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Relationship of the Estrogen Surge and Multiple Mates to Cub Paternity in the Giant Panda (*Ailuropoda melanoleuca*): Implications for Optimal Timing of Copulation or Artificial Insemination

Short Title: Giant Panda Mating and Paternity • Huang et al.

Summary Sentence: Successful siring by giant panda males corresponds to the estrogen surge in females; males who mate earlier hold a competitive advantage, but dual paternity also occurs.

Keywords: giant panda, breeding, estrous cycle, assisted reproductive technology, paternity

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ABSTRACT

The effectiveness of *ex situ* breeding programs for endangered species can be limited by challenges in mimicking mating competitions that naturally occur among multiple mates in the wild. The objective of this study was to evaluate the impact of timed natural matings and/or artificial inseminations (AI) in the context of the urinary estrogen surge on cub production in the giant panda (*Ailuropoda melanoleuca*). We used a large cohort of giant pandas, including 12 females and 17 males. DNA paternity exclusion was used to pinpoint accurately the interval during the estrogen surge that coincided with the ideal sperm deposition time to produce offspring. Of the 31 cubs (in 19 pregnancies), 22 (71.0%; 15 pregnancies) were produced from matings occurring on the day of, or the day after, the maximal urinary estrogen peak. Sixteen of the 19 pregnancies (84.2%) produced at least one offspring sired by the first male mating with the dam. There was a preponderance of twins (12 of 19; 63.2%), and dual paternities were discovered in three of 12 twin sets (25%). These findings indicate a strong relationship between the excreted estrogen surge and sperm deposition to achieve pregnancy in the giant panda. To ensure the production of the most genetically diverse young, it is imperative that the most appropriate male mate first and on the day of, or the day after, the highest detected estrogen

value. There is no advantage to increasing the number of copulations or mating partners within 1 day of the estrogen peak on the incidence of twinning, although this practice may increase the prevalence of dual paternity in cases of multiple births.

INTRODUCTION

Ex situ breeding programs play an important role in the study and recovery of endangered species [1]. One of the many challenges to such programs is establishing a mating protocol that simulates, as closely as possible, what normally occurs in the wild. This is especially important for species where the reproductive norm in nature is for there to be multiple male partners who are highly competitive, particularly for monestrual females that have short periods of sexual receptivity. When these animals are to be propagated in *ex situ* breeding and recovery programs, the situation becomes even more complicated due to the need to mate individuals who are unrelated, thereby ensuring the maximal retention of genetic diversity to retain species integrity and reproductive fitness [2].

The giant panda (*Ailuropoda melanoleuca*) fits this model perfectly. A native to southwestern China, there may be as few as 1,600 giant pandas remaining in the wild, living in fragmented pockets and being vulnerable to continued human threats, especially habitat loss [3]. To provide a hedge for wild counterparts and as a resource for eventual reintroduction, captive breeding programs began for the giant panda in the 1960s, but struggled for 3 decades due to a lack of fundamental knowledge about basic species biology. A more systematic approach to understanding the factors limiting breeding success was initiated in the late 1990s via collaborative and intensive biomedical assessments and proactive research [4]. By applying such newly found information along with a continued aggressive research program in China, the result has been almost a three-fold increase in giant panda numbers *ex situ*, now numbering more than 330 individuals worldwide and spanning eight generations [5].

Because the female giant panda is in estrus for only 24 to 72 h annually, intensive breeding management is the norm and generally combines both natural mating and artificial insemination (AI) using fresh and/or frozen-thawed spermatozoa [6]. Due to the short sexual receptivity window, AI has been especially useful in ensuring that every female is 'exposed' to sperm and, in the case of often-frequent sexual incompatibility [7], can still be inseminated with the most genetically-appropriate mate. Although genetic diversity remains high in this population [2], still ~29% of the total captive population is descended from only four individuals [5].

