone metabolite concentrations, presented results represent daily average values for days where multiple samples were taken.

Nonparametric statistics were our only reliable means of statistical comparison between reproductive years. To this end, Wilcoxon rank-sum-tests (U) were utilized (as Kersey et al., 2016) to compare hormone concentrations and behavioral averages, with tests performed using R Statistical Analysis Software (R Core Team, 2014).

3 | RESULTS

Day 0, in which SB 676 showed a primary increase in urinary progesterone concentrations, occurred on April 27 in 2014, and May 13 in 2015. Despite careful monitoring in 2014, no evidence of fetal presence or development was found, nor did any births occur. Similar monitoring in 2015 led to the detection of two fetuses, and SB 676 subsequently gave birth to two healthy cubs on Day 153 at 0300.

3.1 | Endocrine

SB 676's patterns of urinary progesterone excretion closely matched expected values from the existing literature, providing a clear demarcation of reproductive periods (Figure 1). There was no significant difference between reproductive periods across years ($U = 7498$, $p = .98$ for primary progesterone rise; and $U = 1384$, $p = .79$ for secondary progesterone rise). In both years, the average urinary progesterone concentration was significantly less in the primary rise compared with the secondary rise (11.26 ± 3.74 vs. 32.95 ± 17.630 ng/mg Cr, $U = 501$, $p = 3.46 \times 10^{-16}$ in 2014; 10.21 ± 6.05 vs. 29.60 ± 14.58 ng/mg Cr, $U = 38$, $p = 5.587 \times 10^{-9}$ in 2015).

In 2014, the secondary rise began on Day 180 and lasted 61 days with Day 206 being the highest urinary progesterone metabolite excretion for the year (87.134 ng/mg Cr), 36 days before the luteal phase ended. By contrast, in 2015, the secondary rise began on Day 110 and lasted 47 days. Peak urinary progesterone levels were detected on Day 136, although the maximal value was lower than the previous year (62.074 ng/mg Cr). This occurred 17 days before parturition on Day 153. Progesterone concentrations returned to near baseline levels on Day 157, four days after the birth event.

3.2 | Ultrasound

In 2014, SB 676 had a total of 33 ultrasound examinations of her caudal abdomen between Days 29 and 235. In 2015, she participated

in 19 examinations between Days 14 and 147, the latter date being six days before parturition. In general, the bladder and adjacent uterine body could be identified, as well as the cervix, but often images of one or both of the uterine horns were obscured or obstructed by the large amounts of intestinal gas and feces. The ovaries were never reliably identified. In both years, despite regular ultrasounds occurring for months beforehand, nearly all recorded uterine and fetal developments occurred after the progesterone peak of the secondary rise.

SB 676's 2014 insemination did not produce any cubs, nor were any related structures (i.e., embryo, gestational sac, and fetus) identified during the ultrasound examinations. Instead, after the secondary rise, there appeared to be irregular thickening of the endometrium with the development of significant endometrial edema and folding beginning on Day 212, remaining similar until Day 223, and decreasing by Day 226 with endometrial inactivity noted by Day 257 (Figure 2). No scans were performed between Days 226 and 257. The endometrial folding and edema noted were similar to equine or bovine ultrasound scans performed during estrus with elevated estrogens (Bragg Wever et al., 2002; Fissore et al., 1986). In 2015 on Day 127, 17 days after the secondary rise began, observation of an anechoic structure in the right horn of the uterus was identified and suspected to be an embryo. On Day 135 (18 days pre-parturition, 1-day pre-progesterone peak), a fetus was discovered via ultrasound (Figure 3). Four days later (Day 139, 14 days pre-partum), two fetuses were noted during the ultrasound session, and subsequently located on Days 142, 145, and 147. Significant endometrial edema and folding were noted at each scan with visible fetus(es), first noted at Day 127 with the presumptive embryo.

3.3 | Behavior

In both years of this investigation, SB 676's time budget changed significantly between the primary and secondary progesterone rise (Table 1), though the same general trend remained consistent throughout: while the majority of her time active was spent eating bamboo, overall SB 676 spent more time inactive than in any active behavior (see 24 h observation period in Table 1a). SB 676 spent significantly more time in scratching (primary rise: $U = 1780.5$, $p = 4.40 \times 10^{-12}$ for 9 h, $U = 937$, $p = 1.38 \times 10^{-15}$ for 12 h, $U = 33$, $p = 8.31 \times 10^{-8}$ for 24 h; secondary rise: $U = 423.5$, $p = 3.98 \times 10^{-4}$ for 9 h, $U = 426$, $p = 1.01 \times 10^{-3}$ for 12 h, $U = 54.5$, $p = 2.47 \times 10^{-5}$ for 24 h) and resting (primary rise: $U = 1733.5$, $p = 8.68 \times 10^{-11}$ for 9 h, $U = 1381$, $p = 5.62 \times 10^{-9}$ for 12 h, $U = 109.5$, $p = 1.81 \times 10^{-4}$ for 24 h; secondary rise: $U = 44$, $p = 8.90 \times 10^{-13}$ for 9 h, $U = 31.5$, $p = 5.63 \times 10^{-13}$ for 12 h, $U = 7$, $p = 1.31 \times 10^{-10}$ for 24 h) behavior in 2015 than 2014 (Table 1b).

