ABSTRACT

Title of Document: ASSESSMENT OF MALE GIANT PANDA SEASONAL REPRODUCTION, SEXUAL MATURITY AND SPERM COMPARATIVE CRYOPRESERVATION

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The giant panda is unique as one of the most studied endangered species. Interestingly, despite this, little is known about basic male reproductive physiology. Behavioral problems have posed challenges for reproductive success in the captive population, while geographical isolation and habitat fragmentation threaten the wild population. Techniques such as sperm cryopreservation can improve genetic management and reproductive success within captive populations. But without comparative freeze-thawing studies, diverse methods used for cryopreservation result in inconsistent post-thaw quality, potentially compromising sperm quality and fertility for artificial insemination. This, compounded by limited knowledge of male reproductive physiology, has challenged conservation goals for the giant panda.

Specific objectives of this study include: 1) assess the influence of season on male giant panda reproduction by determining annual changes in testis size, body size, testosterone production, sperm quality and production, and reproductive behaviors throughout the year; 2) characterize the onset of sexual maturity in the male giant panda by documenting the timing of initial sperm production, increasing

testicular size and body weight, rising androgen production and initiation of reproductive behaviors; and 3) identify factors influencing giant panda sperm cryopreservation by comparing the effect of freeze-thawing on sperm motility, acrosomal integrity, forward progressive movement and longevity.

Results provide evidence that 1) reproductive seasonality in the male giant panda is more protracted than the female, with coordinated increases in testes size, androgen production, sperm density, and sexual behaviors; thereby maintaining reproductive competence to prepare for, and accommodate, a brief unpredictable female estrus; 2) sexual maturity in the male giant panda is first observed with consistent sperm production at 5.5 yr, then accompanied by increasing body weight, fecal androgen concentrations, and culminating with the onset of reproductive behaviors, such as scent marking between 7.5 – 8.5 yr; and 3) the giant panda spermatozoon has the capacity to withstand cold stress applied by multiple means, specifically after freezing and thawing using a unique field friendly dry shipper approach. These data greatly improve our understanding of male giant panda reproductive physiology, specifically seasonal rhythms, the cascade of changes associated with sexual maturation, and insights into improved comparative approaches for sperm cryopreservation.

ASSESSMENT OF MALE GIANT PANDA SEASONAL REPRODUCTION, SEXUAL MATURITY AND COMPARATIVE SPERM CRYOTOLERANCE

By

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Preface

The giant panda is one of the world's most widely recognized endangered species and is an international symbol of conservation. This high profile species has long been the focus of international collaboration efforts for conservation. Specifically, the University of Maryland and the Smithsonian's National Zoological Park have been collaborating for years to promote research and conservation of species of special concern such as the giant panda. This rich collaboration has contributed to great opportunities for undergraduate, graduate and even professional students, who have participated in research at University of Maryland at College Park, the Smithsonian Conservation Biology Institute, Smithsonian's National Zoo, and the Chengdu Research Base of Giant Panda Breeding in China. Through these research opportunities and experiences, the next generation of scientists and conservationists will further the preservation of giant panda and other special species.

Dedication

This dissertation is dedicated to my family, Joan Aitken, Rodger Palmer, Wade Aitken-Palmer, Randy Palmer, Jeanetta Palmer, Bev Aitken, George Aitken, Pam Aitken, and Jim Aitken, who have always been supportive of my interest in wildlife conservation, especially the giant panda. All of my work on the giant panda is dedicated to Evelyn Aitken for her recognition that some dreams are possible.

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Giant Panda Conservation Efforts

In situ population

The giant panda is one of the world's most widely recognized endangered species and is an iconic symbol of international conservation. The wild giant panda population consists of an estimated 1600 individuals located exclusively within China [1]. The giant panda is threatened with extinction by habitat loss and fragmentation resulting in interest by worldwide conservation groups to better understand the wild population and ways to optimize conservation. Many of these conservation efforts have focused on habitat conservation [2, 3] and understanding the needs of the wild panda, in situ population [4, 5, 6].

