

RESEARCH ARTICLE



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Comparison of hormone profiles for singleton and twin births in a female giant panda

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ABSTRACT

The breeding of giant pandas is inherently difficult owing to the short oestrous cycles of the females. Determining the exact time of the oestrous cycle and when the females will attempt to mate may increase the chances of reproductive success. Because the female giant panda at the Everland zoo in Korea did not show signs of oestrus in 2019, her oestrous cycle could not be measured. In 2020, we induced oestrous behaviour in the same panda, used oestrogen peak and trough levels to predict her ovulation period, and two natural mating attempts were conducted in two days. We confirmed and monitored the pregnancy using progesterone measurements and ultrasonography, respectively, and the female successfully delivered and raised a single cub. In 2023, the same method was used on the female, with repeated success. Her urine showed a sharp increase in progesterone levels, unlike in 2020, indicating she was pregnant with twins, which she successfully gave birth to in July 2023. Herein, we share our findings on these giant pandas and the potential application of our methods to other endangered animals like rhinoceros and red pandas.

KEYWORDS

oestrogen, progesterone, twin giant pandas, urinary hormones

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) was classified as vulnerable in 2016 owing to the conservation efforts of various research institutions and zoos worldwide, including those in China (Swaisgood, 2015; Swaisgood et al., 2016). Studies have shown that although panda populations had been declining since they were first surveyed, breeding research for species conservation in China and globally has contributed to a population rebound, with populations increasing by approximately 16.8%, and their numbers are now larger than before (Kang and Li, 2019; Leung, 2019). While reproductive research and hormone monitoring have improved the success of ex situ breeding programs, habitat protection and restoration remain equally essential components of in situ conservation for the long-term survival of the species (Martin-Wintle et al., 2019). Giant pandas are only in oestrus for a limited period from February to May, spending two to three days for mating on average. Therefore, determining their ovulation date is crucial for successful breeding (Steinman et al., 2006;

Martin-Wintle et al., 2019). Urinary hormone testing is non-invasive and avoids venipuncture, although blood sampling is routinely conducted without sedation or anaesthesia in many institutions using established training protocols and is minimally stressful for pandas, making it relatively easy to monitor reproduction-related physiological changes in female pandas periodically.

The hormones detectable in urine include oestrone-3-glucuronide (E1G; a urinary oestrogen metabolite), progesterone, and dihydro-15-keto-prostaglandin F_{2α} (PGFM), all of which are analysed on the basis of urinary-specific gravity (USpG) and creatinine levels (Cai et al., 2017; Roberts et al., 2018; Wilson et al., 2019, 2022). E1G, an essential hormone during oestrus, should be measured throughout the oestrous cycle. Notably, mating female and male giant pandas at the time of the oestrous cycle is the easiest way to breed them. Ovulation can be predicted by the E1G level, which starts to decline from its highest peak, with a decrease by less than half of the peak being indicative of egg release from the ovaries (Hama et al., 2008; Wilson et al., 2019). Progesterone levels are continuously measured from the start of the oestrus, throughout pregnancy, and at birth. For giant pandas, distinguishing pregnancy from pseudopregnancy through behavioural and physical changes (e.g., decreased feed intake and progesterone levels) alone is difficult (Zhang et al., 2009; Kersey et al., 2010; Cai et al., 2017; Wilson et al., 2022). Therefore, abdominal ultrasonography was performed once weekly from approximately two months prior to the expected parturition until the period of peak progesterone concentrations to confirm pregnancy in the female giant panda. PGFM is measured in the urine at the time of predicted birth, which usually occurs within 24 h after a sharp rise in the hormone level (Roberts et al., 2018).

In this study, we aimed to learn more about the hormonal changes in female giant pandas during oestrus and ovulation, the timing of births and descriptive observations of behavioural patterns and urinary hormone profiles in a singleton and a twin pregnancy. Such information is important for conserving not only a single species – the giant panda – but also other animals such as red pandas. The first mating was performed in 2018, but the pregnancy was unsuccessful because of the incorrect timing determined for mating. In 2020, with more accurate hormone testing, the first natural mating was successful. Leveraging this experience, the pandas were again mated, resulting in the birth of twins in 2023. The findings from this study are expected to positively impact the conservation of endangered species.