SB 676 showed a significant decrease in non-stereotypic activity from the primary rise to the secondary rise in 2014, but only during the 9-h observation periods ($U = 2874.5$, $p = .038$ for 9 h; $U = 2209$, $p = .054$ for 12 h; $U = 405$, $p = .195$ for 24 h), while a significant increase was observed from the primary into the secondary rise in 2015 ($U = 939$, $p = .020$ for 9 h; $U = 757$, $p = 1.44 \times 10^{-3}$ for 12 h, $U = 81.5$,

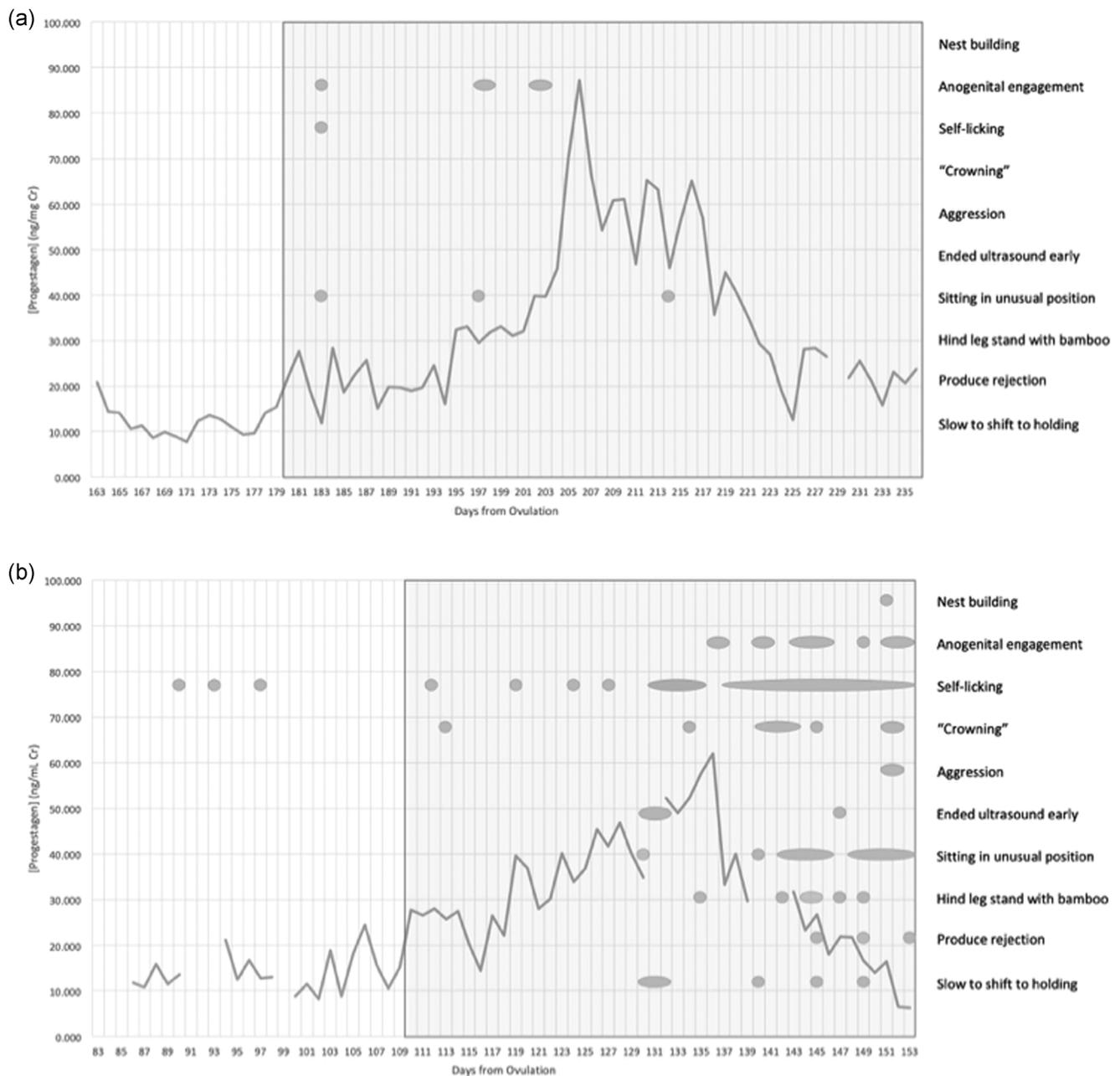


FIGURE 1 Urinary progesterone concentration versus reproductive day (Day of luteal cycle) with occurrence of specific pre-partum behaviors for SB 676 in (a) 2014 and (b) 2015 [Color figure can be viewed at wileyonlinelibrary.com]

$p = 7.60 \times 10^{-4}$ for 24 h). While SB 676's 2014 non-stereotypic behavior values during the primary rise were significantly higher than those in 2015 ($U = 6568.5$, $p = 5.93 \times 10^{-13}$ for 9 h, $U = 4924$, $p = 1.79 \times 10^{-11}$ for 12 h; $U = 420$, $p = .046$ for 24 h), there was no significant difference during the secondary rise except in the 24 h observation period, for which the 2015 values were higher ($U = 907.5$, $p = .137$ for 9 h; $U = 815$, $p = .437$ for 12 h; $U = 119$; $p = .007$ for 24 h). This was contributed to by a considerable increase in non-stereotypic activity overnight from Day 140 through the end of the luteal phase in 2015, with maximal values for the study occurring in the last 2 days pre-partum (for 24 h, 22.47% Day 151, 47.70% for day 152). SB 676's levels of stereotypic behavior varied significantly between years only when considering the

9-h and 24-h timeframes during the secondary rise, with 2014 values being significantly higher ($U = 980$, $p = .025$ for 9 h, $U = 324$, $p = .023$ for 24 h).