Ex situ population

The ex situ, or captive population has also been a focus for conservation efforts. Historically, about 10% of the world's giant panda population has resided in captive breeding centers and zoos. This 10% is a relatively substantial portion of the world's panda population and they provide a critical resource for investigational research on reproduction, health, behavior and physiology. This ambassador population allows the world's scientists to identify and study reproductive challenges such as mating pair behavioral incompatibility, decreased male and female libido, short estrus period and the impact of geographic and genetic isolation. With this ex situ population, Chinese scientists have developed and used assisted reproductive techniques to increase offspring production and ensure that genetically valuable individuals

reproduce. The most commonly used assisted reproductive technologies for the giant panda are sperm cryopreservation and artificial insemination (AI). These techniques have improved genetic management of the ex situ population ensuring that every individual reproduces regardless of temperament or location [7, 8]. These techniques have resulted in more offspring and have driven associated research [8, 9, 10]. Despite advances in assisted reproductive technologies used for the ex situ panda population, there are still unanswered questions about basic giant panda reproductive physiology. Specifically, there is limited information on male giant panda seasonal variation, changes associated with sexual maturity and the comparative cryotolerance of sperm for long term preservation [11, 12, 13]. Further study in these areas has been identified as critical for improved reproductive fitness of the captive population [13]. Before we can evaluate the special reproductive physiology of the male giant panda, one must appreciate the specialized natural history of this unique species.

Natural History of the Giant Panda (Ailuropoda melanoleuca)

Exclusive habitat and unique diet

Giant pandas are found exclusively in the mountains of China. Their natural habitat at elevations of about 8,500 feet [14] historically ranged over much of eastern China. These populations are now reduced to the eastern edge of the Qinghai-Tibetan Plateau in the Chinese provinces of Gansu, Shaanxi and Sichuan, China [15].

High elevation habitat where preferred bamboo species thrive is critical for the giant panda, who are nearly obligate bamboo feeders [4, 14]. Giant panda habitat is identified as an ideal balance of forest cover, slope, elevation and understory

bamboo [4, 16]. The amount of bamboo understory, and the diversity of bamboo species found in this understory is a critical element of giant panda habitat [17].

The giant panda, classified within the order Carnivora, feeds almost entirely on bamboo, a grass [4]. This adaptation is unusual because the giant panda does not have a digestive system capable of fermentation typical in most herbivores that rely on fibrous grassy diets [18]. Instead the giant panda has a relatively short gastrointestinal tract with a simple stomach, degenerate cecum and a simple colon [18]. The giant panda does, however, have adaptations to glean nutrients from this highly fibrous bamboo. These adaptations include large masticatory muscles on their head [14], large molar surface area for crushing bamboo [14], and a sixth digit on their forepaw for bamboo manipulation. These adaptations allow the giant panda to rely on a specialized diet of bamboo for survival.

It seems strange that this large species uses bamboo for nearly 99% of its diet, but perhaps it is due to the historical abundance of bamboo in the natural habitat of the giant panda. Bamboo, as a food source, is relatively constant in accessibility and nutritional level through the year [14, 18]. In times of nutritional stress or lack of quality bamboo, the giant panda eats small rodents, but this is uncommon and usually occurs as the giant panda forages for bamboo [4].

Areas are identified as potential giant panda habitat based on abundance of bamboo. However, the carrying capacity of this habitat depends on bamboo understory, bamboo density, and forest patch size [19]. Because of changes in the wild landscape, there are fewer areas that meet these criteria available to the giant panda. As a result, the wild giant panda population is fragmented and distributed

between 24 isolated populations [15]. These areas and corridors between these areas need to be preserved to sustain the wild giant panda population [15].

Under the observation of world conservation groups, China has made many changes in policy and actions to improve giant panda protection. Specifically, logging of the bamboo forest in the giant panda habitat, has been halted and regeneration has begun [6]. As protection of the remaining habitat has improved, this raises concern about its fragmentation. This habitat fragmentation has resulted in geographical reproductive isolation for many of the remaining wild pandas [3, 5, 6]. Specifically, there is concern about the ability of juveniles to disperse from related pandas to avoid inbreeding [19]. These new concerns, have called for improved habitat preservation and evaluation of methods to connect present fragmented habitat [19].

Giant panda phylogeny

Methods of habitat conservation and sustainability of the wild giant panda are topical, but perhaps the most widely known debate about the giant panda is its phylogenic classification. The giant panda was originally classified within the family procyonidae (same family as raccoons and red pandas) based on habitat and fossil record [14]. Stephen O'Brien [20] at the National Cancer Institute in Fredrick, Maryland has used DNA hybridization to prove the giant panda should be classified within Ursidae (bear family). There are others who still believe the giant panda should be placed in its own family, Ailuropodidae [14]. Despite this debate, it is generally accepted that the giant panda is part of the Ursidae family [21] as it shares many physiological similarities with other bears.

