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Source: Biology of Reproduction, 86(6) 2012.
Published By: Society for the Study of Reproduction
URL: http://www.bioone.org/doi/full/10.1095/biolreprod.112.099044

BIOLOGY OF REPRODUCTION (2012) **86**(6):195, 1–10
Published online before print 4 April 2012.
DOI 10.1095/biolreprod.112.099044

Protracted Reproductive Seasonality in the Male Giant Panda (*Ailuropoda melanoleuca*) Reflected by Patterns in Androgen Profiles, Ejaculate Characteristics, and Selected Behaviors[1]

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ABSTRACT

The female giant panda (*Ailuropoda melanoleuca*) experiences a brief (24–72 h) seasonal estrus, occurring once annually in spring (February–May). Our aim was to determine the existence and temporal profile of reproductive seasonality in the male of this species. The study was facilitated by 3 yr of access to eight giant panda males living in a large breeding center in China. Seasonal periods for the male were defined on the basis of female reproductive activity as prebreeding, breeding (early, peak, late), and nonbreeding seasons. Testes size, fecal androgen excretion, ejaculated sperm density, and frequency of reproductive behaviors (i.e., locomotion, scent marking, vocalizations) increased ($P < 0.05$) from the prebreeding period (October 1–January 31) to the early breeding season (February 1–March 21). Testes volume and sperm concentration were maximal from March 22 through April 15, a period coinciding with maximal female breeding activity. The occurrence of male reproductive behaviors and fecal androgen concentrations began declining during peak breeding and continued from April 16 through May 31 (late breeding period), returning to nadir throughout the nonbreeding interval (June 1–September 30). Reproductive quiescence throughout the latter period was associated with basal testes size/volume and aspermic ejaculates. Our results reveal that testes morphometry, fecal androgen excretion, seminal quality, and certain behaviors integrated together clearly demonstrate reproductive seasonality in the male giant panda. The coordinated increases in testes size, androgen production, sperm density, and sexual behaviors occur over a protracted interval, likely to prepare for and then accommodate a brief, unpredictable female estrus.

Ailuropoda melanoleuca, *giant panda, male sexual function, seasonal reproduction, seasonality, sperm, steroid hormones, testis*

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) is one of the world's most recognized species. A specialist bear that consumes grass (i.e., bamboo [1]), its survival depends on suitable habitat, which now is largely restricted to certain protected and unprotected areas in the Sichuan, Gansu, and Shaanxi provinces of the People's Republic of China [2, 3]. There are an estimated 1600 pandas remaining in nature [4], with the greatest threat being habitat loss and fragmentation [2, 3, 5]. China has placed value on an ex situ collection of this species [6], with the captive population acting as a tangible asset for research, educating the public, and as insurance against a catastrophe affecting giant pandas in the wild (as occurred during recent earthquake events). A self-sustaining ex situ population also serves as a resource for animals to be reintroduced into appropriate wild habitats that now are underutilized by the species [3, 5, 6].

Substantial progress has been made in ex situ giant panda breeding within China. This progress began once limitations to successful reproduction were identified through a cooperative, multidisciplinary biomedical survey conducted by Chinese and U.S. partners [6]. Vast amounts of fundamental data were collected, analyzed, and used to guide husbandry and veterinary health protocols and to enhance the efficiency of assisted reproduction, especially artificial insemination (AI) [7]. As a result, the collective ex situ population of giant pandas has nearly tripled in the last 11 yr from ~120 to >330 individuals [6, 8, 9]. One major outcome has been the availability of more animals for characterizing unique species biology. For example, it is well established that the female giant panda is seasonally reproductive, being sexually receptive from February through May [10, 11]. What is particularly unusual is that there is only one estrus per year, of 24–72 h in duration [10, 11]. Thus, the female of this species devotes less than 1% of its annual lifespan to sexual activity [10]. Most reproductive research has focused on the female, especially interrelating a complicated repertoire of behaviors to temporal fluctuations in gonadal hormones during the periestrual and postovulatory intervals [10, 11] through a pseudopregnancy or parturition [12, 13].

Few studies have examined male reproductive capacity and physiology in similar detail, and none have involved sample sizes larger than one or two individuals. Rather, male studies

[1]Supported by the Morris Animal Foundation, Friends of the National Zoo, University of Maryland Department of Animal and Avian Sciences, and the Chengdu Research Base of Giant Panda Breeding. International travel was supported by United Airlines. Research was conducted in partial fulfillment of Ph.D. requirements for C.A.P. at the University of Maryland.
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Received: 10 January 2012.
First decision: 20 February 2012.
Accepted: 22 March 2012.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

largely have centered on ejaculate characteristics measured during the breeding season and the sensitivity of sperm to cooling/freezing [7, 14]. There is a paucity of physiological information on male reproductive seasonality [7, 14, 15], although there are suggestions that behaviors, testes size, and testosterone appear to change over time and in relation to female breeding season [14–20]. But undertaking a thorough examination of seasonality in the male giant panda has proven challenging because of too few individuals for research managed under the same environmental conditions in one location [14, 19]. Recent advances in giant panda propagation and the availability of the state-of-the-art Chengdu Research Base of Giant Panda Breeding have eliminated this impediment. Improved anesthetic protocols [21] now allow safe, hands-on assessment of body [22] and testes morphometry [14] and semen collection [14]. Emergence of fecal hormone metabolite monitoring for the giant panda also has permitted the noninvasive tracking of gonadal endocrine activity [19, 20].

Our hypothesis was that reproduction in the male giant panda was obligately seasonal. Unlike in the female, where gonadal function onset is abrupt and sexual interest brief, we speculated that reproductive capacity in the male changes gradually over time and in a protracted fashion reflected by variations in testis size, androgen production, semen quality, and overt behaviors. Such a mechanism could be useful in accommodating the short and unpredictable estrus of multiple female conspecifics living in adjacent territories in nature. Besides filling a knowledge gap [6, 14], understanding the regulators of male reproductive function will allow continued enhancement of the ex situ management program to achieve demographic and genetic stability, especially to assist in the near-term reintroductions of this species into remaining viable habitat [3, 5].

MATERIALS AND METHODS

Animals

Eight adult male giant pandas (5–21 yr) were maintained at the Chengdu Research Base of Giant Panda Breeding or at the Chengdu Zoo (a related and nearby institution) in Sichuan Province, People's Republic of China (30°N, 104°E). All were born in captivity, with four being proven breeders as demonstrated by producing living young. Each giant panda was housed individually in an enclosure with combined indoor (3.0 × 3.0 m–10 × 20 m) and outdoor (12 × 12 m–20 × 35 m) areas, all illuminated by natural lighting. Water was provided ad libitum, and freshly cut, seasonally available wild bamboo (10–20 kg daily) was offered to each male throughout the day along with a high-fiber biscuit supplement (~1000 g/day per male; range, 700–1500 g/day per male). Each male was housed within olfactory, auditory, and visual proximity to adult and juvenile conspecific females. A male and female were allowed to physically interact within the same enclosure only during the periestrual interval of the breeding season (February–May) and for brief (2–15-min) periods.