In 2014, SB 676's bamboo feeding time was strongly depressed, and levels of inactivity high, for Days 57 through 128, compared with the surrounding time periods. This strongly influenced the average behavior values for the primary progesterone rise and created the largest differences in time budget for behaviors between her primary and secondary rises for that year (bamboo eating: $U = 492.5$, $p = 6.05 \times 10^{-14}$ for 9 h, $U = 511$, $p = 4.75 \times 10^{-11}$ for 12 h; $U = 184$, $p = 7.89 \times 10^{-3}$ for 24 h; inactivity: $U = 3971$, $p = 8.34 \times 10^{-11}$ for 9 h, $U = 3053$, $p = 7.62 \times 10^{-10}$ for 12 h, $U = 507$, $p = 6.96 \times 10^{-4}$ for 24 h).

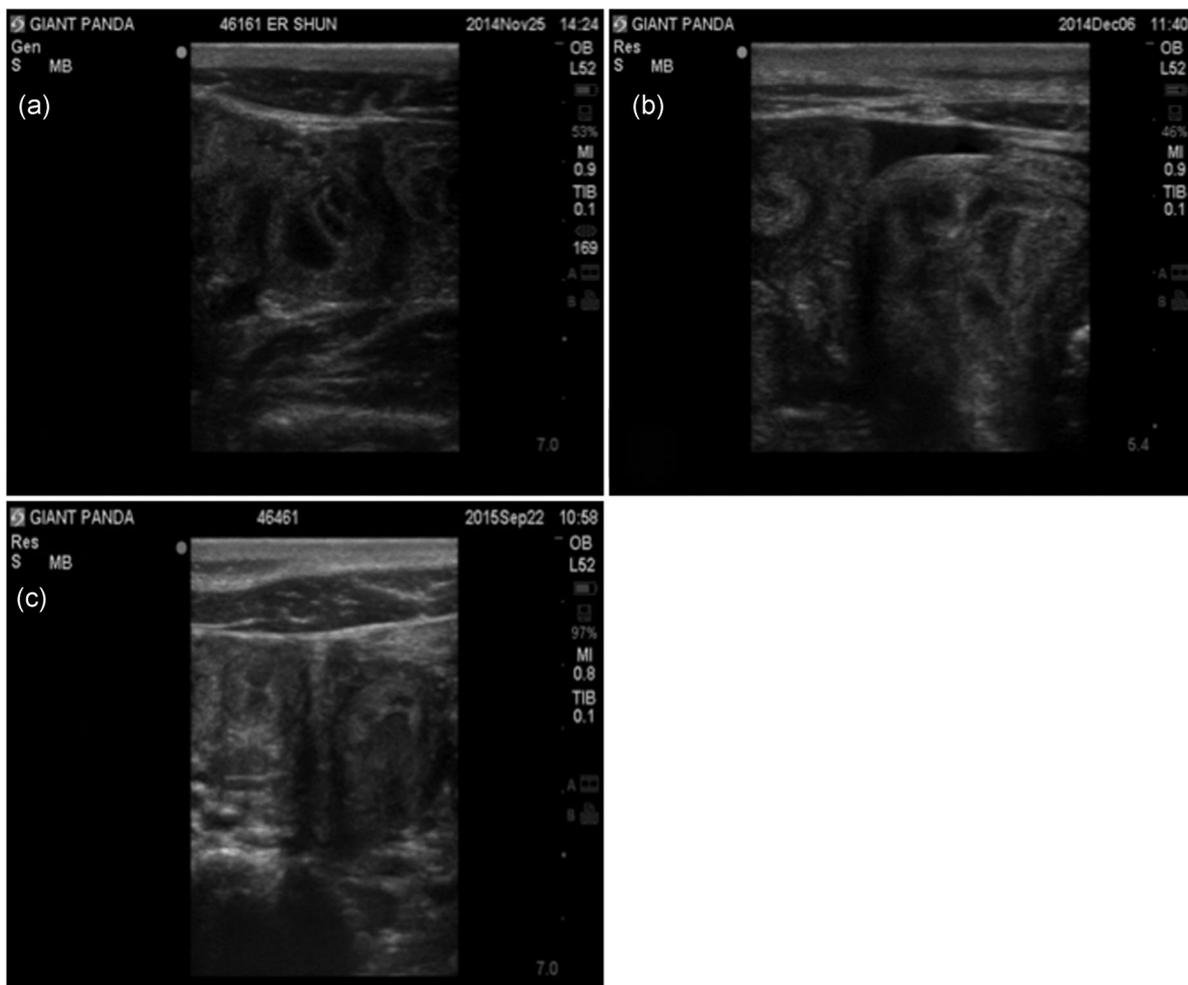


FIGURE 2 Example ultrasound images of uterine structures showing endometrial edemas and folding (a) 2014 Day 212 uterine horn imaged 20 days after secondary rise began, 6 days before peak urinary progesterone; (b) 2014 Day 223 left uterine horn; and (c) 2015 Day 132 for comparison [Color figure can be viewed at wileyonlinelibrary.com]