Improvements in breeding success have resulted from implementing a broad array of strategies, from improved nutrition to health care. However, the most useful have been the skilled combination of monitoring urinary hormone profiles in the context of overt and subtle reproductive behaviors as well as allowing females access to multiple mating partners [7]. Additionally, AI generally is used near the later hours of estrus as 'sperm deposit insurance'. The result often is several matings plus one or more AIs with no understanding of which breeding produced the resulting young. Such information is important to improve the efficiency of the time-breedings and to reduce, or even eliminate, the number of animal 'manipulations'. For example, if the ideal interval to deposit sperm by mating or AI was known, then it would be possible to conserve the sperm that normally are wasted during inopportune inseminations and,

more importantly, reduce manipulating (including anesthetizing) males for sperm collection and females for AI.

Our general aim was to closely pinpoint the ideal time for natural or artificial insemination in the context of the urinary estrogen peak. The hypothesis was that siring success was linked tightly to the timing of the estrogen surge. This testing could be accomplished because of our extraordinary access to a unique dataset representing 19 giant panda pregnancies, each with a known number of natural mating and AI events involving different males. We used DNA paternity testing of the resulting offspring to explore issues related to the ideal timing of mating and the success of this inter-male 'competition'.

MATERIALS AND METHODS

Animals and general strategy

This study occurred at the China Center for Research and Conservation of the Giant Panda (CCRCGP) located in the Wolong National Nature Reserve, the natural habitat for this species. Data were collected from 2000 through 2008 and included 12 giant panda females who produced 19 pregnancies and a total of 31 offspring (7 singletons; 12 sets of twins). These females were of prime breeding age, ranging from 4.5 to 16.5 yr at the time of mating and were housed in an *ex situ* environment that ranged from 15 m^2 indoor enclosures connected to 45 m^2 outdoor areas to semi-natural 1,000 m^2 enclosures. All males were housed similarly, and husbandry for both sexes met both the Chinese Standards and the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction, with specific care protocols, including diets, described elsewhere [7, 8].

The pregnancies and offspring evaluated in this study were derived from a combination of natural mating and AI events occurring on successive days and involving different males. Additionally, there also were data on excreted (urinary) estrogen profiles during the peri-estrual interval for each dam (see below).

Natural mating and AI

Mating and AI occurred from February through May, the natural breeding season for the species [7]. Each female experienced from two to four different male partners in a given year (total males used, n = 17, ages 5.5 – 16.5 y). Each of these breeding males also had mated with multiple females in a given year, including those not included in this study. Additionally, males mated with from zero to two other females during the 7 day interval prior to the copulatory events analyzed in this study. A separate analysis revealed that there was no effect ($P > 0.05$) of these earlier matings on success of the copulations evaluated in our study females. It is the general practice at the CCRCGP to allow natural mating to occur first and then 'supplement' with subsequent AIs to ensure coverage of the unknown ovulation interval. However, in three cases in this study, AI occurred prior to natural mating, largely because these females appeared to be exhibiting strong signs of behavioral estrus, but resisted copulation.

Overt signs of female sexual receptivity have been well described for this species, including at our facility [7] and involve increased vocalization, scent marking and lordosis postures. At the peak of these behaviors (and based on estrogen profiles, see below), each female was allowed to naturally mate with as many males as was feasible during the short 2 to 3 day estrus. On a given day, a female could be exposed to different males one at a time in her

breeding area and then was constantly monitored during the interaction. Generally, females only mated with one male each day, although in five cases a female mated with two males on the same day.

The selection of breeding males was made on the basis of (a) recommendations presented annually by the Conservation Breeding Specialist Group of the International Union for Conservation of Nature that indicated (based on computer modeling) male-female combinations that would retain highest levels of genetic diversity, (b) previous years' abilities to successfully copulate with a female and (c) affinity and receptive behaviors shown by the target female while maintained in an adjacent enclosure. Males that were genetically valuable, but consistently aggressive with conspecifics or ineffectual at natural mating were relegated to being sperm donors for AI.