Study experimental design and methods were approved by the Institutional Animal Care and Use Committees of the Smithsonian's National Zoological Park, the University of Maryland, the Chinese Association of Zoological Gardens, and the Chengdu Research Base of Giant Panda Breeding. Transport of fecal samples (collection protocol below) from China to the United States was approved and monitored by the Convention on the International Trade of Endangered Species, the U.S. Department of Agriculture, and the U.S. Fish & Wildlife Service.

Characterization of Seasonality in the Female Giant Panda

To identify potential intervals of male reproductive seasonality, we first examined the fluctuating reproductive patterns of female giant pandas at our research location. Seasonality has been well described for the female of this species, usually using a combination of changes in behavior [17, 18] and steroid metabolite profiles voided in urine [10, 11]. We used these highly reliable approaches to measure the prevalence of female sexual activity, specifically peak estrus, throughout the year. This was accomplished by monitoring behaviors and estrogen profiles in 16 adult female pandas housed at the same locations and over the course of 4 yr (n = 46 monitored cycles). The preponderance of combined peak sexual behaviors and estrogen concentrations (23 of 46 cycles; 50%) occurred from March 22 through April 15 (Fig. 1), which then was designated as ''peak breeding season.'' Some females entered estrus (14 of 46; 30%) from February 1 through March 21, an interval labeled as ''early breeding season'' (Fig. 1). There also were females (9 of 46; 20%) that displayed peak sexual behavior and estrogen from April 16 through May 31, a period deemed ''late breeding season'' (Fig. 1). No giant panda had more than one estrus per year, and none produced behavioral or hormonal indications of reproductive activity from June 1 through January 31, a period designated the ''nonbreeding season.'' Therefore, based on these data and criteria, we objectively identified four distinctive reproductive periods for female giant pandas: 1) nonbreeding, 2) early, 3) peak, and 4) late breeding season (Fig. 1). These findings were used as a baseline for relating changes in reproductive activity in conspecific males (see below).

Testicular and Body Morphometry and Ejaculate Characteristics

Each male was conditioned to walk across an electronic scale (Shanghai Yitai Electromechanical Equipment Co.) to collect body mass data monthly. The anesthetic protocol involved using 10–12 mg/kg ketamine hydrochloride (Sinceta International Trading Co.) delivered by intramuscular injection [21]. Isoflurane gas (Ningbo Samreal Import and Export Co.) was delivered via a face mask or intubation, as necessary, to increase depth and/or duration of anesthesia. While each male was under anesthesia, testicular and body morphometry measurements were collected as previously described [14, 22]. Specifically, the length and width of each testicle were measured using a digital caliper (Model #500-196-20 Absolute Digimatic Calipers; Mitutoyo Corp.), and volume of each testis then was calculated using the formula volume = 4/3 π ab^2 (a is 1/2 length; b is 1/2 width) [14]. Total testicular volume per male was determined by combining the right and left testis volumes. Calipers and a flexible tape measure were used to ascertain chest girth, abdominal girth, contour length of body from tip of nose to base and tip of tail, right foreleg circumference at elbow, and axial skin fold thickness, all as described previously [22].

Electroejaculation also was conducted during anesthesia using a well-described approach [14]. In brief, this involved use of a 2.6- to 3.2-cm-diameter rectal probe with three longitudinal electrodes and a 60-Hz sine-wave stimulator (P.T. Electronic). Standardized sets of low voltage stimulations (2–5 V) over three series of 30 stimuli each were delivered over 20 min to elicit an erection and ejaculation into a temperature-controlled (23°C) container [14]. Seminal volume was measured and pH determined using pH indicator strips (Colorphast; EM Science). A 2–5-μl aliquot of ejaculate was examined for a subjective estimate of sperm motility (0%–100%) and forward progressive motility (scale, 0–5; 5 = fast forward cellular trajectory) under phase-contrast microscopy (200–400×) [14]. Sperm concentration was calculated using a standard hemocytometer method [14]. Total sperm per ejaculate was calculated (sperm concentration/ml × total volume of ejaculate) and recorded.

A 10-μl sample of undiluted semen was placed in 100 μl of fixative solution (0.3% glutaraldehyde in PBS) and sperm morphology (200 sperm per sample) assessed using phase contrast microscopy (1000×) [14]. In cases of multiple malformations for a given spermatozoon, each was classified according to the most serious malformation [14]. Individual defects then were compiled into three general categories of sperm pleiomorphisms (head, midpiece, or flagellar), with deformities related to the head being considered the most serious. Sperm head morphology was further delineated by detailed evaluations of acrosomal integrity using rose bengal/fast green stain [14]. Briefly, an aliquot of raw, unfixed semen was diluted in Ham F10 medium (1:10 semen:medium; Irvine Scientific) and then 1 μl of this mixture added to 9 μl of rose bengal (high-purity biological stain; Cole-Parmer) and fast green stain (certified biological stain; Sigma-Aldrich), incubated for 90 sec, and smeared on a glass slide. A minimum of 100 sperm acrosomes per sample was assessed by bright-field microscopy (1000×) for a 1) normal intact apical ridge (uniform staining of the acrosome over the anterior half of sperm head), 2) damaged apical ridge (nonuniform staining with ruffled or folded acrosome), 3) missing apical ridge (lack of staining due to acrosome absence), or 4) loose acrosomal cap (loose membrane protruding above the level of the sperm head) as previously described [14].

Fecal Androgen Assessment

Enzyme immunoassays (EIAs) have been validated recently for monitoring alterations in fecal concentrations of estrogens and progesterone in female [10, 11] and androgens in male [19] giant pandas. We collected a fresh (<1 h

