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Urine collection conditioning in determining the oestrous cycle of a captive female giant panda *Ailuropoda melanoleuca*

Trisha Tay Ting Ni[1], Josephine Kawi[1], Li Desheng[2] and Huang Yan[2]

[1]*Wildlife Reserves Singapore, 80 Mandai Lake Rd, Singapore*
[2]*China Conservation and Research Center for the Giant Panda (CCRCGP), Sichuan, China*

Correspondence: Trisha Tay Ting Ni, email; trisha.tay@wrs.com.sg

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Abstract

The breeding of giant pandas *Ailuropoda melanoleuca* in captivity can be a challenge, especially if natural mating is unsuccessful and artificial insemination is the only way to achieve fertilisation. The best chance of successful artificial insemination is when the female is inseminated within 24 hours of ovulation, right after oestrogen levels peak and start declining during her oestrous period. Hence, determining the exact point at which the female reaches her oestrous peak is crucial. Besides monitoring behavioural and physical changes in the female, urinary oestrogen is a common non-invasive biomarker in determining the female's oestrogen hormone profile. However, urine may be hard to collect in a naturalistic environment when the female is out on display. Conditioning a female panda to urinate on cue allows for direct urine collection and hence accurate monitoring of oestrogen levels at a consistent rate while ensuring that the sample collected is fresh and clean. This has led to improvement of the oestrogen hormone profile over recent years, allowing for a more accurate determination of the female's oestrous peak and a better grasp of timing for artificial insemination.

Background

Wildlife Reserves Singapore (WRS) received a pair of subadult giant pandas *Ailuropoda melanoleuca* from China in September 2012. The female (Jia Jia—studbook number 734) was age 4 and the male (Kai Kai—studbook number 690) was age 5 on arrival to Singapore.

Giant pandas are seasonally mono-oestrous, with oestrus generally occurring during the breeding season that lasts from February through June, with a peak in April (Steinman et al. 2006). Female giant pandas only have a brief fertile window of about 24–48 hours annually (Kersey et al. 2016). As most zoos outside of China have only one pair of giant pandas, breeding in captivity remains the most challenging aspect of managed care.

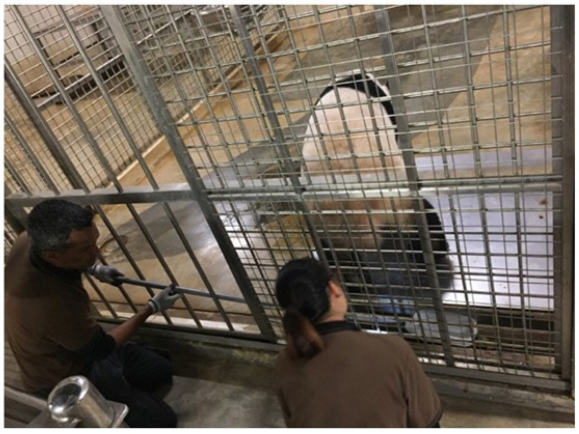
The female started showing signs of her first oestrus in 2015. In 2015–2017, efforts have focused on monitoring behavioural and physical changes in the female (Lindburg et al. 2001; Swaisgood et al. 2003), manipulating photoperiod and temperature to simulate seasonal changes (Tay et al. 2018), training exercises to strengthen the male's hind legs for mating, exposing the male to giant panda reproduction videos as recommended by Chinese reproduction experts, swapping of dens and enclosures to increase the male's sexual motivation via the female's scent prior to mating introduction (Swaisgood et al. 1999; Swaisgood et al. 2000; Swaisgood et al. 2002; Swaisgood et al. 2004) and monitoring hormonal changes in the female via urinary oestrogens (Czekala et al. 2003). However, a combination of the above methods did not result in any successful birth as the male was unable to position

Step 1: Keeper calling female to the weighing scale where she usually urinates

Step 2: Keeper giving verbal cue for urine collection to female

Step 3: Keeper checking to determine that female has urinated and bridges with a clicker

Step 4: Keeper rewards the female with food



Step 5: Keeper introduces urine collection container

Step 6: Modified urine collection container to allow for quick and easy collection of urine at other areas of the den as well

Figure 1. Conditioning steps carried out to train giant panda female for urine collection.

for copulation, a common reason for mating failure (Zhang et al. 2004), and artificial insemination (AI) was delayed beyond the ideal window for success due to attempts at natural mating.