MATERIALS AND METHODS

Urine sample collection

The female giant panda was born in 2013, and the male giant panda was born in 2012. During the pregnancy preparation period, the urine of the female panda was collected twice daily, once in the morning and once in the afternoon. However, samples were collected frequently and

stored when her oestrus behaviour became evident. Because pandas tend to urinate immediately after waking up, urine was collected from the floor using a syringe and then frozen at –18°C or below. Urine samples were collected weekly as part of routine hormonal monitoring prior to the onset of the oestrus, and the pre-oestrus period was defined retrospectively. The collected urine was tested for E1G, progesterone, and PGFM. Before testing, the frozen samples were thawed to ambient temperature and centrifuged. USpG was measured, and the samples were diluted 20–100 times, depending on the duration of the oestrous period. When ovulation was imminent, the samples were diluted 100 times.

Measurement of urinary-specific gravity

USpG was measured using a portable digital refractometer (Pal-USG(Cat); Atago™, Fukaya, Japan). According to the manufacturer's instructions, the instrument was calibrated with 300 µL of sterile physiological saline before measuring the first sample (300 µL). Urinary hormone levels were calculated as follows (Millet et al., 2004): USpG-corrected concentration = raw hormone concentration × [(USpG mean value – 1.000)/(USpG sample – 1.000)].

Measurement of oestrone-3-glucuronide

Urinary E1G levels were measured using a DetectX® enzyme immunoassay (EIA) kit (K036-H5; Arbor Assays, Ann Arbor, MI, USA). The assay and wash buffers were diluted 5 (1:4) and 20 (1:19) times, respectively, with sterilised distilled water. Standard solutions were prepared as follows: 450 µL of assay buffer was added to microtube #1 and 200 µL to tubes #2–#7, respectively. Then, 50 µL of E1G stock solution was added to microtube #1 and the mixture was vortexed. Subsequently, 200 µL was transferred from microtube #1 to microtube #2, and the mixture was vortexed. These serial dilutions were repeated in microtubes #3–#7. All standards and samples were measured in duplicates to ensure experimental accuracy. In brief, 50 µL of the respective standards and samples was dispensed into the wells of a plate and 75 µL of assay buffer was added to the well for non-specific binding. Then, 50 µL of assay buffer was dispensed to B0 (maximum binding). Next, 25 µL of DetectX E1G conjugate was dispensed into all wells, and 25 µL of DetectX E1G antibody was added to all the wells except for the one with non-specific binding.

The edges of the plate were tapped gently to ensure thorough mixing of the reagents. The plate was then covered with a sealer and shaken at 160 rpm for 2 h at ambient temperature. Subsequently, the cells were washed four times with 200 µL of wash buffer. Next, the plate was patted with an absorbent towel and dried, following which 100 µL of 3,3',5,5'-tetramethylbenzidine substrate was added to every well. After incubation for 30 min at ambient temperature, 50 µL of stop solution was added to terminate the reaction. The absorbance at 450 nm wavelength was recorded using a spectrophotometer (BioTek Epoch Microplate Spectrophotometer; Agilent Technologies, Santa Clara, CA, USA). The results were interpreted using a four-parameter logistic curve.

Measurement of progesterone and dihydro-15-keto-prostaglandin F2 α

The progesterone and PGFM levels were measured using a DetectX EIA kit (K025-H5; Arbor Assays) and DetectX enzyme-linked immunosorbent assay (ELISA) kit (K022-H5; Arbor Assays), respectively, according to the protocols described in the manufacturer's product documentation, which were similar to the above-mentioned methods. The absorbance of each hormone was measured at a specified wavelength using a BioTek Epoch microplate spectrophotometer (Agilent Technologies).

Data analysis

The absorbance outputs were analysed using ELISA software (<http://elisaanalysis.com>). The results, which were based on the USpG levels, were summarised using Microsoft Excel.

RESULTS

Hormone levels and mating in 2020

Both neither the female nor the male giant panda showed reproductive behaviour in 2019, which should have been observed following a false pregnancy in the same female in 2018. We continuously measured the E1G and progesterone levels when the female vocalised or exhibited repetitive behavioural changes, which were consistent with established behavioural indicators of oestrus in giant pandas. E1G concentrations in the female giant panda showed a gradual increase beginning approximately 10 days before mating. Following the E1G peak, the E1G concentration declined to less than 50% of its peak value, which was used as an indicator of ovulation; therefore, we concluded that the female and male were ready to mate. Mating took place over two days (from March 21st to 22nd), and the duration of each session was measured to ensure that it was successful (Fig. 1). During the first breeding season in 2020, nine mating attempts were observed. Following the guidance of experienced Chinese panda breeding specialists, we defined a successful mating attempt if the pair maintained a typical