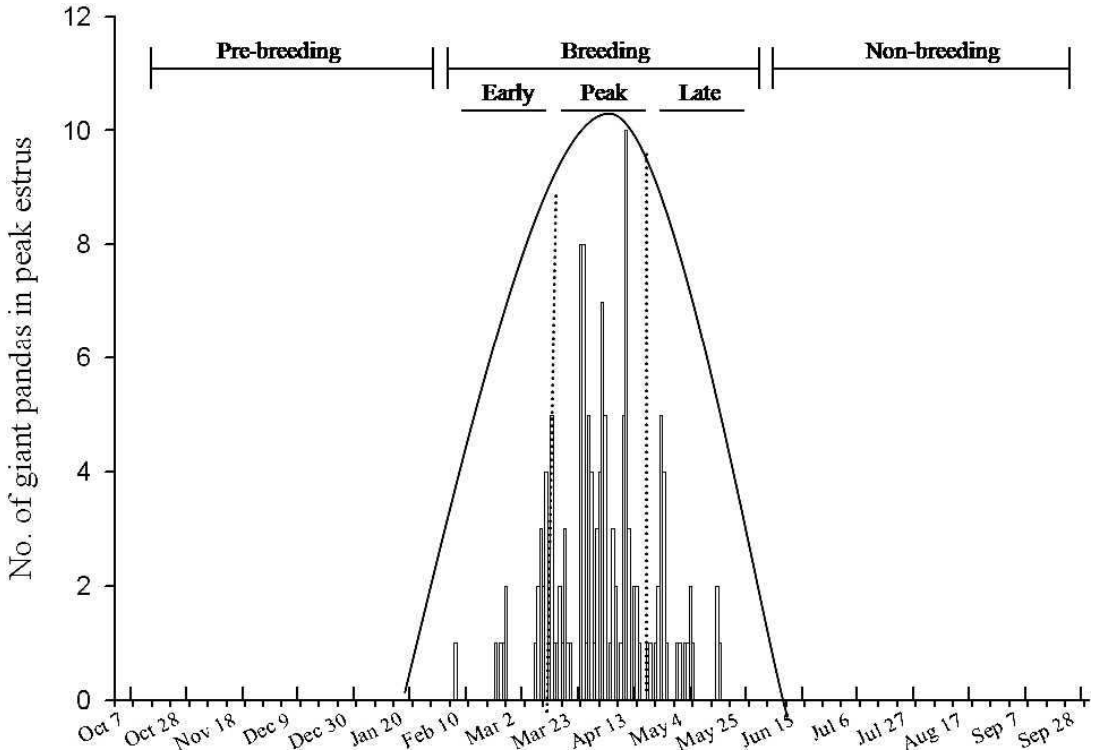

FIG. 1. Distribution of ex situ female giant pandas (n = 16) in peak estrus throughout the year. Peak estrus defined as behavioral signs of estrus and/or maximum urinary estrogen concentration. Bars represent total number of females in peak estrus that were naturally mated and/or artificially inseminated on specific dates (February 1–May 30). Reproductive seasons divided into categories based on number of females in peak estrus from February 1 through March 21 (early), March 22 through April 15 (peak), and April 16 through May 31 (late).

postexcretion) fecal sample from each of our eight studied males every 48–72 h over the course of 3 yr. Each sample was placed in a plastic bag labeled with the animal's number and date of collection, which then was sealed and stored frozen (−20°C) until processing. Samples were batch shipped on dry ice to the Smithsonian Conservation Biology Institute (Front Royal, VA) for processing and hormonal analysis.

Before hormone evaluation, samples were freeze-dried (Lyophilizer; Labconco), crushed, and hormone extracted using previous methods [19]. After 0.1 g of fecal powder was placed in 90% ethanol, extracts were vortexed and centrifuged to remove particulate, sonicated with glass beads, dried under air, and then resuspended in 1 ml of BSA-free phosphate buffer before freeze-storage. The EIA [19] relied on a polyclonal anti-androgen R156/7 antibody (C. Munro, University of California, Davis, CA) added to 96-well microtiter plates (Nunc-Immuno, Maxisorp; Fisher Scientific) that were allowed to equilibrate for 12–18 h (4°C). Unbound antiserum was removed with wash solution, and samples (processed fecal extract, equivalent 0.0005–0.005 ml) in duplicate and standards in triplicate (0.05 ml; 47–12 000 pg/ml; 17β-hydroxy-4-androstein-3-one; Steraloids) were added to the EIA microtiter plate. A peroxidase enzyme-conjugated testosterone (0.05 ml; C. Munro) then was added to each well containing standard or sample and incubated (2 h, 23°C) before unbound components were removed. A chromagen solution was added (0.1 ml) to each well and incubated (~30 min, 23°C) before optical densities were determined using a microplate reader (Dynex MRX, reading filter 405 nm, reference filter 540 nm). Intra-assay and interassay correlations of variation were <10% and 10%, respectively [19].

Baseline concentrations of fecal androgen metabolites were determined through an iterative process [19]. In brief, baseline values were assessed yearly in each male, then for each male over all years of study for all males. Values in excess of two standard deviations of baseline were removed from the dataset until no values exceeded two standard deviations of the baseline mean. This iterative mean was considered the androgen baseline and was expressed as mean ± SEM.

Behavioral Assessment

Male behavioral data were collected based on a modified ethogram for this species [17]. These well-documented behaviors include those associated with scent marking (i.e., handstand mark, leg cock mark, reverse mark, squat mark, handstand urine mark, leg cock urine mark), affiliative interactions, barrier interaction, olfactory behaviors (i.e., scent anoint, open-mouth olfactory, lick olfactory), vocalization, investigate/explore, nonmotile stereotypy, and total motile stereotypy (locomotor stereotypy, pace) [17]. The behavior of each male was evaluated during two consecutive 30-min focal observation periods conducted twice weekly with a balanced number of mornings (0800–1130 h) and afternoons (1300–1700 h) throughout the 3-yr study. The 11 data observers were trained for 2 wk in behavioral identification and recording. Consistency among observers was monitored by comparing responses for the same observation period and found to be ≥80% during the study interval. On the rare occasion that observations were perturbed by an animal-keeper interaction (e.g., unscheduled shifting of an animal to an adjacent enclosure or a breeding event), data associated with that episode were deleted and a replacement observation period was conducted within 24–48 h.

Behaviors were summarized in two ways: 1) all-occurrence (number of behavior occurrences/visible min); and 2) instantaneous sampling (percentage of time behavior occurred/60-min observation period). Rate of occurrence for a behavior was determined by dividing the frequency of the behavior by the number of minutes in the focal period (time when the animal was actually visible). Instantaneous behaviors (feeding, resting, stationary alert, and locomotor activity) were recorded at the end of each minute during the focal period. Percentage of time engaged in a particular behavior then was determined for each animal.

Statistical Analysis

Data for each male and each year of the 3-yr study interval were assessed for influence within the model. Because there was no effect, as determined by using fixed effects influence diagnostics (SAS 9.1.3; SAS Institute), data were pooled for each male and across years. Each reproductive characteristic (body morphometrics, androgen concentration, sperm metrics, and behavior) was tested for normality. Body morphometrics were log transformed, whereas spermic trait percentiles (percentage normal morphology, percentage abnormal acrosomes) were arcsin transformed. Morphometric, androgen, and seminal traits were summarized for each seasonal period and analyzed using ANOVA followed by a Tukey-Kramer test of multiple comparisons to determine differences among assessed metrics and time intervals (SAS 9.1.3). Behavioral data were compared using GLMIX (SAS 9.1.3). Based on the preliminary

TABLE 1. Seasonal body and testicular morphometry in giant pandas in China during a 3-yr study.