In 2015, SB 676's behavior showed little change from the primary rise until Day 129, more than 2 weeks after the beginning of the secondary rise, and 24 days before parturition. On Day 129, SB 676 sharply decreased her time spent feeding on bamboo and concurrently increased her inactivity. Her feeding levels continued to decrease, and her inactivity increase, through the end of her luteal phase, creating significant statistical differences between the time budget for these behaviors during the secondary rise and that of the previous year (bamboo: $U = 1350$, $p = 3.34 \times 10^{-9}$ for 9 h; $U = 1311$, $p = 2.21 \times 10^{-10}$ for 12 h; $U = 424$, $p = 2.23 \times 10^{-7}$ for 24 h; inactivity: $U = 67$, $p = 1.56 \times 10^{-15}$ for 9 h, $U = 96$, $p = 1.62 \times 10^{-13}$ for 12 h; $U = 45$, $p = 1.37 \times 10^{-6}$ for 24 h). On Day 152, SB 676 ate for only 8 min total and then did not eat again until Day 156, 3 days after parturition. Overall SB 676's behavior in the secondary rise was significantly different from that during her primary rise for all behaviors (non-stereotypic activity: $U = 939$, $p = .020$ for 9 h, $U = 757$, $p = 1.44 \times 10^{-3}$ for 12 h, $U = 81.5$, $p = 7.60 \times 10^{-4}$ for 24 h; enrichment use: $U = 842.5$, $p = 3.07 \times 10^{-3}$ for 9 h; $U = 773$, $p = 2.08 \times 10^{-3}$ for 12 h; $U = 121.5$, $p = 1.81 \times 10^{-2}$ for 24 h; bamboo eating: $U = 2065.5$, $p = 4.57 \times 10^{-7}$ for

9 h; $U = 1930.5$, $p = 1.41 \times 10^{-6}$ for 12 h; $U = 404$, $p = 1.35 \times 10^{-7}$ for 24 h; inactivity: $U = 532$, $p = 5.91 \times 10^{-7}$ for 9 h, $U = 567$, $p = 7.27 \times 10^{-6}$ for 12 h, $U = 53$, $p = 1.21 \times 10^{-5}$ for 24 h) except stereotypic activity ($U = 1241$, $p = .716$, for 9 h; $U = 1174$, $p = .729$ for 12 h; $U = 218$, $p = .970$ for 24 h) in 2015.

SB 676 performed few parturition-associated behaviors in her early secondary rise in 2014 (Figure 1a), but a far greater diversity and abundance of such behaviors in the same period the following year, notably increasing on Days 130 and 140, surrounding the PdG peak at Day 136 (Figure 1b).

A great proportion (average $15.96 \pm 11.72\%$ for 24 h observations) of SB 676's non-stereotypic activity in the secondary rise of 2015 included sitting with her front feet up, with much of this behavior involving engagement with the anogenital region (Figure 1b). She enacted such behavior for over 3 h on the day before parturition, mostly overnight. Much of this behavior was performed while positioned in a corner of a holding pen, facing the corner with her head tucked down but active, precluding reliable detailed categorization of her actions.