Urinary estrogen analysis

A peak in urinary estrogen concentration is an established cue for confirming maximal sexual receptivity behavior and presumed follicular activity in the female of this species [9, 10]. We measured urinary estrogen levels according to the protocol of Czekala et al. [9]. In brief, beginning at onset of first signs of annual sexual behavior, 1 to 2 ml of urine was collected from the indoor enclosure floor once daily (09:00 – 11:00 hr) per female and then stored frozen (-20ºC) in individual, labeled, plastic vials. All samples were subsequently evaluated for estrone-3-glucuronide content using a, sold-phase and validated enzyme immunoassay [11]. E1G antiserum (100 µl 1:5,000, from C. Munro, University of California-Davis) was combined with E1G HRP (Munro) and E1G standard (3.1 – 400 pg), and tetramethylbenzidine (TMB, Sigma, St. Louis, MO) was the substrate for the colorimetric endpoint change. To control for between-sample differences in urine dilution, we indexed concentrations by creatinine (Cr), as measured via a colorimetric assay [12]. The intra-assay and inter-assay variations were 10.1% and 11.6%, respectively. The urinary estrogen peak was determined by visual inspection of patterns (E1G versus time).

Semen collection, assessment, storage and AI

Semen was collected via electroejaculation according to previously detailed methods for this species [13, 14]. In brief, animals were anesthetized using an intramuscular injection of ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA, USA; 6 – 8 mg/kg of body weight) followed by maintenance on isoflurane gas (0.3 – 0.5 kg/mg of body weight), as necessary. To stimulate ejaculation, we used a rectal probe (2.6 cm diameter) with three electrodes and a 60-Hz sine-wave stimulator (P. T. Electronics, Boring, OR, USA). Generally 20 to 30 stimulations at a low voltage (2 – 8 V) resulted in semen production that was assessed immediately for subjective estimates of sperm motility (at 40x) and the type of forward cellular progression (on a 0 – 5 scale, with a rating of 5 being rapid, forward cell movement [13, 14]). Sperm concentration per ml and total ejaculate were calculated using a standardized hemocytometer procedure. Only fresh ejaculates with a > 80% sperm motility and 3 forward status rating and a minimum of 100×10^6 spermatozoa/ml were used for AI.

As necessary, spermatozoa were cryopreserved according to methods detailed in Howard et al. [6] and Huang et al. [14]. In brief, raw semen was first diluted with TEST egg yolk buffer (Irvine Scientific, Santa Ana, CA, USA) and glycerol (to a final concentration of 5% glycerol). After cooling at 4°C in a refrigerator, samples were pipetted into 0.25 ml plastic straws and then subjected to a cooling rate of -40ºC per min (while held 7.5 cm above a bath of liquid nitrogen

[LN] for 1 min) and then -100ºC min (while held 2.5 cm above LN for 1 min). After plunging into the LN, straws were stored immersed and long-term in a LN dewar. For thawing, straws were placed in a 37º C water bath for 30 sec and then the thawed liquid transferred to 5 ml plastic tubes after diluting with equal volume of Ham's F10 medium (Irvine Scientific, Santa Ana, CA), with 5% fetal calf serum (Irvine Scientific) and 25 mM HEPES (Irvine Scientific). Generally, thawed spermatozoa were maintained in the vial in the water bath for ~30 min before AI. Usually three straws of semen were used for each AI procedure (AI dose volume = 1.4 ml; range in sperm number per insemination, 80 – 120 x 10^6 cells/ml).

AI was conducted as described previously [6, 14]. Briefly, each female was anesthetized as explained for males above. A stainless steel catheter (outer diameter, 25.5 mm; length 125 mm) was inserted into the external cervical os (~18 cm from the vulva). The semen injector located inside the catheter was a 300 mm long stainless steel pipe (inner and outer diameters, 1 and 3.5 mm, respectively). A syringe was used to transfer the fresh or thawed semen from the water bath to the catheter. The semen was deposited into the uterus with the female in a supine position, and her hindquarters were sustained at an ~45ºangle for 5 min to prevent backflow from the vaginal vault.