	Prebreeding (Oct 1–Jan 31)	Early breeding (Feb 1–Mar 21)	Peak breeding (Mar 22–Apr 15)	Late breeding (Apr 16–May 31)	Nonbreeding (Jun 1–Sep 30)
No. of males	7	8	6	8	6
No. of evaluations	10	19	9	19	8
Body morphometry*					
Body weight (kg)	114.3 ± 4.1	119.1 ± 3.2	127.9 ± 4.1	117.1 ± 3.8	107.5 ± 5.1
Chest girth (cm)	108.1 ± 4.2	109.0 ± 1.7	114.3 ± 2.2	108.4 ± 2.2	120.4 ± 9.0
Abdominal girth (cm)	113.3 ± 3.8	112.1 ± 2.3	119.3 ± 3.5	118.2 ± 6.8	112.5 ± 3.3
Testicular morphometry*					
Total testicular volume (cm^3)	246.9 ± 24.2^{a}	325.8 ± 22.7^{a,b}	369.6 ± 31.4^{b}	279.0 ± 15.7^{a,b}	125.3 ± 8.8^{c}
Right testis volume (cm^3)	129.3 ± 13.2^{a}	169.6 ± 13.9^{a}	175.1 ± 12.5^{a}	136.1 ± 8.3^{a}	60.4 ± 4.6^{b}
Right testis length (cm)	7.4 ± 0.4^{a,b}	8.3 ± 0.2^{a}	8.7 ± 0.4^{a}	8.2 ± 0.2^{a}	6.4 ± 0.3^{b}
Right testis width (cm)	5.7 ± 0.2^{a}	6.1 ± 0.2^{a}	6.2 ± 0.2^{a}	5.6 ± 0.1^{a}	4.2 ± 0.1^{b}
Left testis volume (cm^3)	117.6 ± 11.7^{a}	156.2 ± 10.4^{a,b}	194.4 ± 20.6^{b}	142.9 ± 9.5^{a,b}	64.9 ± 4.6^{c}
Left testis length (cm)	7.5 ± 0.3^{a}	8.3 ± 0.2^{a,b}	9.0 ± 0.6^{b}	8.1 ± 0.2^{a,b}	6.2 ± 0.2^{c}
Left testis width (cm)	5.4 ± 0.2^{a}	5.9 ± 0.1^{a,b}	6.3 ± 0.2^{b}	5.8 ± 0.2^{a,b}	4.4 ± 0.1^{c}

* Values are means ± SEM.
^{a,b,c} Within a row, values with different superscripts denote differences among seasons ($P < 0.05$).

evaluation of the prevailing times of peak estrus during the year, we initially focused on the four specific intervals defined in Figure 1 (i.e., nonbreeding, early, peak, and late breeding season). However, based on collective results (see below), we found it necessary to include another reproductive activity period that was essential for the male panda, a ''prebreeding'' season that was factored into statistical analyses. Differences were considered significant with a confidence interval of $P < 0.05$. Data are presented as means ± SEM.

RESULTS

Body and Testicular Morphometry

General body morphometrics did not differ ($P > 0.05$) across reproductive seasons. Data from example traits including body mass, chest girth, and abdominal girth are presented in Table 1. In contrast, there were changes ($P < 0.05$) in testis size among the designated reproductive seasons. Total testicular volume increased nearly twofold ($P < 0.05$) from the nonbreeding to prebreeding season. A further increase ($P < 0.05$) was observed by the peak breeding period that was sustained through the late breeding interval (Table 1). There were no differences ($P > 0.05$) between the size of the right and left testis with the exception of a larger ($P < 0.05$) left gonad during peak breeding season. Changes in testis size over time reflected both increased gonadal length and increased gonadal width (Table 1).

Androgen Concentrations and Patterns

Mean fecal androgen concentrations in male giant pandas differed ($P < 0.05$) over time, with basal levels (74.2 ± 3.9 ng/g dry feces; n = 414 samples) measured during the late breeding season (Fig. 2). Values peaked ($P < 0.05$) during the prebreeding (160.6 ± 4.4 ng/g dry feces; n = 790 samples) and early breeding (158.0 ± 8.2 ng/g dry feces; n = 335) periods. Once the interval of peak female sexual activity was reached, the amount of excreted androgen in the male giant panda was in decline (108.6 ± 7.9 ng/g dry feces; n = 196 samples), a trend that continued to the late period mean nadir (74.3 ± 3.9 ng/g dry feces; n = 427 samples) (Fig. 2).

Temporal fluctuations in fecal androgen concentrations were best reflected in weekly means for the entire male cohort (Fig. 3). The iterative mean showing an androgenic baseline was calculated (Fig. 3) within two standard deviations (indicated by the shaded area). In all cases, nadir androgen excretion was observed in the late breeding season (mid-April–May) with variable concentrations remaining near baseline until October (Fig. 3). The prebreeding interval (October–January) was associated with increased androgen production that was sustained from February through mid-April (early and peak breeding periods; Fig. 3), intervals when most females were in estrus (Fig. 1). Interestingly, by April 15 (when 19% of females still had not displayed estrus), androgen production was declining and reached nadir about 6 wk before onset of the female nonbreeding season (Fig. 3). There were subtle, but nonsignificant ($P > 0.05$), variations among males (Fig. 4). Although there were clear trends in androgen fluctuations within individuals over time (Fig. 4), these changes were not significant ($P > 0.05$). Additionally, onset and rate of increased and decreased androgen metabolite production was consistent ($P > 0.05$) within an individual from year to year (data not shown).

Ejaculate Characteristics

None of the six males produced seminal fluid (or spermic ejaculate) during the nonbreeding season, and only three of six individuals did so during the prebreeding season (Table 2). In contrast, all males produced spermic ejaculate during the early, peak, and late breeding seasons. The sperm concentration of ejaculates varied throughout the year and was consistent with increases in total testes volume (Table 1) and mean weekly androgen excretion (Fig. 3). Ejaculate volume, sperm concentration, and total sperm per ejaculate were lower ($P < 0.05$) in the prebreeding compared to the early, peak, and late breeding periods (Table 2). Time of year had no influence ($P > 0.05$) on ejaculate pH or sperm motility traits (Table 2).

Most ejaculates contained more than 50% structurally normal spermatozoa, with most malformations occurring in the midpiece or flagellum (Table 2). Head pleiomorphisms generally comprised 5% or less of total spermatozoa analyzed. Flagellar deformities were more prevalent ($P < 0.05$) during the prebreeding than any period of the breeding season (Table 2). The majority of structural abnormalities observed from October through January were associated with proximal cytoplasmic droplets (35.5% ± 12.4% of all recovered spermatozoa). Ejaculates also contained high percentages of spermatozoa with normal acrosomes (Table 2). Even during the prebreeding season, at least 80% of the sperm acrosomes had a normal apical ridge. There was a modest decrease ($P < 0.05$) in percentage of normal acrosomes as males transitioned from the peak to late breeding season (Table 2). Ejaculates recovered in