In 2018, the focus shifted to artificial insemination. For this, determination of the exact period when the female hits her oestrus peak is crucial, as the likelihood of successful fertilisation is highest when the female is inseminated within the first 24 hours after oestrogen levels peak and start declining (Czekala et al. 2003; Howard et al. 2008; Huang et al. 2012). Hence, determining the most accurate hormone profile in the shortest time possible is critical. Since urinary oestrogens are being used to determine the female panda's hormone profile, fresh and clean urine samples are needed at a consistent rate to accurately monitor her oestrogen levels to identify the exact peak period (Czekala et al. 2003).

During the first three breeding seasons from 2015–2017, urine samples were routinely obtained from the den floor whenever the female urinates. These samples may have been contaminated by faeces passed out together with her urine. Moreover, during park operational hours when the female was out in the exhibit, she may have chosen to urinate in the exhibit, resulting in no urine being collected for approximately 24 hours. Overnight urine samples obtained from the den floor in the morning could have been passed anytime within the window of 1900–0845 hours (after operational hours), which made it hard to accurately determine the oestrogen levels during such periods. Additionally, these samples may too have been contaminated by faeces nearby, thus affecting the accuracy of the data generated.

A more efficient method of collecting fresh and clean urine samples thus had to be developed.

Action

After breeding efforts did not result in any successful parturition in 2016, conditioning efforts were focused on training the female to urinate on cue after the breeding season in 2017. This would enable the team to obtain fresh and clean samples of urine directly from the female, allowing for an efficient method of accurately monitoring her oestrogen levels at a consistent rate. Samples can be obtained approximately every 4 hours (if the female is responsive) and more data can be collected in order to generate a clearer oestrogen hormone profile.

The female has been trained for multiple husbandry and medical procedures using positive reinforcement training methods. The clicker was used as a bridge to reinforce the desired behaviours when specific cues were presented. The primary reinforcer used on the female were diced apples and carrots or high fibre biscuits that were part of her daily diet.

Using operant conditioning with the same positive reinforcers, the desired behaviour (urinating) was coupled with a verbal cue as well as the bridge to encourage a repeat of the behaviour. It