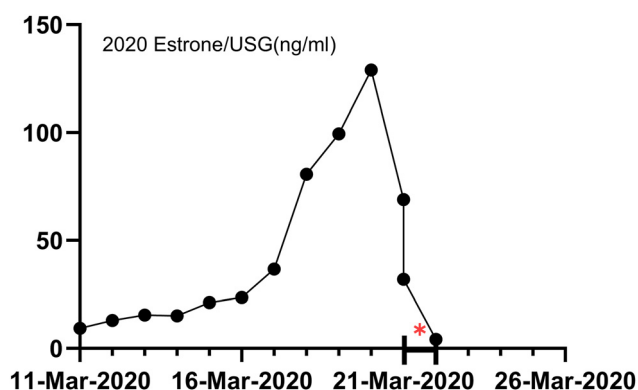


Fig. 1. Oestrus Estrone (E1G) changes and mating days (Red star) in the female giant panda in 2020

copulatory posture with confirmed intromission for more than 1 min. The first lasted 1 min and 40 s between 15:32 and 16:31 h, and the second lasted 4 min and 30 s the following day between 08:59 and 09:09 h. After successful mating, the progesterone level of the female slowly increased, peaking 18 d before parturition. One pup was born after a gestational period of 120 d (Fig. 1).

The USpG values ranged from 1.001 to 1.018 (mean: 1.009). The E1G levels were measured accordingly, showing a 50–400-fold increase in urinary dilution as oestrus progressed. The oestrone/USpG value (adjusted for USpG) peaked in the afternoon of March 20 and then began to decrease in the evening, reaching approximately 25% of the peak value approximately 24 h later. On March 21, mating was successfully achieved.

The PGFM level was measured during parturition because the timing of labour is difficult to determine on the basis of the behaviour of the female only. The PGFM level tended to increase until progesterone peaked, then decreased with decreasing progesterone levels, and subsequently increased again at approximately 120 d, just before delivery, and increased sharply at 17:57 h. However, in 2018, when oestrus did not occur, the PGFM level similarly increased and then decreased with increasing and decreasing progesterone levels, respectively, but no sharp increase was observed thereafter (Fig. 2).

Hormone test results in 2023

After separating the first-born baby panda from its mother in 2022, the mother was expected to show signs of oestrus again by 2023. Similar to the procedures performed in 2020, we assessed the female by measuring her urinary E1G and progesterone levels using ELISA and EIA kits. The E1G level increased slightly and rose sharply 14 d before mating (February 16, 2023), reaching a peak on February 18. It then decreased to less than 50% of the peak value on the afternoon of February 19, at which point the female and male were introduced for mating. The oestrus USpG values

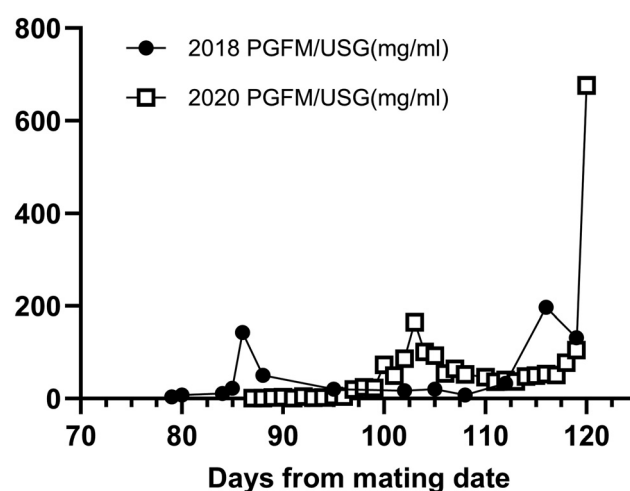


Fig. 2. Changes in urinary PGFM in a female giant panda from 2018 (non-pregnant) to 2020 (pregnant)

ranged from 1.005 to 1.018 (mean: 1.009). The E1G level showed a 50–100-fold increase in urinary dilution as oestrus progressively intensified. The oestrone/USpG values (adjusted for USpG) peaked on February 18 and then decreased rapidly, reaching approximately 25% or less of the peak value 24 h later (Fig. 2).