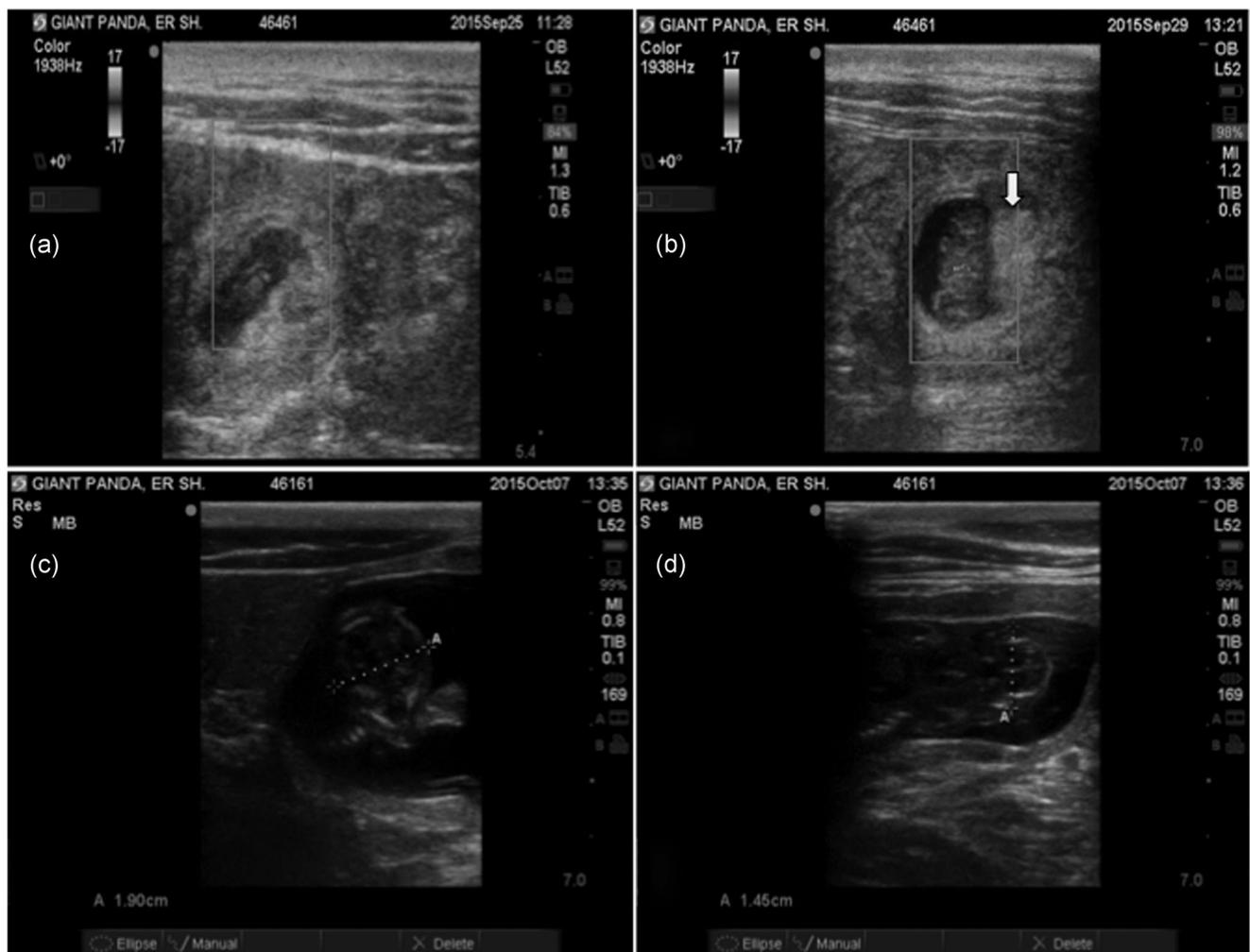


FIGURE 3 Example ultrasound images of fetal development. (a) 2015 Day 135 first confirmation of fetal development. (b) 2015 Day 139, second identified fetus; note the discoid of appearance of the placenta. (c,d) 2015 Day 147 separate images of both cubs born Day 153 [Color figure can be viewed at wileyonlinelibrary.com]

Where no significant difference was seen between reproductive periods in 2014 for “sitting front feet up” behavior, the following year SB 676 significantly increased this behavior from the primary rise into the secondary rise ($U = 672.5$, $p = 3.43 \times 10^{-5}$ for 9 h; $U = 546$, $p = 2.82 \times 10^{-6}$ for 12 h; $U = 59.5$, $p = 8.54 \times 10^{-5}$ for 24 h). This led to a significant difference in time spent sitting front feet up in the secondary rise between years ($U = 376.5$; $p = 1.10 \times 10^{-4}$ for 9 h; $U = 300$, $p = 6.58 \times 10^{-6}$ for 12 h; $U = 48$, $p = 1.22 \times 10^{-5}$ for 24 h). In 2015, the difference in resting time over 24 h was significantly higher in the secondary rise over the primary rise ($U = 34$, $p = 5.1 \times 10^{-6}$), but this was not detected in 2014 ($U = 285.5$, $p = .42$).

4 | DISCUSSION

While this investigation represents a case study and wider application of trends observed here should be done carefully, our observations add to and in some instances provide valuable contrast to what is currently reported in the literature for this species. In this study, a

combination of endocrine analysis, transabdominal ultrasound imaging, and behavioral observations was used to differentiate the luteal phases of a panda experiencing a pseudopregnancy and a twin pregnancy in consecutive years. There were no significant differences in the amplitude of urinary progestagens between these states, but behavior and ultrasound assessments showed significant divergence between the two. The differences between these data and those reported in instances where natural abortion or fetal reabsorption were suspected, lead us to believe that SB 676's 2014 reproductive cycle may represent a pseudopregnancy (Chaudhuri et al., 1988; Steinman et al., 2006; Willis et al., 2011; G. Zhang et al., 2004). For unclear reasons, SB 676 showed significant variation between her 2 reproductive years, contrasting with the reports of other facilities that witnessed high degrees of similarity between suspected pseudopregnant and truly pregnant female pandas (Chaudhuri et al., 1988; Narushima et al., 2003; Owen et al., 2005; Sutherland-Smith et al., 2004; Swaisgood et al., 2003). Given her age (6.75 years in 2014 at the time of insemination) and that her first insemination occurred after her first recorded strong season of estrus, it is possible

TABLE 1 (a) Average time budget (% total observed time) and (b) average time values for behavioral sub-categories of interest (# minutes in behavior) for SB 676 in different reproductive periods, across different observation timeframes and reproductive periods