It is believed that the progenitor that gave rise to the giant panda diverged from the other bears within Ursidae 25-18 million years ago followed by the spectacled bear (*Tremarctos ornatus*) [14, 20, 21]. This makes the giant panda the oldest species within the Ursidae family. As a result of this early divergence from other bears, the giant panda possesses unique physiologic adaptations.

The giant panda diverged from other bear species before Ursidae developed hibernation [21] and is one of the species of bear that do not hibernate. They also possess anatomical and dietary differences from the rest of the family [21] allowing classification within its own subfamily, Ailuropodinae [20, 21].

Captive population management

The giant panda consumes a diet predominately of bamboo. All species of bamboo are classified as grasses. Unlike typical grasses that flower and produce seeds annually, most bamboo species flower gregariously. This means that a particular species of bamboo will flower, produce seeds and then die synchronously [14]. During the 1970's, there was a massive natural bamboo flowering, resulting in the drastic reduction of multiple bamboo species [4]. The giant panda relied on many of these species of bamboo for survival causing the giant panda to forage for other bamboo species at lower altitudes. Unfortunately, many giant pandas died of starvation during this period. Others were rescued by the Chinese Ministry of Forestry [14]. These rescued pandas were placed in captivity, and were the progenitors of the first giant panda breeding programs within China. These breeding programs had two main locations including: 1) Conservation and Research Center for the Giant Panda in the Wolong Nature Reserve (commonly called the 'Wolong

Breeding Center'); and 2) Chengdu Research Base of Giant Panda Breeding (commonly called the 'Panda Research Base'). Over the past 30 years, these initial populations have served as founders for the current captive giant panda population around the world.

Because the giant panda is threatened with extinction, this global captive population is recognized as a vital component of the giant panda recovery strategy, making it essential that a viable, self-sustaining captive population is maintained as a research resource, ambassadors in educational programs and as a reservoir for initial reintroduction plans [10, 22].

Currently, there is a self-sustaining captive population of > 300 individuals that reside in breeding facilities and zoos globally. The location and status of these individuals are recorded in the 2010 Giant Panda Studbook (a pedigree of the captive population). Historically, this population was not as successful and faced challenges, such as poor reproductive success largely due to behavioral incompatibility and a lack of competent breeding males [22, 23].

Giant panda breeding facilities have made progress over the past decade by producing more surviving young, but many of these young are still sired by only a few males, leaving the population genetically skewed [24]. For the moment, there is ample genetic diversity in the captive giant panda population if the current captive adults can produce progeny [5]. The use of natural breeding in combination with assisted reproduction can ensure that all of these genes are carried to subsequent generations. Sperm cryopreservation and artificial insemination (AI) are used in combination for genetic management and has been targeted for the establishment and

management of a Giant Panda Genome Resource Bank [22, 25, 26], a repository of cryopreserved biomaterials such as sperm, embryos and DNA.

The Giant Panda Genome Resource Bank has been identified as a high priority for collaboration by our Chinese colleagues [13, 22]. Because of the success of this effort, sperm cryopreservation has been used in the giant panda for over 10 years [13, 25, 27] with various success at different breeding centers and zoos [13, 27, 28].

Reproductive physiology

The female giant panda is highly seasonal, with estrus and breeding primarily occurring from February through May of each year [4]. The female has behavioral [29, 30, 31] and hormonal changes [29, 32] that occur prior to the breeding season with peak reproductive behaviors and peak estrogen concentrations occurring during estrus [33]. Peak estrus in the giant panda is a very short interval lasting only about 48-72 h [33, 34, 35]. It is during this short period, that the female is receptive to mating as indicated by rising levels of estrogen with a sharp decline and corresponding behavioral changes such as presentation of genitalia and altered vocalization [31, 32, 36, 37].

The male giant panda is known to produce sperm [38, 39], increase testicular size [39] and exhibit reproductively related behaviors [40] during the female's breeding season. Changes in reproductive capacity before the breeding season or after the breeding season in the male giant panda, has not been described in the literature, but are anticipated to be similar to that of other seasonally breeding bears.