Paternity testing

Paternity of offspring was determined by amplification of microsatellite DNA recovered from blood samples and buccal swabs. Cubs produced from this study were restrained at 6 to 18 mo of age for collecting a 5 to 6 ml blood sample from the front leg vein using a syringe containing ethylenediaminetetraacetic acid. At the same time, a buccal sample was collected from each animal using a cotton-tipped swab rolled on the inner mouth cheek area. The raw blood and buccal swab samples were stored at -80°C in individual labeled and sealed vials.

Subsequent DNA extraction, amplification and paternity assessments were conducted as previously described in collaboration with others [15] and by our own laboratories [16]. Genomic DNA was extracted from samples via standard phenol/chloroform methods [17] and then PCR amplifications performed using 16 loci [15, 18]. Amplification products were loaded on 6.5% denaturing polyacrylamide gels and analyzed on an automated DNA sequencer (Li-Cor 4200, size standard= 50-350 bp). SAGA^{GT} version 3.2 software (LI-COR) was used to analyze gel images, and paternity exclusion (PE) testing was performed in a user-designed Microsoft Excel program.

Data analysis

We calculated the time between the urinary estrogen peak in the female and the time of first mating. This interval was expressed in days rather than hours because, due to management constraints, it was logistically possible to only secure one urine sample per day per female for estrogen evaluation. As a result, an interval indicated as '0 days' reflected that the mating (or AI) occurred < 24 hr from the estrogen peak. Likewise, a '1 day' and '2 day' interval meant that the sperm deposition and maximum estrogen value had occurred within 48 hr and 72 hr, respectively, of each other. Using paternity analysis results, we identified the sire of each cub, and then we assumed that the day of mating for that male coincided with day of conception. Gestation duration then was calculated on the basis of the number of days from the date of conception to the date of parturition. All data were summarized and presented on the basis of offspring produced in the context of (a) the explicit sire identified by paternity assessment and (b) timing of breeding on the basis of peak urinary estrogen expression and sperm deposition via natural

mating or AI using different males. We also summarized number of twinning events and specifically the prevalence of multiple paternity within a set of twins. Mean values are ± standard deviation of the mean.

RESULTS

Every first copulation for all 19 pregnancies occurred within ± 2 days of the maximally-detected urinary estrogen peak. Fifteen of 19 first matings (78.9%) occurred on the day of, or the day after, the maximal estrogen value (Table 1). On two occasions (10.5%), mating did not occur until 2 days after the estrogen peak. In one case each, the initial copulation occurred 1 or 2 days before the detected urinary estrogen peak.

In 16 of the 19 pregnancies (84.2%), the paternity of one or both cubs was attributable to the male who mated or was artificially inseminated first with the female (Table 1). In the remaining three cases (female 476 [2006], 432 [2005] and 474 [2007]), the sire was the male naturally mating either 1 or 2 days after the first sperm deposition event by copulation or AI. Dual paternity was detected in three of the 12 sets of twins (25.0%), with differing sires for each cub (female 476 [2006], 477 [2003] and 474 [2006]) (Table 1). One set of dual paternity twins (from female 477 [2003]) was derived from a combination of one natural mating following one AI using fresh spermatozoa. The three dual paternity cases occurred when the first successful mating was 2 days before (female 474 [2006]), 1 day before (female 477 [2003]) and 1 day after (female 476 [2006]) the detected estrogen peak. Having a higher number of matings or mating partners within ± 1 day of the estrogen peak did not increase the chance of twinning, since the majority of twins (67%) resulted after one or zero matings occurring during this period. However, having more mating events and partners within 1 day of the estrogen peak did appear to influence the chance of experiencing a double paternity in a twinning event. The three 'split' paternities within a cub pair resulted from the only three cases where there were more than two mating events occurring within the 1 day estrogen peak interval (with two, three and four different male partners, respectively). In all three cases, the offspring were the same sex (female). Of the nine other sets of twins, eight were comprised of opposite sex cubs with only one as same sex (male).