		9-h observation timeframe		12-h observation timeframe		24 h observation timeframe	
		Primary rise	Secondary rise	Primary rise	Secondary rise	Primary rise	Secondary rise
a							
2014	% Non-stereotypic Activity	12.4%	9.9% ^a	11.9%	10.1%	7.2% ^{b,c}	6.4% ^{b,c}
	% Stereotypic Activity	2.2%	3.6% ^a	1.1%	3.1% ^a	0.7%	2.1% ^a
	% Enrichment Engagement	2.6%	3.2% ^a	2.5%	3.2% ^a	1.1% ^{b,c}	1.6% ^{a,b,c}
	% Bamboo Feeding	33.1%	50.0% ^a	36.6% ^b	51.7% ^a	30.4% ^c	37.5% ^{a,b,c}
	% Inactivity	49.7%	33.3% ^a	47.9%	31.9% ^a	60.6% ^{a,b,c}	52.4% ^{a,b,c}
2015	% Non-stereotypic Activity	7.2% ^d	9.2% ^a	7.7% ^d	10.4% ^a	5.9% ^{b,c,d}	11.2% ^{a,d}
	% Stereotypic Activity	1.5%	1.5% ^d	1.7%	1.6%	0.7%	1.0% ^d
	% Enrichment Engagement	2.4%	3.1% ^a	2.4%	3.2% ^a	1.2% ^{b,c}	1.9% ^{a,b,c}
	% Bamboo Feeding	43.3% ^d	26.1% ^{a,d}	45.4% ^d	27.6% ^{a,d}	40.8% ^d	16.6% ^{a,b,c,d}
	% Inactivity	45.7% ^d	60.1% ^{a,d}	42.8% ^d	57.2% ^{a,d}	51.3% ^{c,d}	69.3% ^{a,b,c,d}
b							
2014	Scratching	0.69	0.63	0.43	1.02 ^a	0.87 ^c	2.35 ^{a,b,c}
	Sitting with front feet up	1.97	1.63	2.69 ^b	1.95	4.08 ^{b,c}	2.29
	Rest	27.64	30.41 ^a	30.43	34.05 ^a	41.87 ^{b,c}	43.71 ^{b,c}
2015	Scratching	3.43 ^d	4.49 ^d	3.97 ^d	5.03 ^d	5.31 ^{b,c,d}	10.33 ^{a,b,c,d}
	Sitting with front feet up	1.66	6.03 ^{a,d}	2.10	9.44 ^{a,d}	3.13 ^b	33.89 ^{a,b,c,d}
	Rest	42.79 ^d	110.3 ^{a,d}	47.29 ^d	121.61 ^{a,d}	73.69 ^{b,c,d}	175.63 ^{a,b,c,d}

Note: Statistical significance between values was determined by Wilcoxon rank-sum tests ($p = .05$).

^aIndicates statistically significant difference between a value and the period of primary progesterone rise for that observation timeframe.

^bIndicates a statistically significant difference between the value and the value for the same behavior category during the 9-h observation timeframe of similar year and reproductive period.

^cIndicates a statistically significant difference between the value and the value for the same behavior category during the 12-h observation timeframe of similar year and reproductive period.

^dIndicates statistically significant differences between years.

that sexual maturation accounts for some of this unknown variability (Kleiman, 1974, 1983). Ideally, future studies will be able to examine every reproductive period of the lives of pandas to examine reproductive patterns relating to individual age and experience.

Reports to date have shown that significant variability exists in the timing of panda reproductive cycles, and while SB 676's 2015 reproductive cycle occurred later in the year and had a longer than average luteal phase length, it fit within reported values for both parameters (Durrant et al., 2003; Howard et al., 2006; Huang et al., 2012; Kersey et al., 2010b; Kleiman, 1983; Schaller et al., 1985; Wang et al., 2004; H. Zhang et al., 2009; Zhu et al., 2001). Total time from insemination to parturition in pandas has been reported anywhere from 85 to over 185 days (Hodges et al., 1984; Howard et al., 2006; Huang et al., 2012; Kleiman, 1983; H. Zhang et al., 2009; Zhu et al., 2001). Therefore, the differences between the timings of SB 676's 2014 and 2015 returns to baseline urinary progesterone concentrations were not remarkable. Previous studies have suggested that following delayed implantation, the initiation of the

secondary rise in urinary progesterone levels (on Day 180 [2014] and Day 110 [2015] in this study) represents the implantation stage of embryonic development (Kersey, 2008; Kleiman, 1983; Monfort et al., 1989; Steinman et al., 2006; H. Zhang et al., 2009). If correct, this would mean that SB 676 had a postimplantation pregnancy length of 44 days in 2015, which is consistent with the existing literature (Kersey et al., 2010b; Steinman et al., 2006).

4.1 | Endocrine

The patterns of urinary sex steroid hormone excretion in pandas for both the peri-estrus and peri-partum periods are well documented, and SB 676's general hormone profiles fit well with the existing literature (Chaudhuri et al., 1988; Czekala et al., 2003; McGeehan et al., 2002). Most recent endocrinological investigations of panda reproduction have sought not to map basic trends but isolate specific chemical signaling markers, pheromones, or sexual information

carriers during the peri-estrus period (Liu et al., 2013; Steinetz et al., 2005), and differentiators between pregnancy and pseudo-pregnancy peri-partum (Roberts et al., 2018; Willis et al., 2011).