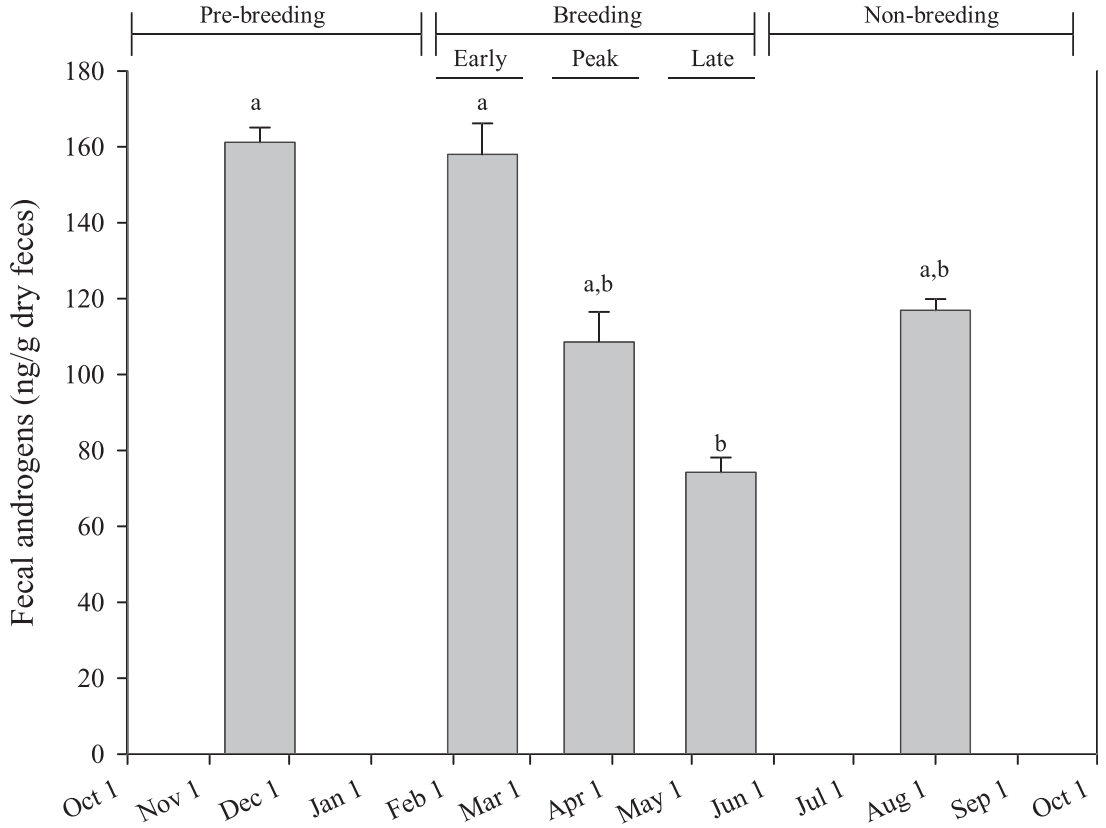

FIG. 2. Total androgen concentrations in adult male giant pandas (n = 8) in China over a 3-yr period. Bars represent mean (±SEM) total androgens within a season during the prebreeding (October 1–January 31), early breeding (February 1–March 21), peak breeding (March 22–April 15), late breeding (April 16–May 31), and nonbreeding (June 1–September 30) seasons. Means with different superscripts represent differences among periods ($P < 0.05$).

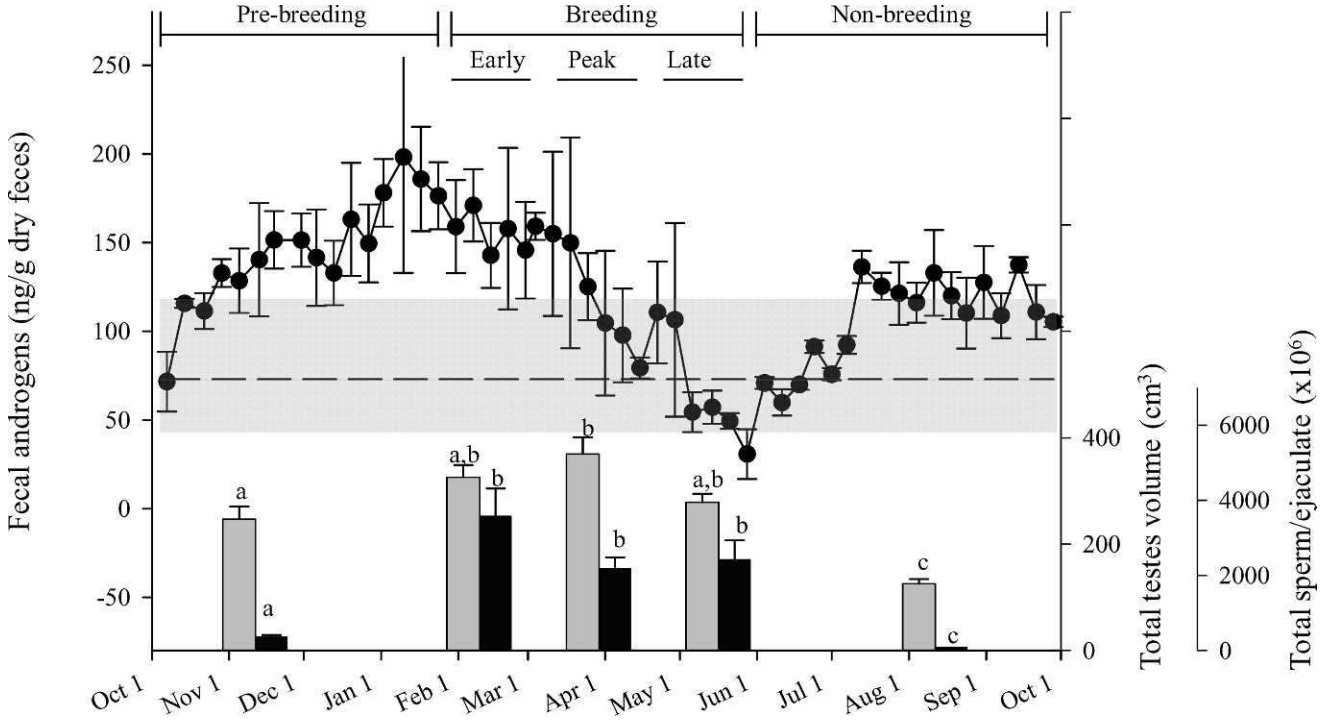

FIG. 3. Fecal androgens, testicular volume, and total sperm per ejaculate in male giant pandas (n = 8) in China over a 3-yr period. Mean (±SEM) fecal androgens (solid circles) during prebreeding (October 1–January 31), early (February 1–March 21), peak (March 22–April 15), and late (April 16–May 31) breeding seasons, as well as the nonbreeding (June 1–September 30) season. Baseline (±SEM) androgen in the dashed, shaded area. Mean (±SEM) values for testicular volume are presented by gray bars and total sperm per ejaculate by black bars. Bars with different letters within each trait represent differences among seasons ($P < 0.05$).