Of the 31 offspring, 29 (93.5%) were produced from natural mating. The remaining two were confirmed from AI, one with fresh and the other with frozen-thawed spermatozoa. The higher success of natural mating over AI may have been largely due to the common strategy of using natural mating first and AI as 'insurance' for later during estrus. In fact, in 15 of the 19 pregnancies (78.9%) natural mating was used first before AI. Therefore, on all but one occasion, natural mating sperm deposition occurred closer to the female's urinary estrogen peak than when AI was used. The two AI successes were among the four exceptions when the 'artificial' sperm deposit occurred first. Among the 23 AI failures, 68% occurred 2 or more days after and 88% occurred 1 or more days after the maximal estrogen value. For the total of 19 pregnancies, gestation length ranged from 101 to 168 days (typical of the species; [19]) and appeared unrelated to the timing of estrogen peak or probability of twinning. The magnitude of the estrogen peak ranged from 67 to 198 ng/mg Cr (Table 1) and appeared unrelated to both the probability of twinning and the timing of successful matings.

The overall sex ratio for the 31 cubs was 16 males and 15 females. Of the seven singleton births, six of seven cubs were males, whereas in the 12 sets of twins, there was one pair of male offspring, three pairs of females and eight pairs of mixed gender.

DISCUSSION

This is the first study to inter-relate the urinary estrogen rise and fall (indicative of impending ovulation) in the giant panda with timing and number of sperm depositions and then paternity of resulting cubs. This type of investigation is important because this species oddly produces only a single and short estrus and estrogen surge once per year, thereby devoting less than 1% of its annual lifespan to reproduction. Our findings were bolstered by having special access to 19 pregnancies from 12 females exposed (by mating or AI) to 17 adult males and the resulting 31 cubs. Empirical information on this topic has been limited to-date because generally panda females have been mated randomly with one or more males, thereby making it impossible to associate the detected estrogen peak with a mating event from a specific male [9, 14]. The present study was unique in that different males were rotated with a given female so that the effectiveness of a particular mating could be traced to the timing of the estrogen surge as well as sire through DNA analysis of the resulting cubs. It became clear that the urinary estrogen surge was indeed an excellent marker of impending ovulation, with most pregnancies (and cubs) produced from matings (or AIs) occurring within 1 day before or after the peak in this hormone. Only rarely (16% of the time) did pregnancies result from matings that occurred earlier or later than the 48 hr period before or after the estrogen peak. Secondly, > 70% of the pregnancies resulted in twins, with three instances of dual paternity (different sires). Although most were derived from the first mating, a few cubs were produced from mating as late as 3 days after the detected estrogen peak.

It has been long-established that giant panda females experience a rapid increase and then decline in urinary estrogen concentration during the peri-estrual interval [9, 10]. More specifically, estrogen metabolites rise gradually from baseline to peak excretion over 1 to 2 weeks (depending on female) and then decline precipitously at the presumptive time of ovulation [20]. More recently a similar pattern had been described in panda feces, with the estrogen rise occurring over 5 days and then declining to nadir over the next 4 days, the overall profile being 2 to 9 days shorter in feces than urine [21]. Regardless, it is apparent that secreted estrogen is metabolized rapidly because both urinary and fecal estrogen concentrations decrease within a few days. Most progestagen then is detected in urine and feces as a protracted primary (61 – 122 days) and then 20-fold higher secondary (28 – 63 day) phase [20, 22]. The trigger for the transition to the secondary phase is unknown, but is believed to be related to the timing of the delayed implantation of the embryo after a free-floating period in the uterus [19]. Nonetheless, the diversity in length of the primary phase pre-implantation no doubt is responsible for the wide variation in gestation length for this species.