Sexual maturity and the process of sexual maturation are not well understood in the male or female giant panda. There are reports of female giant pandas giving birth at the age of 4.5 yr in China (Howard, personal communication), but the majority of young females in the Giant Panda Studbook have given birth at 5.5 yr and 6.5 yr. It is likely that the males and females of the species go through puberty at a similar age, but there is little documentation for this. Anecdotal evidence from our Chinese colleagues indicates males 5.5 yr are capable of successful natural breeding, however this has not been the case for individual males in zoos within the United States.

There have been some studies on male sexual maturation in other bear species. Specifically, changes in testicular size and presence of sperm in the epididymis have been used to indicate sexual maturity in the Hokkaido brown bear [41], Alaskan brown bear, grizzly bears [42] in the Yukon and grizzly bears in the continental United States [43]. Using those parameters, it is accepted that the Hokkaido brown bear reaches sexual maturity at 2 to 5 yr [41], whereas the Alaskan brown bear is mature after 4.5 yr [42], the Yukon grizzly bear matures 5-7 yr [43] and grizzly bears in the continental United States attain puberty as young as 3.5 to 5.5 yr [44]. These data from other bear species demonstrate a range of sexual maturity between 2 to 7 yr within the family Ursidae. Changes in hormone profiles, behavior, body mass index, testicular size and presence of spermatozoa have been parameters used to understand puberty in these bear species, but information for these parameters is previously undocumented in the peri-pubertal male giant panda.

Behavior

Animal behavior has been studied in many species. In giant pandas, behavioral observations has been used to understanding communication [31, 36], reproduction [23, 46], visual capability [45] and social interactions [40]. Behavioral research also has been used for management and husbandry applications to identify areas for improved captive management for the giant panda and to identify areas of stress influencing health and reproductive success [45, 47, 48].

Aspects of behavior, particularly reproductive behavior, are hormone dependent [34, 49], and understanding these behaviors is necessary for promoting reproductive success. In other bear species, reproductively related hormonal changes are associated with testicular size or sperm quality alterations [50, 51-53] are influenced by natural light patterns [54].

Influence of season on male reproduction

The integration of photoperiod by seasonal breeding species involves complex pathways controlled by the pineal gland [55]. This gland is relatively poorly understood, but is believed allows day/night information to be relayed biologically through rhythmic melatonin secretion. This process allows biological systems to change in concert with alterations in melatonin signaling [55]. The melatonin signal modulates seasonal changes in various physiological functions. These self-sustained circannual rhythms play a critical role in determining seasonal variations in reproduction, by fine-tuning the breeding season to correspond with environmental constraints [55]. Despite knowledge accumulated on the basis of melatonin signaling,

there are unanswered questions on how the same melatonin signal produces opposite effects in animals with different breeding seasons.

Seasonal reproduction in bears

Examples of different seasonal strategies also are found in the bear family Ursidae. The black bear (*Ursus americanus*) and polar bear (*Ursus maritimus*) are two species of bear known to hibernate. Increased levels of testosterone occur during the breeding season compared to the non-breeding season, and this elevated testosterone coincides with an increase in testicular size [51, 56]. The Japanese black bear (*Ursus thibetanus japonicus*), also a hibernating bear, shows a similar increase in testosterone production and corresponding increase in LH receptors during the breeding season [53], with corresponding changes at subcellular level as indicated by increased mitochondrial function in Sertoli cells during the breeding season [52]. In the black bear, sperm production increases 2-3 months prior to the breeding season to prepare the male for female receptivity [50].

The tropical Malayan sun bear (*U. malayanus*) does not hibernate and does not display seasonal patterns in androgen, gonad or behavioral changes like other bears [57]. Instead, they go through a consistent period (3-4 months) of testicular regression with variable timing of onset [54], with no pattern in peak circulating testosterone concentration [58]. Females have been seen to breed throughout the year, with a breeding peak during Nov to Jan [58]. In the tropics, the cues for breeding season are less likely due change in photoperiod, and more likely due to changes in rainfall or nutrient availability [58].