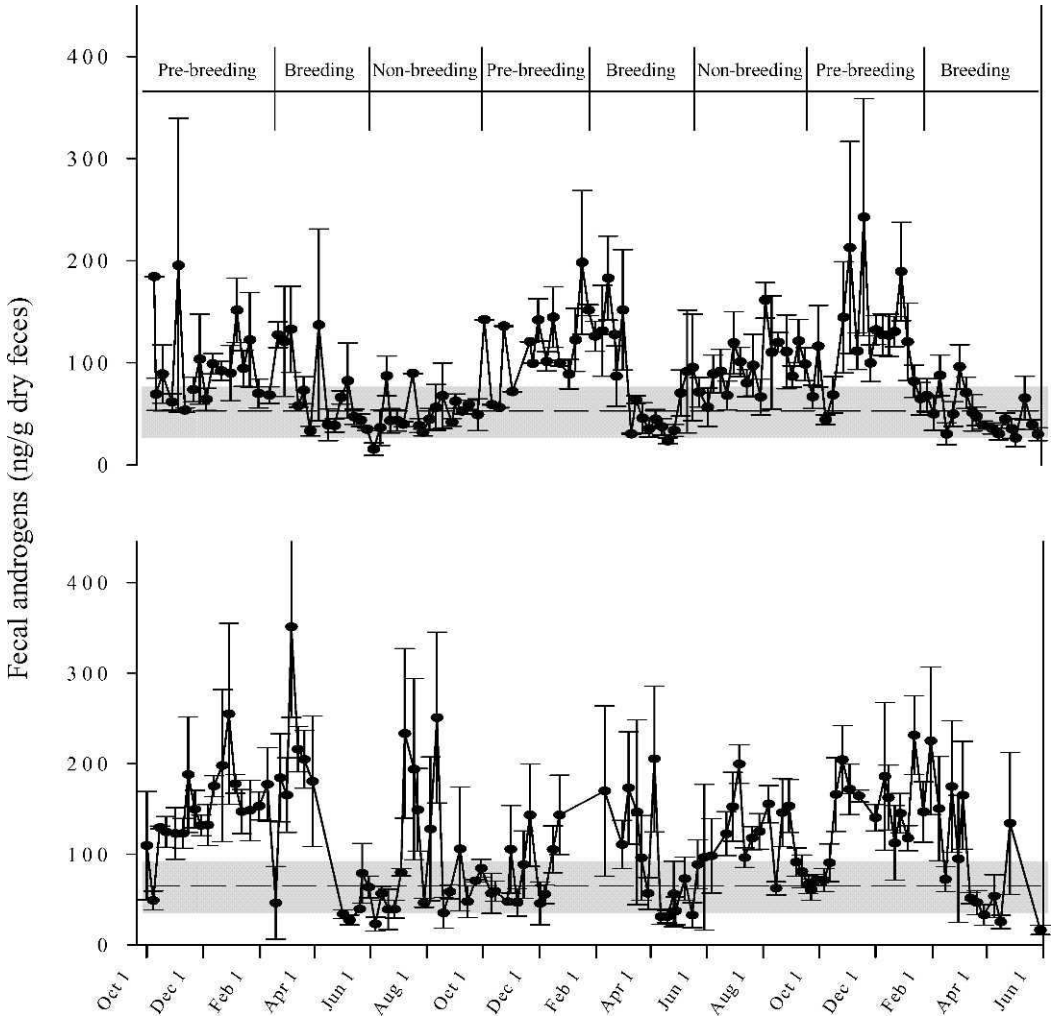

FIG. 4. Representative weekly (±SEM) androgen profiles for two male giant pandas during the prebreeding (October 1–January 31), early (February 1–March 21), peak (March 22–April 15), and late (April 16–May 31) breeding seasons, as well as the nonbreeding (June 1–September 30) season, over 3 consecutive yr. Baseline androgen (dashed line) was determined by hormone iterations using all fecal androgen samples for each male. Two standard deviations above and below baseline androgen are represented by the shaded area.

the late breeding season contained a higher ($P < 0.05$) number of sperm with a damaged apical ridge compared to those during the early and peak breeding seasons. Otherwise, there was no variation in acrosomal integrity (Table 2).

Behaviors

The frequency of behaviors associated with scent marking, vocalization, and pacing differed ($P < 0.05$) with season (Table 3) with the first acceleration in activities occurring within 30–45 days of elevated androgen patterns. There was variation among males in the intensity or frequency of these behaviors, but trends within individuals were clear. For example, there was an increased frequency ($P < 0.05$) of males demonstrating handstand, squat, and handstand urine markings in the prebreeding or breeding seasons compared to the nonbreeding season (Table 3). Prevalence of these behaviors tended to decline over the three phases of the breeding season, but this was nonsignificant ($P > 0.05$) because of variations among males. Vocalizations and pacing increased ($P < 0.05$) from nadir during the nonbreeding period to maximal values during the early and peak reproductive season when most females entered estrus (Table 3).

DISCUSSION

The female giant panda is an unusual example of a mammal that devotes minimal time (only 24–72 h annually) to sexual interaction with a male [10, 11, 15, 18]. As revealed by our findings here and those of earlier investigators [1, 10, 15], the female of this species is clearly an obligate seasonal breeder; 100% of the 46 cycles we monitored resulted in a single overt estrus occurring sometime from February through May. By contrast, we discovered that the male of this species has evolved a markedly different strategy. From monitoring morphological, physiological, and behavioral traits simultaneously, it was apparent that males also expressed fluctuations in reproductive traits and activities, but over a protracted interval. When all data were interrelated, male gonadal function was only briefly in quiescent abeyance. In fact, there were subtle increases in androgen excretion beginning within 6–8 wk of the end of the active female breeding season (circa mid-July). This androgen production remained modest until late October, when a more marked rise coincided with the first successful collection of spermic ejaculates. At least 60 days before the first female demonstrated estrus, testis volume and corresponding sperm production were elevated significantly.

TABLE 2. Seasonal ejaculate and sperm traits in giant pandas in China during a 3-yr study.

	Prebreeding (Oct 1–Jan 3)	Early breeding (Feb 1–Mar 21)	Peak breeding (Mar 22–Apr 15)	Late breeding (Apr 16–May 31)	Nonbreeding (Jun 1–Sep 30)
No. of males	6	8	5	8	6
No. of semen collections	11	17	7	20	8
No. of spermic ejaculates	4	16	6	19	0
Ejaculate volume (ml)*	0.2 ± 0.1[a]	2.3 ± 0.4[b]	2.9 ± 0.8[b]	1.3 ± 0.2[b]	0.0[c]
Ejaculate pH*	7.8 ± 0.9	8.6 ± 0.1	8.5 ± 0.1	8.6 ± 0.1	n/a
Sperm concentration/ml ($\times 10^6$)*	601.3 ± 586.4[a]	1955.3 ± 371.8[b]	1669.7 ± 508.8[b]	1780.4 ± 241.0[b]	0.0[c]
Total sperm/ejaculate ($\times 10^6$)*	61.9 ± 58.5[a]	3571.2 ± 744.6[b]	2178.2 ± 299.5[b]	2414.9 ± 519.8[b]	0.0[c]
Sperm motility (%)*	58.8 ± 7.2	78.5 ± 2.2	85.4 ± 2.7	77.0 ± 4.7	n/a
Sperm forward progression*†	2.6 ± 0.6	3.8 ± 0.2	4.3 ± 0.2	4.0 ± 0.2	n/a
Sperm morphology (%)*					
Normal sperm	42.3 ± 13.8	60.0 ± 5.1	74.4 ± 3.6	58.7 ± 4.1	n/a
Abnormal sperm	57.7 ± 13.8	40.0 ± 5.1	25.5 ± 3.6	41.3 ± 4.2	n/a
Head defects	5.0 ± 4.3	1.8 ± 0.5	3.2 ± 2.4	1.2 ± 0.3	n/a
Midpiece defects	13.3 ± 7.0	25.2 ± 4.2	15.2 ± 3.5	21.4 ± 3.4	n/a
Flagellar defects	39.4 ± 11.7[a]	13.0 ± 2.6[b]	7.1 ± 1.7[b]	18.7 ± 2.6[b]	n/a
Acrosomal integrity (%)*					
Normal apical ridge	80.7 ± 7.4[a,b]	87.9 ± 1.6[a]	89.4 ± 3.3[a]	71.1 ± 3.4[b]	n/a
Damaged apical ridge	18.0 ± 6.1[a,b]	10.8 ± 1.1[a]	9.2 ± 2.3[a]	24.4 ± 3.3[b]	n/a
Missing apical ridge	1.3 ± 1.3	1.1 ± 0.3	2.6 ± 1.3	5.2 ± 1.5	n/a
Loose acrosomal cap	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	n/a

* Values are means ± SEM. No available ejaculate is represented as n/a.
† Scale 0–5; 5 = best.
[a,b,c] Within a row, values with different superscripts denote differences among seasons ($P < 0.05$).