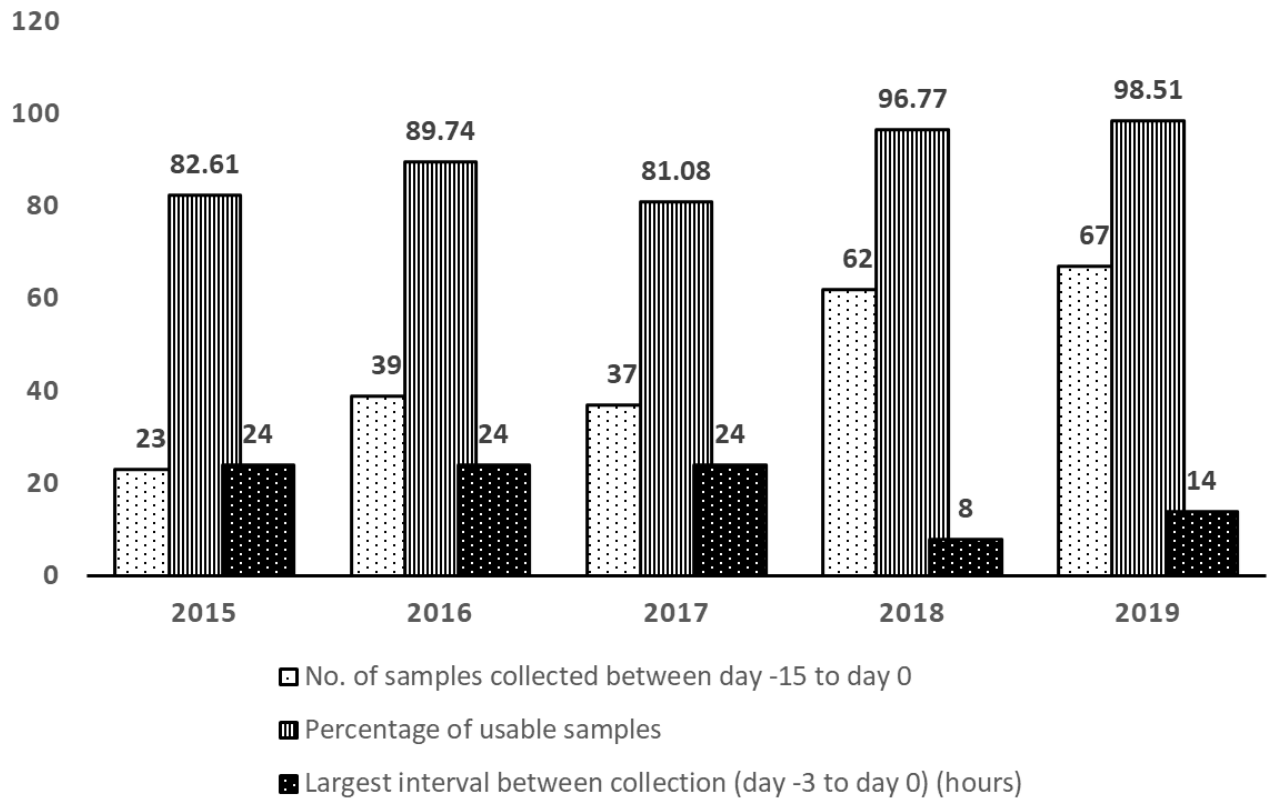


Figure 2. Usable urine samples collected during day -15 to day 0 of each oestrus period from 2015 to 2019.

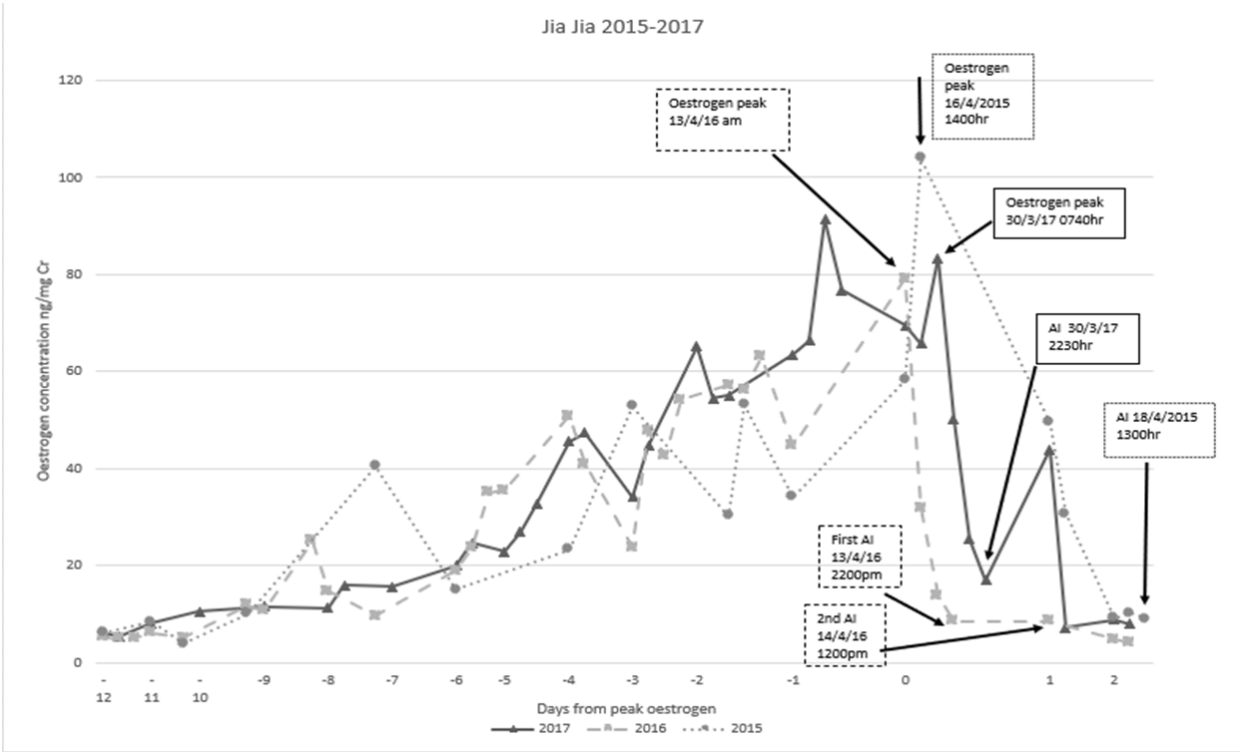


Figure 3. Oestrogen profile of female giant panda from day -12 to day +2 of each oestrus period from 2015 to 2017.