Mating took place over 2 d (the afternoon of February 19 to the morning of February 20), and the mating duration was measured each time to ensure success. Seven mating attempts were made in 2023, with the first mating lasting 3 min 50 s and the second lasting 1 min 46 s. After successful mating, the progesterone level in the female increased gradually, peaking 20 d before parturition, and the PGFM level increased sharply just before birth, similar to the levels observed in 2020 (Fig. 3). The total gestation period was 138 d, after which twin pandas were delivered.

Differences in hormonal changes between 2020 and 2023

During the first parturition in 2020, the E1G levels were significantly higher than those in 2023, resulting in the birth of one female cub (Fig. 4). However, the progesterone peak was much higher in 2023 (when the twins were born) than in 2020 (Fig. 4B). The increase in PGFM within 24 h before parturition was the same, and the time from the progesterone peak to parturition was not significantly different between days 17 and 20 in 2020 and 2023, respectively.

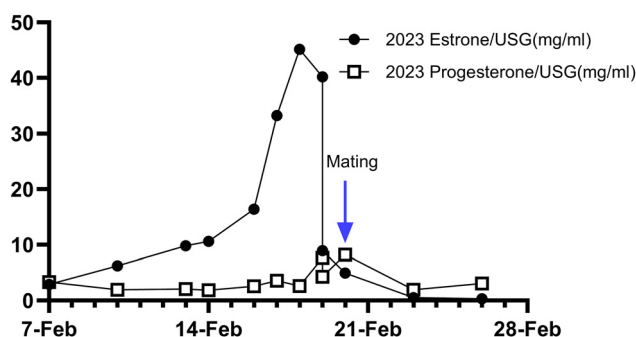


Fig. 3. E1G and progesterone level changes and mating day (blue arrow) of the female giant panda in 2023

DISCUSSION

In 2016, the China Panda Wildlife Conservation Center gifted a male and female giant panda pair to Korea. Since then, as a result of continuous healthcare and breeding research in Korea, this pair has successfully given birth to the first female cub in July 2020 and twins in 2023. Blood and urine samples were collected regularly every year to check for changes in the concentrations of hormones such as E1G and progesterone and to observe behavioural changes during oestrus to determine the appropriate mating date.

Female giant pandas are characterised by a once-yearly oestrous cycle, with ovulation occurring one or two weeks after an increase in oestrogen and mating being performed within 1–2 d of the oestrogen peak (Steinman et al., 2006; Huang et al., 2012). In 2018, the female showed signs of oestrus for the first time. Her bamboo intake decreased, and water play and marking behaviours were observed within the enclosure. However, this is typical of giant panda oestrus, and no mating behaviour was observed. The breeding age of female giant pandas is typically six years, although the female in this study was only five years old in 2018 (Kersey et al., 2010). Urinary hormone analysis showed little change in her E1G and progesterone levels, suggesting that normal ovulation and oestrus behaviour did not occur in 2018. Normal oestrus can be induced by indirect methods, such as olfactory stimuli (e.g., female and male urine and pheromones) and inducing curiosity by visual blocking (only sound) as in a wild environment, which increase the probability of ovulation and mating, as observed in other studies (Zhang et al., 2021). Traces left by the opposite sex, such as urine, faeces, and leftover bamboo, also induce oestrus and mating. Changes in hormonal levels are also observed during this period. During oestrus, females exhibit behavioural changes as their E1G levels increase, such as lifting their tails and turning their bodies to approach males after peak behaviours, similar to mating. Vulvar opening is also observed during this period, with a distinct change occurring immediately before mating, indicating that the female is ready to copulate. As the vulva opens, the amount of urine produced also increases.

In giant pandas trained for medical procedures, blood sampling can be minimally invasive; however, urinary