Physiology of mammalian male puberty

Organization of the male reproductive system occurs prenatally, with sexual differentiation of the hypothalamic-pituitary-gonadal axis (HPG axis). At the time of puberty, the reproductive axis activates, with the gonads initiating spermatogenesis and steroidogenesis [59]. The onset of puberty is preceded by increasing production and release of hypothalamic gonadotropin releasing hormone (GnRH) [60]. These events reflect changes in the synthesis and/or alteration in GnRH associated with the pubertal rise of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). As the testes become more steroidogenic, there is a positive effect on the response of testicular cells to the actions of FSH and LH, which enhance spermatogenesis and steroidogenesis, respectively. Testosterone is essential for spermatogenesis during and after the onset of puberty, and circulating testosterone is critical for the development of accessory sex organs, hormone dependent behavior, and metabolic function.

The onset of sperm production in dogs and bulls is accompanied by increased testicular size, rising hormone (FSH, LH, testosterone) concentrations and initiation in male behavior patterns [61, 62, 63].

Body size and weight appears to be a conserved change that occurs during sexual maturation across a number of species from mammals to fish [64, 65]. But it is difficult to determine if body weight triggers the onset of puberty or if increased body mass is a result of pubertal increases in testosterone. While weight gain and changes in body morphology may be associated with puberty, these physical changes are only a portion of the changes occurring in the male during puberty.

Circulating testosterone levels are closely linked with reproductively related behaviors, such as mounting, intromission and ejaculation [49]. Rising levels of circulating testosterone that occur during sexual maturation modulate neural systems in brain areas for sexual behavior [66]. For this reason, monitoring reproductive behavior in addition to gonadal steroids provides a marker of reproductive function and a biomarker for the onset of puberty.

Assisted Reproductive Technology for Giant Panda Conservation

Assisted reproductive technology is widely used for domestic [67-72] and wild animal species [73-76]. Specifically, the most commonly used techniques are electroejaculation [7, 13], artificial insemination (AI) [8, 13, 77, 78], sperm cryopreservation [7, 13, 25, 79] and hormone monitoring [73, 80]. These techniques are becoming increasingly common for wild species conservation and management [79].

Sperm collection by electroejaculation [7], sperm cryopreservation [12, 13, 27] and AI [8, 13] have been used for captive management of the giant panda over the past two decades. In conjunction with assisted reproduction, research has been conducted to assess giant panda sperm ultrastructure [81], capacitation [82], acrosome reaction [83] and oocyte penetration [82], but all studies have used freshly-collected spermatozoa. While there are reports of successful progeny using cryopreserved giant panda sperm [9, 84, 85], only one study examined sperm physiology and function in this species before and after the freeze/thaw process [27]. Clearly there

are gaps in our knowledge of the fundamental biology of male giant panda reproductive physiology.

Sperm cryopreservation

Giant panda semen cryopreservation methods utilize rapid freezing rates (~ -40 to 100ºC/min), in which semen is frozen in pellets [13, 85] or straws [27, 84] manually over liquid nitrogen vapor or using an automated programmable freezing unit (Forma Cryomed® [13]). While these freezing methods are rapid, current thawing rates occur at intermediate rates (exposure to 37ºC) [27]. As demonstrated with other species, rapid to ultra-rapid thawing by exposing frozen sperm to temperatures of 50ºC to 75ºC yields optimum results after rapid cryopreservation rates. Optimal conditions have been established for canine [86], porcine [87], bovine [87] and human [88] sperm. Slow to intermediate rates of thawing by exposure to 20ºC or 37ºC have been found optimal following semen cryopreservation at slow rates (10°C/min) in humans [89]. It is likely that optimal thawing rates depend on the rate of cryopreservation applied, and it is possible that rapid thaw rates provide optimal sperm survival following rapid cryopreservation, while slow thaw rates should be applied to sperm cryopreserved at a slow rate. The comparative cryotolerance of giant panda sperm is poorly understood.

Freezing giant panda sperm manually over liquid nitrogen vapor or with an automated computerized method, such as the Cryomed® is used most commonly [13, 27]. But these techniques pose challenges for some breeding centers and zoos in China. The manual liquid nitrogen vapor technique requires access to liquid nitrogen at the time of sperm cryopreservation [7]. Because of the remote location of many

wildlife centers in China, access to liquid nitrogen is often inconsistent and unreliable.

Another alternative, the automated Cryomed® technique, is used frequently in domestic species and allows for large volumes of sperm to be cryopreserved using a computerized standard freezing rate. But this technique also requires access to liquid nitrogen and expensive computers and equipment. For this reason, the Cryomed® is cost prohibitive for most breeding centers and zoos with giant pandas.