Optimal sperm quality with highest cell concentration was reached in April, about 3 mo after peak androgen excretion in January and coinciding with the majority of females in estrus. The frequency of scent marking was greatest after October 1 through the peak breeding period, perhaps indicating this is a critical time for olfactory communication with conspecifics, and before the breeding season. Other behaviors associated with breeding, especially frequency of vocalization and pacing, remained high at least 30 days after detecting maximal androgen production.

Particularly interesting was that the waves in male giant panda reproductive activity (testes volume, androgen patterns, sperm density, and sexual behaviors) occurred 3–5 mo before the interval when most females displayed their brief estrus. This prebreeding physiological change in male reproductive activity was consistent with previous findings in the brown bear (*Ursus arctos*) [23], Asiatic black bear (*U. thibetanus*) [24], polar bear (*U. maritimus*) [25, 26]), and American black bear (*U. americanus*) [25, 27], all of which seemed to experience gradually rising androgens, sperm production, and specific sexual behaviors 1–5 mo before peak female reproductive activity. However, the shift in timing and temporal patterns appears species dependent. For example, reproductive recrudescence in the female American black bear begins while the male is still hibernating [25] with testosterone in the latter peaking 3 mo later, coincident with leaving the den [25]. Elevated androgens in the male occurring 2–3 mo before the female black bear achieves peak estrual activity are believed to trigger the spermatogenic cycle [27]. Also as in the black bear, there was no need for the giant panda to sustain

TABLE 3. Seasonal frequency of behaviors in male giant pandas in China during a 3-yr study.

	Prebreeding (Oct 1–Jan 31)	Early breeding (Feb 1–Mar 21)	Peak breeding (Mar 22–Apr 15)	Late breeding (Apr 16–May 31)	Nonbreeding (Jun 1–Sep 30)
No. males observed	8	8	8	8	8
Total scent marking ($\times 10^{-3}$)*	23.5 ± 2.0[a]	27.6 ± 2.8[a]	22.5 ± 3.1[a,b]	14.0 ± 1.9[a,b]	9.3 ± 1.2[b]
Handstand mark ($\times 10^{-3}$)	1.8 ± 0.3[a]	1.7 ± 0.4[a]	0.4 ± 0.3[a,b]	0.3 ± 0.2[a,b]	0.1 ± 0.1[b]
Leg cock mark ($\times 10^{-3}$)	2.4 ± 0.4	2.0 ± 0.5	1.2 ± 0.5	0.4 ± 0.2	1.8 ± 0.5
Reverse mark ($\times 10^{-3}$)	4.2 ± 0.7	3.6 ± 0.8	5.2 ± 1.5	2.1 ± 0.6	1.1 ± 0.3
Squat mark ($\times 10^{-3}$)	8.0 ± 1.0[a,b]	11.9 ± 1.9[a]	6.4 ± 1.6[a,b]	5.0 ± 1.1[a,b]	4.8 ± 0.8[b]
Handstand urine mark ($\times 10^{-3}$)	5.7 ± 0.6[a]	7.4 ± 0.9[a]	8.2 ± 1.2[a]	4.5 ± 0.8[a]	0.5 ± 0.7[b]
Leg cock urine mark ($\times 10^{-3}$)	1.4 ± 0.3	1.0 ± 0.3	1.1 ± 0.4	1.7 ± 0.5	1.0 ± 0.2
Affiliative interaction ($\times 10^{-3}$)*	0.1 ± 0.0	2.8 ± 1.2	2.3 ± 1.1	0.1 ± 0.1	0.5 ± 0.3
Barrier interaction ($\times 10^{-3}$)*	9.3 ± 1.0	25.5 ± 2.2	33.0 ± 4.6	14.8 ± 1.8	4.7 ± 0.7
Total olfactory behaviors ($\times 10^{-3}$)*	15.5 ± 1.8	15.6 ± 2.9	41.4 ± 10.1	27.1 ± 4.8	9.4 ± 1.2
Scent anoint ($\times 10^{-3}$)	3.6 ± 1.1	2.4 ± 0.8	4.7 ± 1.8	3.2 ± 1.4	1.4 ± 4.9
Open-mouth olfactory ($\times 10^{-3}$)	6.1 ± 0.9	6.1 ± 1.0	21.3 ± 4.6	16.3 ± 2.7	6.0 ± 0.8
Lick olfactory ($\times 10^{-3}$)	5.6 ± 0.7	7.1 ± 2.2	15.4 ± 6.2	7.6 ± 1.8	1.8 ± 0.5
Vocalization ($\times 10^{-3}$)*	119.5 ± 16.3[a,b]	237.3 ± 36.5[a]	491.0 ± 91.8[a]	171.2 ± 27.2[a,b]	86.0 ± 12.2[b]
Investigate/explore ($\times 10^{-3}$)*	42.5 ± 2.2	42.5 ± 4.4	57.9 ± 7.3	50.6 ± 4.4	22.2 ± 1.3
Nonmotile stereotypy ($\times 10^{-3}$)*	44.7 ± 10.0	23.7 ± 6.5	10.6 ± 2.4	17.9 ± 8.3	36.7 ± 10.4
Total motile stereotypy ($\times 10^{-3}$)*	40.2 ± 3.0	49.2 ± 3.8	44.1 ± 5.2	31.2 ± 3.7	20.1 ± 2.5
Locomotor stereotypy ($\times 10^{-3}$)	19.2 ± 6.9	16.3 ± 2.0	7.9 ± 1.8	4.8 ± 1.1	5.9 ± 1.3
Pace ($\times 10^{-3}$)	21.0 ± 2.1[a]	32.9 ± 3.3[b]	36.2 ± 4.6[b]	26.4 ± 3.3[b]	14.2 ± 1.9[a]

* Values are means ± SEM. Frequency of each behavior was recorded as the number of times behavior occurred per visible minute.
[a,b] Within a row, values with different superscripts denote differences among seasons ($P < 0.05$).

androgens at a high level throughout the entire breeding season. We observed a decline in androgen excretion in the panda by mid-April (when 19% of females had not yet displayed estrus) and baseline concentrations by 6 wk before the end of the female's reproductive season. Although being somewhat reproductively similar to the male black bear, the giant panda is the most phylogenetically distant of the eight species in the Ursidae family [15, 28]. Rising testosterone appears to stimulate the black bear's metabolism, so torpor stops, and then the animal leaves the den [25]. As the giant panda does not hibernate, gonadal change is more rapid and shorter in duration than that of the black bear [25, 27]. The male polar bear, like the panda, does not undergo hibernation [25, 26]. Thus, these two species regain full reproductive activity over a shorter interval than the black bear. But of these three species, the polar bear experiences the most protracted decline in gonadal hormone activity, with androgen activity sometimes not reaching nadir until ~6 mo after the end of the breeding season [25, 26]. This more prolonged gonadal activity may be related to the female polar bear's propensity to hibernate or experience denning, thereby resulting in a longer breeding season compared to the female panda [25, 26]. Despite the giant panda's sharing some reproductive similarities with other bears, our findings were unequivocal in revealing a cessation of spermatogenesis in August in the giant panda, a phenomenon that has been documented in only one other ursid, the Japanese black bear (*U. thibetanus japonicas*) [29, 30]. The latter is another temperate-zone bear that is known to produce increasing numbers of spermatozoa 1–2 mo before the onset of female receptivity [29–31].