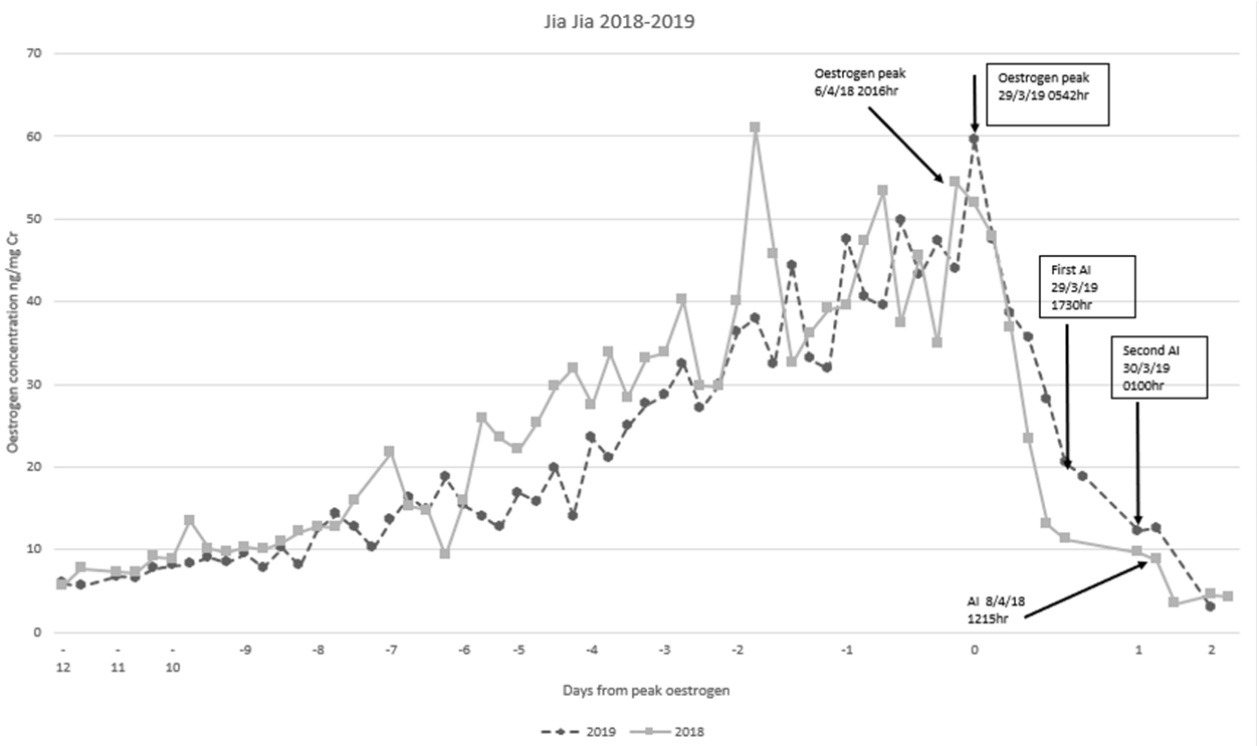


Figure 4. Oestrogen profile of female giant panda from day -12 to day +2 of each oestrus period from 2018 to 2019.

was observed that the female would urinate on the weighing scale in the den after resting in between feeds. Therefore, training was timed to coincide with the female's wake time.

Training began by providing a specific verbal cue just before the female urinated on the weighing scale. As the desired behaviour (urinating) was being performed, the bridge was delivered intermittently to encourage the good behaviour performed, and primary reinforcers were presented to her after the urine samples had been collected. Progressively, the female learned to recognise the cue and associated it with the desired behaviour intended. The

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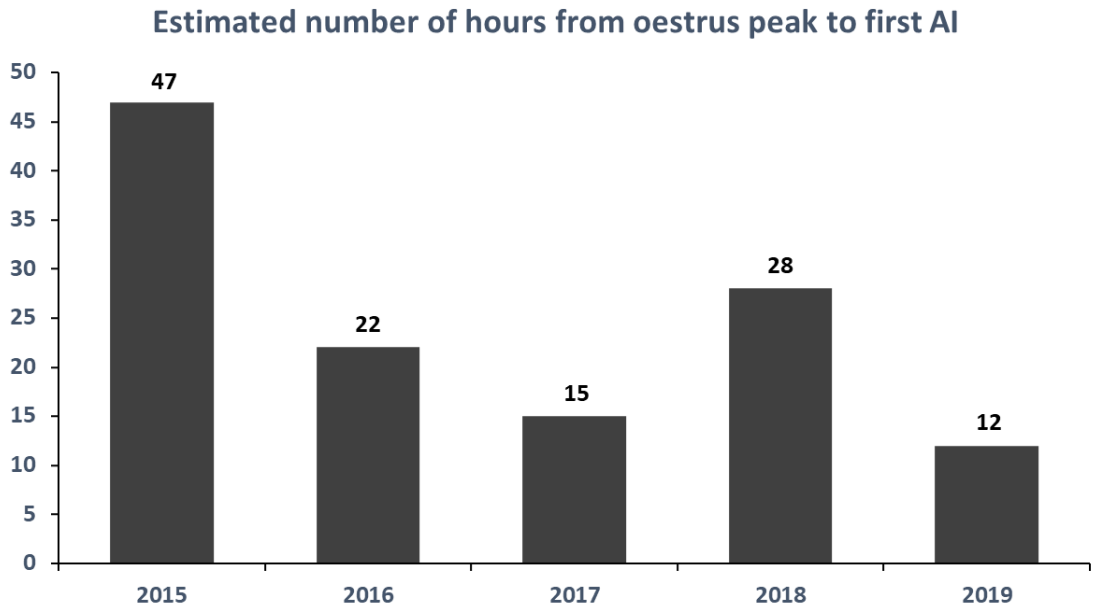