Liquid nitrogen dry shippers are used as biological shippers that maintain -196ºC for an extended period of time (about two weeks) before additional liquid nitrogen is needed. The canisters are easily transported and do not require access to liquid nitrogen in the field or at the time of sperm freezing. While dry shippers are often used to store or transport biological samples, there are few reports of liquid nitrogen dry shippers used to freeze sperm [90]. The dry shipper approach could be a good alternative sperm cryopreservation in field settings or at remote breeding centers, if giant panda sperm are cryotolerant to this approach.

Successful sperm cryopreservation requires careful techniques designed to minimize cellular damage. Damage during freezing does not occur when the sperm is exposed to ultra low (-80 to -196°C) temperature [91]. Rather, damage is incurred during the intermediate temperature zone (-15 to -60°C) endured during freezing and thawing. Sperm survival requires an appropriate cryodiluent, cryoprotectant, cooling rate, equilibration period, storage package; freezing rate, thawing rate, and post-thaw dilution to remove cryoprotectant.

Most sperm processed for cryopreservation are placed in a cryodiluent in the absence or presence of cryoprotectant, such as glycerol, and then cooled to 5° to 0°C. Many compounds are cryoprotective, but glycerol (a penetrating cryoprotectant) is most widely used for mammalian sperm [91]. The cooling process itself can be critical. Most damage occurs during the rapid cooling phase causing reversible or irreversible disruption of membranes and cell motility [92]. An ideal cryodiluent is isosmotic to the cell. Adding a cryoprotectant such as glycerol, either at room temperature or during initial cooling, causes a transient osmotic imbalance because water moves out of the spermatozoon faster than the glycerol moves in [93].

The packaging of the diluted semen also can affect the cooling rate, as different approaches (straws, pellets, and vials) vary the surface-to-volume ratios. Smaller surface areas (straws and pellets) permit faster freezing rates than when samples are packaged in vials. The cooling and freezing rate is a function of methods that can range from exposure to refrigerator temperatures and liquid nitrogen vapor to controlled-rate freezing in electronic, programmable cryopreservation units. Cooling of sperm suspensions below 0°C (-5 to < -10°C) triggers extracellular ice crystal formation, causing an increase in the extracellular solute concentration [93]. During this time, water within the spermatozoon is supercooled, but remains unfrozen. Because of the different concentration gradient of solute across the plasma membrane, water flows out of the cell until the internal osmolality matches that of the surrounding media and freezes externally. Loss of intracellular fluid dehydrates the spermatozoon allowing cryopreservation without cell lysis as a result of internal ice formation. If sufficient intracellular water is not removed, irreversible cell damage

occurs due to ice crystal formation. Thus, the rate of cooling to the intermediate temperatures (-12 to -40°C) is very important. A slow cooling rate (< 10°C/min) allows the formation of large extracellular ice crystals and subsequent flow of water from the cell resulting in little or no intracellular ice formation. Rapid cooling (10 to 100°C/min) inhibits water movement from the cell which results in the formation of some microcrystals inside the cell. Likewise, very rapid cooling (several hundred degrees/min) further reduces the time for water to exit from the cell, resulting in both internal and external small ice crystal formation [93].

The warming/thawing phase dictates how ice crystals melt and influences the rate of solute concentration change which affects sperm cell volume and survival post cryopreservation [94]. Slow thawing frequently results in local intracellular microcrystal formation that can extensively damage organelles. If thawing is too rapid, the unbalanced efflux of cryoprotectant and influx of water causes cellular expansion and rupture. Thus, optimal thawing occurs when cryoprotectant efflux and water influx occur at a rate minimizing microcrystals formation and cellular ballooning and bursting. Finally, since glycerol removal has been shown to cause a loss in plasma membrane integrity, it is essential that glycerol be removed slowly after thawing to minimize osmotic membrane rupture [95].

A spermatozoon, as a cell, contains three functional units: 1) plasma membrane and the underlying acrosome (for initial oocyte binding and penetration facilitated by the acrosome reaction); 2) mitochondria and flagellar network (responsible for metabolism and motility); and 3) nucleus (for DNA storage). All intracellular organelles in mammalian sperm are covered by a membrane. During

cryopreservation, the plasma membrane represents the primary site of damage to sperm [96]. Vital staining has demonstrated that the plasma membrane is compromised by both the cooling and freeze/thawing process [96]. Recent studies have revealed that cooling sperm to temperatures above freezing results in capacitation-like membrane changes [97]. Investigations also have shown that membrane damage from cooling/freezing results in premature release of acrosomal contents [88, 98-100].