In studies to-date, generally fewer than half of monitored females present a clear luteal rise in progestagen during the first 20 days after the estrogen peak, thereby providing no useful information on the actual timing of ovulation. Therefore, in the absence of an unambiguous marker of ovulation, our study was relevant to confirming the importance of the easy-to-identify estrogen surge as a useful indicator for timing matings or AI. As observed in earlier investigations [20], the short time interval associated with near-maximal estrogen excretion was

associated with the distinctive behavioral signs of estrus, including the expression of lordosis and distinctive vocalizations [23]. While clearly most conceptions occurred when mating (or AI) occurred on the day of, or within 1 day after, the estrogen peak, our findings also revealed the potential of achieving pregnancy with a sperm deposition 1 day pre-peak. Thus, an 'early' copulation when the female was sexually receptive in advance of maximal estrogen excretion could be productive. Likewise, our data also indicated the potential of achieving conception when the first sperm deposit was delayed to as much as 2 days post-peak. Despite the substantial size of our research population, we still had two few samples to determine if these later matings were less efficient. Based on our collective information, it was prudent to recommend that sperm deposition be focused on the interval encompassing the day of, and the day after, the maximally detected urinary estrogen concentration.

There was a strong, unequivocal advantage to being the first male with the opportunity to mate. Nearly 75% of the offspring produced were derived from first copulating males. There is absolutely no information available in the giant panda (or other bears) for the duration of longevity of spermatozoa *in vivo* or for the significance of sperm competition. Most adult male pandas at the peak of the breeding season are well known to produce prodigious numbers of spermatozoa [6], and the quality of these ejaculates in terms of concentration, sperm motility and morphology are relatively similar [24]. Therefore, the benefit of being the first male probably was most related to timing and ensuring sperm presence in the oviduct in the peri-ovulatory period. Our observations also were interesting in light of what little is known about breeding behavior in wild giant pandas. In nature, females approaching estrus attract as many as five males that travel long distances and then assemble in the female's territory to sometimes violently compete for copulatory opportunities [25]. The winner mates first, but the female also will breed with secondary and even tertiary males [25]. Clearly, our findings with this *ex situ* panda collection have discovered a physiological advantage to this reproductive strategy. Males in our controlled, captive environment who mated first produced the most cubs. This explains the need for wild males to be highly competitive, assertive and combative, thereby gaining first access to a female during that brief estrual window, translating into a vastly improved chance of passing along genes to the next generation.

More than half the pregnancies monitored in our study (63%) resulted in twins, a phenomenon well established for the giant panda, including in the wild where one of the pair is commonly abandoned by dam [26]. The speculated cause for this desertion is that these cubs are extraordinarily altricial, requiring intensive care by a panda dam who (unlike in other ursid species; [27]) appears unable to manage dual cubs. Interestingly, the recent exponential increase in size of the current *ex situ* panda population [28, 29] has been due to a combination of improved reproductive knowledge, health care and husbandry as well as developing sustainability methods for twins. Especially important has been the strategy of alternating (or 'swapping') cubs daily between the dam and a human care/incubator system with a minimal need for artificial (bottle) feeding [30, 31]. Thus, a high twinning capacity plus a dual cub survival rate of >90% has played a major role in the contemporary species recovery program. We determined in our study that, of the 12 sets of twins, 25% had a cub pair with different sires. This incidence was higher than the findings from David et al. [18] who examined 13 sets of twins and found only one with split fatherhood. Multiple paternities have been documented in the black bear [32] and brown bear [33], occurring at a rate of ~18 and 29%, respectively, which is similar to what we observed in the giant panda. As all the dual paternities occurred coincident with females copulating with multiple males during the high peri-estrogen interval, it may be that

more matings at this time increased the prevalence of cubs with differing sires. However, our sample size was too small to make a conclusive argument and, by contrast, a female having more than a single partner did not ensure multiple paternities. For example, there were six pregnancy cases when there were multiple breeding partners within 1 day of the urinary estrogen peak, and the three singletons and three sets of twins were sired by only one of the males. In the context of the wild population, our findings suggested that it could be possible for males that secure a secondary breeding opportunity in nature to sire a cub, but the chance probably is low unless the neonate resulting from the initial breeder is abandoned. Regardless, confirming the existence of dual paternity of giant panda twins explains how the female can contribute to enhancing genetic diversity by allowing copulation by less assertively successful, secondary males.