Figure 5. Timing between oestrus peak and first AI.

female is well motivated by the reinforcers to continue to perform the urinating behaviour each time it is required. Subsequently, a container to collect the urine was introduced so that the fresh urine can be collected simultaneously as she was urinating (Figure 1).

This process took five months before she started urinating on cue occasionally (there was a two-month break in between as she was non-responsive during the pseudopregnancy phase of August to September), and nine months for her to do so on a regular basis multiple times a day. Since then, training has been done at least once a day, and depending on the breeding or parturition season, she can be cued to urinate as frequent as every 4 hours, if responsive.

The verbal cue for urine collection evolved as she started anticipating or getting the verbal cue mixed up with a previously trained behaviour, 'cub retrieval'. Prior to the cues, the same sliding mesh at her den was opened for keepers to either place the urine collection container in the den, or to get her to retrieve the 'toy' that she 'adopts' during the pseudo-pregnancy period which she would bring to keepers during 'cub retrieval'. A combination of a verbal cue and hand signals were carried out to help her differentiate the two required behaviours. The container used for urine collection also evolved as speed and accurate placement of the urine container was required to collect the urine stream promptly, while avoiding any faeces she might simultaneously release (Figure 1).

Consequences

As the male was consistently unable to position for copulation or did not show strong interest in the female during peak oestrous over the years, artificial insemination was key in trying to breed the pair during the crucial window of the first 24-hour post-oestrous peak.

Before the urine collection conditioning (breeding seasons from 2015–2017), generating accurate oestrogen hormone profiles for the female panda was difficult (Figure 3). This was because there were fewer samples, and samples were either contaminated or too diluted as they were mixed with water from the drinking water troughs that the female would play with during the oestrous period (Figure 2).

From 2018 onwards, the female was able to urinate on cue consistently and urine can be collected every 4 hours during the day. During the last week of the breeding season leading up to peak oestrous, keepers stayed overnight and cued her to urinate whenever she was responsive. This resulted in a greater number of accurate samples (Figure 2), which in turn gave us a clearer oestrogen hormone profile for both 2018 and 2019 (Figure 4).

As keepers and the endocrine team were able to identify the peak oestrus within a shorter time frame, the veterinary team was able to sedate the male for electroejaculation to collect a fresh semen sample and use that same sample for artificial insemination immediately to ensure the highest chance of successful insemination and fertilisation.

The estimated time from oestrus peak to first AI showed significant improvement in 2019 due to the more accurate hormone profile (Figure 5) and a second AI was also done within 19 hours of the oestrogen peak (Figure 4). However, even with a more accurate hormone profile in 2018, due to an unforeseen delay in the process of gathering the AI consultant team, the AI was setback that year. Evidence of the benefits of this training method can therefore be observed in 2019. This resulted in a prompt AI, in spite of a slight delay due to inaccurate hormone results on day 0 and issues with the male's sedation for electroejaculation.

In summary, teaching the female panda to urinate on cue was a viable method, however, the execution of such training was difficult with the use of cues needing to be discriminative between urination and other behaviour. Although, a lengthy and

iterative process, the training regime was successful and gives other breeding centres options for efficient and uncompromised collection of urine, as well as the opportunity to teach the panda more behaviours to aid in husbandry regimes. With the success of urine collection conditioning, a clearer oestrogen hormone profile for the accurate determination of the oestrus cycle of the female giant panda can be obtained. With this knowledge, chances for successful AI can be increased, and will hopefully lead to the eventual birth of a cub.

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