The timing and general pattern of SB 676's pregnancy were similar to that reported in the literature (Chaudhuri et al., 1988; Hodges et al., 1984; Kersey et al., 2016; Monfort et al., 1989; H. Zhang et al., 2009), but her pseudopregnant luteal phase was far longer, most notably during the period of primary progesterone rise. Kersey et al. (2010b) found high levels of consistency in the luteal phase length between years for the same panda, though with a general trend for longer luteal phases in suspected pseudopregnant years. We could not find published evidence of a successful birth occurring more than 198 days after ovulation (Howard et al., 2006). This is corroborated by similar findings in red pandas (*Ailurus fulgens*) in which fecal progesterone metabolite analysis identified a prolonged, but not statistically significant, luteal phase rise in progesterone in pseudopregnant females compared to proven pregnant females (Mastromonaco, unpublished). These data suggest the possibility of an upper limit to pregnancy length in pandas, such that any luteal phase exceeding this mark is indicative of a nonpregnant state.

Despite this temporal variation, there was no significant quantitative distinction between urinary hormone concentrations in pregnant and nonpregnant years. Studies on other bears (*Melursus ursinus*, *Tremarctos ornatus*, *Ursus arctos*, and *Ursus thibetanus*) have shown a similar lack of differentiation in progesterone profiles (Göritz et al., 1997; Quest, 2010). Kersey et al. (2010b) recorded that in both parts of the luteal phase non-parturient female pandas demonstrated consistently higher fecal progesterone metabolite concentrations than those who gave birth, a trend that SB 676 followed, though not at a statistically significant level. Similarly, in domestic dogs, progesterone concentrations in peripheral blood are not useful in differentiating pregnant from pseudopregnant luteal phases and the luteal phase is often longer in pseudopregnant cycles (Johnston et al., 2001).

4.2 | Ultrasound

The endometrial edema and hyperplasia observed in 2014 might have been indicative of unsuccessful fertilization or implantation, or of the initiation of embryo(s) which then failed to fully develop, resulting in subsequent resorption or termination. The full length of both uterine horns displayed endometrial edema and folding rather than focal nidation site(s). Since SB 676 was not examined during a reproductive cycle where she was not bred, we are unable to confirm whether these endometrial changes occur only following embryonic loss or in all nonpregnant luteal phases following the secondary progesterone rise. Hildebrandt et al. (2006) report that a 1998 Zoo Berlin investigation found "an embryonic resorption site... which caused a temporary edematous degeneration of the endometrium" (p. 431) during an ultrasound session about 180 days after AI (progesterone profile not reported). The edematous structures that characterized SB 676's pseudopregnancy did not emerge until well after the secondary rise in PdG excretion, first identified on Day 220 in 2014, well after the peak

progesterone value (Day 206). Göritz et al. (1997) found that trans-rectal ultrasounds of other bear species (*M. ursinus*, *T. ornatus*, and *U. arctos*) revealed no difference between suspected pseudopregnant and pregnant bears during embryonic diapause.

In their multi-disciplinary study of panda reproduction, Kersey et al. (2016) suggested that embryonic diapause ends not at the start of the secondary rise, but approximately 3 weeks before parturition, coinciding with the progesterone peak; an idea that is supported by others (Chaudhuri et al., 1988; H. Zhang et al., 2009). This contradicts Steinman et al.'s (2006) report of a female panda aborting a fetus on the day of her progesterone peak. Though lacking ultrasound evidence, other sources support the idea that nidation occurs at the start of the secondary progesterone rise (Steinetz et al., 2005; Willis et al., 2011). Our study demonstrated potential uterine embryo development on Day 126, 4 days before diverse pre-partum behaviors emerged; and confirmation of a well-developed fetus with a heartbeat on Day 135; both dates before peak urinary progesterone levels (Day 136). These data support the idea that in pandas embryo implantation occurs before the luteal progesterone peak. It is unclear, however, what physiological or extraneous event triggered the distinctive change in SB 676's behavior mid-way through the period of secondary progesterone rise (Day 129).

In 1998, a study at Zoo Berlin reported ultrasound evidence of the free-floating blastocyst (>1 mm across) in the uterus 1 month after AI (Hildebrandt et al., 2006). Identifying a panda pregnancy before embryonic implantation is otherwise undocumented, though Göritz et al. (1997) were able to detect developing fetuses via ultrasound in other bear species before the secondary progesterone increase. Typically, the first identification of embryonic development is multiple weeks after the secondary progesterone rise begins (Kersey et al., 2016; Willis et al., 2011; H. Zhang et al., 2009). In a study with nine female pandas, H. Zhang et al. (2009) found that the period between 15 and 20 days pre-partum is when the fetus first became visible via ultrasound, an average of 107 days after insemination, a range that is supported by other reports (Hildebrandt et al., 2006; Kersey et al., 2016; Willis et al., 2011). Our study first confirmed a fetus 18 days before parturition, on Day 135, with a potential earlier identification on Day 127 (26 days before parturition). In this study, the operators were always temporally limited by the panda's behavior to only a few minutes per scan. The unique gas-filled intestines of the panda made scanning of both uterine horns more difficult on some days. Furthermore, unlike the pseudopregnant year when no resistance to scanning occurred during the late luteal phase, scanning time permitted by the panda decreased substantially as parturition approached. This may have been due to a decrease in appetite during pregnancy to the extent that the apple "treats" provided during scanning would not hold her attention.