Although the findings from this study did not support a high efficacy for AI in the giant panda, we believe the advantage shown by natural mating was due to timing of the sperm depositions, with copulations generally occurring during the day of, or day after, peak urinary estrogen excretion. By contrast, 17 of the 25 AIs conducted (68%) were performed 2 or more days away from the maximal estrogen value. Of the two AI successes, both were conducted before natural mating and 0 and 1 day, respectively, from the estrogen peak. Furthermore, the overall effectiveness of transcervical AI (without concomitant mating) using fresh, cooled (4ºC) or frozen-thawed spermatozoa has been reviewed by Howard et al. [6] who reported an average success of 57%, which agrees with more recent estimates by Huang et al. [14]. Regardless, findings in the present study have implications for implementing AI as an assisted breeding tool in the genetic management of giant pandas [2]. Although the goal is to ensure that every genetically valuable female reproduces, it also is essential to minimize animal manipulations (especially inducing anesthesia for AI) and expensive procedures. Our study here has determined that sperm deposition for high conception in the giant panda (whether by AI or natural copulation) must be tightly linked to identifying the estrogen peak, which is easily discernible via non-invasive, urinary monitoring. Further, it is essential that the highest priority male preferentially have the first copulatory (or AI option), since it is this initial sperm deposition that most frequently results in offspring. Lastly, these findings would advise against using valuable frozen-thawed sperm for an ‘insurance’ deposition late in estrus (specifically 48 hr beyond the estrogen peak) as this practice fails to result in cubs.

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Table 1. Summary of natural mating (NM) and artificial insemination (AI with fresh or thawed spermatozoa) events for 12 giant panda females (19 pregnancies) at the China Center for Research and Conservation of the Giant Panda, Wolong National Nature Reserve, China.

Female studbook no.	Age (yr)	Breeding type	Date	Male studbook no.[a]	Days from urinary estrogen peak to first breeding event[b]	Peak urinary estrogen (ng/mg Cr)	Gestation length (days)[c]	Date of giving birth	Number of cubs (sex)
446	12.5	NM	May 13, 2000	**329**	1	134	136	Sept 26, 2000	1 (♂)
		AI [fresh]	May 14, 2000	357					
		AI [fresh]	May 15, 2000	357					
	13.5	NM	May 13, 2001	**308**	0	67	138	Sept 28, 2001	2 (♀, ♂)
		AI [fresh]	May 14, 2001	357					
		AI [thawed]	May 15, 2001	357					
397	16.5	NM	Apr 1, 2000	**394**	1	94	168	Sept 16, 2000	2 (♂, ♂)
		NM	Apr 2, 2000	308, 329					
		AI [fresh]	Apr 3, 2000	305					
374	12.5	NM	Feb 23, 2002	**394**	2	79	144	July 17, 2002	1 (♀)
		NM	Feb 24, 2002	308					
		AI [thawed]	Feb 25, 2002	415					
414	12.5	NM	Apr 7, 2003	**308**, 399	2	170	127	Aug 14, 2003	2 (♀, ♂)
		NM	Apr 8, 2003	399					
		AI [thawed]	Apr 9, 2003	394					
476	5.5	AI [thawed]	Mar 28, 2004	**327**	0	69	107	July 13, 2004	1 (♂)
		NM	Mar 29, 2004	455					
		NM	Mar 30, 2004	455					
	7.5	NM	Apr 22, 2006	369	0[d]	94	110	Aug 10, 2006	2 (♀,♀)
		NM	Apr 23, 2006	369, **503**					
		NM	Apr 24, 2006	**502**					
		AI [thawed]	Apr 25, 2006	357					