Beyond having a bountiful sperm number, the giant panda ejaculate also was notable for high cellular motility and percentages of structurally normal spermatozoa, including good acrosomal integrity. The giant panda appears to produce far greater total sperm per ejaculate (by 20-fold) compared to, for example, the Japanese black bear ($84.0 \pm 32.2 \times 10^6$ motile sperm/ejaculate) [31], but similar to the Hokkaido brown bear (*U. arctos yesoensis*) ($1387 \pm 2160 \times 10^6$ motile sperm/ejaculate) [32]. Clearly, it would be adaptive and advantageous for the giant panda to produce a sperm-rich ejaculate when competing with other males for access to females with extraordinarily brief and unpredictable windows of sexual activity. This trait also is highly useful in the genetic management of the ex situ population because AI is used commonly in breeding centers. As AI pregnancies have been achieved with as few as 100 million motile spermatozoa [7], a single ejaculate can be used to inseminate multiple females and remaining fractions cryopreserved for later use.

Our findings revealed a 3-mo interval between the peak in androgens (January) and consistently successful, high-quality sperm collections (April), suggesting that reproductive recrudescence requires ~90 days for spermatogenesis to fully reinitiate. This protracted improvement in sperm quality included fewer numbers of flagellar defects in the early, peak, and late breeding compared to the prebreeding interval. Interestingly, acrosomal integrity was optimal during the early and peak breeding season, suggesting that perhaps this metric is a sensitive indicator of sperm quality or even fertility potential.

Measured changes in male giant panda reproductive events fit well with current knowledge of mammalian reproductive biology. For example, elevated excreted androgen occurred before measuring volumetric increases in testes size. This observation confirmed other recent findings from our laboratory demonstrating the utility of monitoring gonadal and adrenal steroidal activity in male giant panda feces [19]. The latter study [19] involved only five males (in three locations) and generally a less frequent and shorter fecal sampling interval. Yet the resulting rise and fall in androgen patterns were similar to the more extensive, multidisciplinary approach taken in the present study. Kersey et al. [19] also discovered that excreted (fecal) glucocorticoid patterns almost exactly mimicked the seasonal increase and decrease in androgens, and suggested that rising adrenal hormones served to mobilize energy reserves to prepare males for the rigorous battles to gain access to estrual females in the wild. Regardless, steroidal metabolites can clearly be monitored even in the highly fibrous feces [19] of the giant panda, thereby offering many research opportunities [19], including correlating hormone patterns and sperm production.

In nature, innate communicative behaviors are essential for allowing a male to locate a sexually receptive female. Although managed in captivity, our study male giant pandas were only one to two generations removed from the wild. When we related a wide array of frequently monitored giant panda behaviors to physical and physiological metrics, only those associated with scent marking, vocalization, and pacing increased before and/or during the breeding intervals. These traits are known to occur in nature [1, 17, 33]. For example, free-living male giant pandas rely on handstand marking to deposit scent from an anogenital gland to communicate presence to females and to maintain a territory, even by advertising size and dominance information [1, 34, 35]. Our findings confirmed earlier observations [16, 17, 34] that this behavior is retained in captivity with frequency increasing with nearness to breeding season. But additionally, we discovered a close parallelism between this behavior and excreted androgen patterns. Although prevalence of handstand and squat marking was sustained throughout the breeding season, total scent marking had declined by late season, suggesting a waning interest in communicating to receptive females. This finding was in complete alignment with declining androgen production that also occurred before the end of the female season, likely indicating that testosterone was the endocrine driver of scent marking behavior.

We suspect similar cause and effect relationships between our circannual androgen fluctuations and the prevalence of vocalizations and pacing. Vocal communication is well known to convey important information among individual wild giant pandas and potentially over long distances [1, 36–38]. More recently, females managed under ex situ conditions have been found to perceive vocal signatures of different males that may be related to body size, of course an important indicator of reproductive fitness [36, 37]. This ''verbal capacity'' also involves the ability of females to identify novel (not previously encountered) males, an apparently heritable trait [36, 37]. Giant pandas also are known to travel long distances in nature, and outside of home territories, to find and compete for a female [1]. Clearly, we were able in the present study to confirm that patterns in these two behaviors followed other morphological and physiological elements of the male cycle. Whereas pacing did not increase significantly until onset of the early breeding period, the vocalization profile was aligned tightly to the androgen pattern, including declines prior to onset of the nonbreeding season. It may well be that scent marking, vocalizations, and even pacing all play a role in ability to select, win, and copulate with a mate. Regardless, our collective data demonstrated the remarkable convergence of varied morphological, physiological, and behavior elements over a protracted timeline to achieve male reproductive fitness.

Besides documenting a remarkable gender difference in onset and duration of gonadal activity between the male and

female giant panda, our findings have application to the genetic management of this species in ex situ collections. Since the late 1990s, interdisciplinary studies, led by new data in the reproductive sciences combined with improvements in animal husbandry and preventative medicine, have been used to create a self-sustaining giant panda population of more than 300 individuals. This has included increased use of AI to ensure that all individuals (including those that fail to breed naturally) have the opportunity to reproduce [5, 7, 8]. In this context, the systematic banking of spermatozoa has become common [5, 7, 9]. Our results here have demonstrated conclusively that the male giant panda varies throughout the year in reproductive fitness, including the capacity to produce high-quality spermatozoa. From an applied perspective, collection and cryopreservation of spermatozoa for contributing to genetic and demographic management should be focused solely on the active months of January through June.

ACKNOWLEDGMENT

The authors thank the dedicated staffs of the Chengdu Research Base for Giant Panda Breeding and the Chengdu Zoo, with special appreciation extended to Luo Li, Luo Lan, Zhang Liang, Liu Yuliang, and Yang Bo. Thanks also go to our international research assistants Margaret Cooney, Richard Lang, Gabriella Dancourt, Nathaniel Watson, H. Kasen Whitehouse, Jon McRoberts, Emily King, Rebecca A. Wilson, Meghan Martin, Corianna Bazlett, Sarah Wakamiya, Jenny Keatinge, and Pan Xuan; to Lena May Bush and Jenny Santisteven for technical support; to Steven Monfort, Janine Brown, and David Kersey for intellectual and laboratory support in the endocrine analyses; and to Rebecca Snyder and David Powell for guidance on the intricacies of measuring giant panda behaviors.

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