4.3 | Behavior

The behavior of wild pandas shows significant annual variation according to the growth stage of their bamboo diet (Hu & Wei, 2004;

Nie et al., 2015; Schaller et al., 1989). This combined with the seasonal nature of panda reproduction makes it challenging to tease apart reproductive from environmental effects on panda behavior (Owen et al., 2005). In summer of 2014, SB 676's bamboo feeding was low through the early portion of the primary progesterone rise and increased steadily through the fall months into the early portion of her secondary progesterone rise. This lines up well with both Schaller et al.'s (1985) observations of wild pandas, which were least active July through September, as well as additional data recorded from 2013 to 2016 for SB 676 (Magnus, unpublished). However, SB 676 behaved differently in the summer of 2015, with higher bamboo consumption through her shorter primary rise in progesterone, decreasing 24 days pre-partum; at a similar date post-insemination as the beginning of an increase in bamboo feeding the previous year. It is possible that increased primary progesterone rise feeding may be an intentional effort by an expecting female panda to store energy in preparation for a period of fasting surrounding the birth event (Brambell, 1976; Owen et al., 2005). While this pattern does not appear to be universal (Mainka & Zhang, 1994), the general trend of this data set fits well with such a hypothesis. The zoo's male panda, SB 732, generally spent less time eating than SB 676, but showed similar temporal trends in feeding patterns (excluding the pre-partum feeding decrease), suggesting the possible influence of additional nutritional, seasonal, environmental, or management factors on this behavior, that future authors would do well to account for if possible.

A high degree of variation has been documented in the behavior of female pandas during the period of secondary progesterone rise; however, some major trends do exist (Chaudhuri et al., 1988; Kersey et al., 2016; Kleiman, 1983; Narushima et al., 2003; Schaller et al., 1985; Zhu et al., 2001). In 2014, despite the apparent progesterone profile of a pseudopregnant female, SB 676 exhibited the behavioral traits of an acyclic female (Hildebrandt et al., 2006; Kersey et al., 2010b, 2011; Steinman et al., 2006), showing very few parturition-associated behaviors, in stark contrast to much of the literature describing suspected pseudopregnant female pandas (Chaudhuri et al., 1988; Narushima et al., 2003; Owen et al., 2005; Sutherland-Smith et al., 2004; Swaisgood et al., 2003). However, in 2015, SB 676 demonstrated a variety of parturition-specific behaviors that are consistent with the literature; notably, beginning around Day 129 (19 days after the beginning of the secondary rise, 24 days before parturition), a decreased appetite (Owen et al., 2005; Swaisgood et al., 2003; Zhu et al., 2001) linked with greatly increased lethargy (Masui et al., 1989; Narushima et al., 2003; Steinman et al., 2006), and an increasing disinterest in participating in ultrasound examinations, and favored foods (Kersey et al., 2016; Sutherland-Smith et al., 2004). At this time, SB 676 demonstrated a greater affinity for the more sheltered areas of her holding, and while secluded in this way, she greatly increased her self-licking behavior, particularly toward her anogenital region, as reported in denning and pregnant female pandas (Kersey et al., 2016; Swaisgood et al., 2003). Furthermore, although not extensively reported in the literature as an indicator of pending parturition, an increase in sitting behavior was

observed. Certain other behaviors, such as lifting bamboo while standing on her hind legs, also appear unique to this individual.

Owen et al. (2005) documented cases of females fasting for multiple weeks pre-partum, but SB 676 continued to eat until the day before she gave birth (Brambell, 1976). Swaisgood et al. (2003) reported that increased urination may be an indicator of true pregnancy within 48 h of parturition. We found no specific increase in either defecation or urination concurrent with the secondary progesterone rise and impending parturition (Magnus, unpublished); variation in these behaviors appeared to be linked more closely to food and water consumption than hormone levels for SB 676 (Kersey et al., 2016; Swaisgood et al., 2003).

5 | CONCLUSIONS

In seeking to elucidate distinctions between the pseudopregnant and pregnant states within the same individual for *A. melanoleuca*, we examined one animal (SB 676) in her early years of sexual maturity; in 2014, she was reproductively unsuccessful (the data suggested a pseudopregnancy), and in 2015, she gave birth to twin cubs. Across these reproductive years, SB 676 showed a strong disparity in luteal phase length, as observed with urinary-endocrine analysis, endometrial development in relationship to fetal development, as observed via ultrasound, and behaviors, both in terms of time budget of typical behaviors, and frequency and diversity of parturition-associated behaviors enacted. Our results suggest that for giant pandas a lack of pre-partum behavior, extended ultrasound evidence of endometrial edema and folding without observation of a fetus, or a luteal phase extending beyond an as-yet-unknown specific limit, may provide early indication to invested zoological facilities of a failed breeding attempt. The discovery of a fetus via ultrasound before the peak urinary PdG levels in the secondary rise informs the discussion of the as-of-yet undetermined relationship between urinary progesterone and the timing of fetal implantation for the giant panda. We recommend that future studies increase sampling to multiple individuals, and additional breeding seasons. Such investigations would be able to examine effects of individual age, maturity, sexual experience on the physiological and behavioral signals of a panda's reproductive status, and changes in study design may reveal potential effects of additional factors related to nutrition, season, environment, and management. Increased sample size and broader reviews of reproductively similar Carnivorans may provide further insight into whether delayed implantation and pseudopregnancy are limited by a maximum timeframe for fetal implantation and development.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

This investigation was conducted meeting all guidelines of AZA animal husbandry and the Canadian Council on Animal Care.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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