Female studbook no.	Age (yr)	Breeding type	Date	Male studbook no.[a]	Days from urinary estrogen peak to first breeding event[b]	Peak urinary estrogen (ng/mg Cr)	Gestation length (days)[c]	Date of giving birth	Number of cubs (sex)
544	10.5	NM	Apr 29, 2005	**424**	1	126	101	Aug 8, 2005	1 (♂)
		NM	Apr 30, 2005	399					
		AI [thawed]	May 1, 2005	413					
432	8.5	AI [fresh]	Mar 10, 2005	357	0[d]	198	149	Aug 6, 2005	1 (♂)
		AI [fresh]	Mar 11, 2005	357					
		NM	Mar 12, 2005	**455**					
		AI [fresh]	Mar 13, 2005	357					
	11.5	NM	Mar 26, 2008	**503**	0	170	135	Aug 8, 2008	1 (♂)
		NM	Mar 27, 2008	502					
		AI [thawed]	Mar 29, 2008	502					
516	4.5	NM	Apr 4, 2005	**369**	0	126	129	Aug 11, 2005	1 (♂)
		NM	Apr 5, 2005	503					
		NM	Apr 6, 2005	503					
		AI [thawed]	Apr 7, 2005	327					
	5.5	NM	Apr 5, 2006	**503**	1	81	140	Aug 23, 2006	2 (♀, ♂)
		AI [fresh]	Apr 6, 2006	467					
	6.5	NM	Apr 21, 2007	**479**	1	145	125	Aug 24, 2007	2 (♀, ♂)
		NM	Apr 22, 2007	503					
		AI [thawed]	Apr 23, 2007	542					
487	7.5	NM	Mar 9, 2007	**502**	1	110	130	July 16, 2007	2 (♀, ♂)
		AI [thawed]	Mar 10, 2007	467					
		NM	Mar 11, 2007	479					

Female studbook no.	**Age (yr)**	**Breeding type**	**Date**	**Male studbook no.**[a]	**Days from urinary estrogen peak to first breeding event**[b]	**Peak urinary estrogen (ng/mg Cr)**	**Gestation length (days)**[c]	**Date of giving birth**	**Number of cubs (sex)**
477	4.5	AI [fresh]	Apr 15, 2003	**357**[e]	-1	151	143	Sep 6, 2003	2 (♀,♀)
		NM	Apr 16, 2003	**424**					
		AI [thawed]	Apr 17, 2003	357					
		AI [thawed]	Apr 18, 2003	357					
	6.5	NM	Apr 11, 2005	**424**	1	143	126	Aug 16, 2005	2 (♀, ♂)
		NM	Apr 12, 2005	369					
		AI [thawed]	Apr 13, 2005	327					
511	5.5	NM	Apr 27, 2006	**424**, 369	1	92	107	Aug 12, 2006	2 (♀, ♂)
		NM	Apr 28, 2006	503					
		NM	Apr 29, 2006	369					
		AI [thawed]	Apr 30, 2006	386					
474	7.5	NM	Apr 16, 2006	**369**[e]	-2	81	146	Sep 11, 2006	2 (♀,♀)
		NM	Apr 18, 2006	**424**, 369					
		NM	Apr 19, 2006	503					
		AI [thawed]	Apr 20, 2006	357					
	8.5	AI [thawed]	May 23, 2007	467	1[d]	85	128	Sep 28, 2007	2 (♀, ♂)
		NM	May 24, 2007	**502**					
Average	8.75					116.6	130.7		
SD[f]	3.4					38.6	16.3		

[a] Male studbook number highlighted in bold corresponded to sire of resulting offspring (determined by paternity testing via DNA microsatellite amplification of blood and buccal swab samples).

[b] Estrogen peak in the female was determined using an enzyme immunoassay for estrone-3-glucuronide concentration performed on daily urine samples.

[c] Gestation length was determined as number of days from first copulation or AI to birth of offspring.

[d] In these three cases, the first breeding event did not produce the resulting offspring. For all other cases, except those identified below in [e], the first breeding event resulted in one or more cubs.

[e] In these two cases, the exact date when the offspring was conceived was unknown, because this male copulated with the female on multiple days.

[f] Standard deviation of the mean.