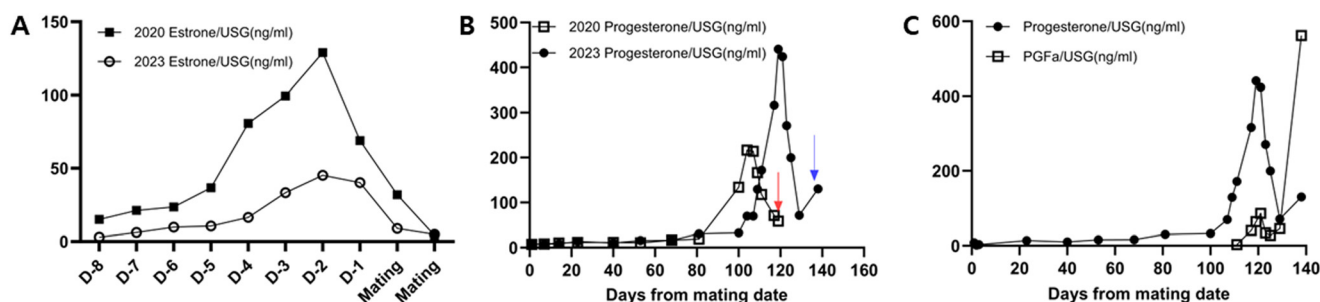


Fig. 4. Urinary hormonal changes associated with reproduction in a female giant panda during 2020 and 2023 pregnancies (A) Urinary E1G concentrations and mating days in 2020 and 2023, (B) Gestational progesterone concentrations with birth dates indicated (red arrow: 2020; blue arrow: 2023), (C) Progesterone and PGFM concentrations in 2023

hormone monitoring avoids venipuncture entirely and reduces handling-associated stress. For pandas with short breeding windows, the method is useful for determining ovulation dates more accurately. Various organisations have measured and calibrated urinary hormone levels (Hama et al., 2008; Willis et al., 2011; Roberts et al., 2018; Wilson et al., 2019). Additionally, detecting pregnancy using ultrasonography requires prenatal training, which can be distracting and stressful for female pandas as they approach labour. Therefore, various alternative methods have been proposed to detect pregnancies. Although ultrasonography was performed approximately one month before delivery, we were unable to perform another because the animal was uncooperative. Therefore, we aimed to diagnose pregnancy on the basis of urinary hormonal changes. Several studies have shown that progesterone levels are not suitable for distinguishing between pregnancy and fertility. Therefore, to compare non-pregnant and pregnant animals, both progesterone and ceruloplasmin levels were measured, and an increase in pregnancy was observed (Steinman et al., 2006; Zhang et al., 2009; Willis et al., 2011).

Research on the first pregnancy of the giant panda in Korea was used to assist with the second mating attempt in 2023, whereupon the animal became pregnant and gave birth to twins. However, there were several differences between the twin and singleton births. First, the behavioural changes in oestrus and peaks in the E1G level were lower than those of the first oestrus. This may have been due to the rapid behavioural changes in the female in 2020 after the non-oestrus period in 2019 and the rapid hormonal changes occurring along with those behavioural changes. Additionally, the change in E1G levels in 2023 reflected the first oestrus for the female which had been lactating through 2022 and was then separated from her offspring. The hormonal and behavioural changes during oestrus were milder than those in 2020. Although this is not clear in giant pandas and more research is required, we postulate that the relatively sharp increase in the progesterone level in 2023 may have been due to more of this hormone being released from the placenta by the twins compared with that released in 2020 when the mother was carrying the singleton. This phenomenon has been observed in other animal species. In humans, maintaining adequate progesterone levels during twin pregnancies has been suggested to increase the sustenance of the gestation process (Ikoma et al., 2017; Pasciu et al., 2022). On the basis of this evidence, we speculate that the high progesterone peak in the mother when she was pregnant with twins and the relatively high level maintained until just before birth may have been for foetal stability, as the growth period of a normal panda after implantation is relatively short (~1 month) (Zhang et al., 2009).

In summary, a giant panda in South Korea successfully gave birth for the first time in 2020. Although she exhibited signs of non-breeding and non-pregnancy in 2018 and 2019, oestrus induction techniques—such as visual blocking, reciprocal olfactory exposure and enclosure exchange—combined with monitoring of urinary E1G levels, facilitated the identification of optimal breeding timing. Pregnancy was

subsequently confirmed through progesterone profiling and ultrasonographic examination, with parturition occurring 120 days post-conception. Building on these findings, a second successful breeding occurred in 2023, resulting in the birth of twins. Although only two pregnancies were compared, slight differences in urinary hormone patterns were observed relative to the singleton pregnancy. These observations should be interpreted with caution and warrant further investigation to determine whether twin gestation may be associated with distinct hormonal characteristics. Despite these promising results, this study was limited to observations from a single female, thereby restricting the generalisability of the findings. Nevertheless, the ability to detect potential twin pregnancies in advance could contribute significantly to perinatal management and improve neonatal outcomes in captive breeding programs. These findings underscore the value of hormonal monitoring not only for timing oestrus and confirming pregnancy, but also for anticipating the nature of the gestation. Continued collaborative research and transparent data sharing across institutions are essential to refine reproductive management protocols and to strengthen both in situ and ex situ conservation strategies for giant pandas and other endangered species.

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