Thus, any successful sperm cryopreservation protocol must maintain membranes to ensure acrosomal, mitochondrial and nuclear stability. There are surprisingly few detailed, systematic studies of the influence of cryopreservation on membrane integrity and comparative cryo-approach studies for giant panda sperm.

Non-invasive hormone monitoring

Hormone fluctuations are an integral part to understanding reproductive changes and physiological changes in all animal species. Traditional approaches to endocrine monitoring have focused on serial blood sampling. This technique is often not possible for use with wild animal species. Captive wild animals have been trained for blood sampling, but this also includes potential dangers and is not an option for long term hormone monitoring. Alternatives such as non-invasive hormone monitoring through the collection and processing of feces or urine are widely used as an alternative for wildlife species, such as the giant panda [33, 76, 80, 101].

Specifically, fecal steroid monitoring has been used in other bear species such as the American black bear [102] and Asiatic black bear [103] indicating this is a valuable technique for monitoring reproductive physiology. More recently, fecal

androgens have been monitored in the male giant panda indicating that excreted steroid hormones and their metabolites are easily measured in feces [76]. Monitoring fecal and urinary hormones provides additional advantages over serum measurements because they represent averaged hormone concentrations over several hours rather than the pulsatile changes measured in serum [73, 76].

Kersey et al [76] indicated that androgens are elevated in the giant panda fibrous feces early in the year (Jan) corresponding with the initiation of the panda breeding season (Feb – May). These fecal androgens were correlated with serum and urinary androgen production of testosterone and androstenedione proving fecal androgens are reflective of physiologic androgen profiles [73]. This was the first study using male fecal androgens to evaluating male giant panda physiology.

In summary, decades of study on the giant panda set the stage for the following studies. Despite diverse research on the giant panda, there were three areas with a surprising paucity of information. The knowledge gaps in the areas of male seasonal reproduction, sexual maturity and comparative sperm cryopreservation were closed with the following three studies in dissertation.

Chapter 2:

Protracted reproductive seasonality in the male giant panda (*Ailuropoda melanoleuca*) reflected by patterns in testicular morphometry, androgen patterns, ejaculate characteristics and behavior

Abstract

The female giant panda experiences only a brief (24-72 h) seasonal estrus, occurring once between February and June. 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 years of access to eight male giant panda living at one of the world's largest, most successful breeding centers in China. Seasonal changes were characterized as reproductive quiescence occurring June 1 through September 30. This was associated with basal testes size/volume and aspermic ejaculates (non-breeding interval). Periods off reproductive change occurred from October 1 through January 31 (pre-breeding) and from February 1 through March 21 (early breeding season), with increasing ($P < 0.05$) testes size, fecal androgen excretion, ejaculated sperm density, and frequency of reproductive behaviors (i.e., locomotion, scent-marking and vocalizations). Testes volume and sperm concentration were maximal from March 22 through April 15, a period that coincided with maximal female breeding (peak breeding interval). Decreasing incidence of male reproductive behaviors and fecal androgen concentrations began during peak breeding and continued to decline from April 16 through May 31 (late breeding period), returning to nadir for the non-breeding interval. Our results reveal that testes morphometry,

androgen excretion in feces, seminal quality and certain behaviors integrated together and 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.

Introduction

The giant panda is one of the world's most recognized endangered species. A specialist bear that consumes grass (i.e., bamboo [4, 14]), its survival depends on suitable habitat in China, which now is largely restricted to certain protected and unprotected areas in the Sichuan, Gansu and Shanxii Provinces of China [3, 6, 15]. Broadly, there are an estimated 1,600 pandas remaining in nature [15, 103], with the greatest theat being habitat loss and fragmentation [6]. Because of uncertainty about the status of wild populations, China always has placed value on creating an ex situ collection of this species [104]. A captive population is a tangible asset with multiple values for research, educating the public and as insurance against a catastrophe affecting giant pandas in situ. A self-sustaining ex situ population also serves as a resource for animals to be reintroduced into appropriate wild habitats that now are under utilized by the species [10, 104].

Substantial progress has been made in enhancing the viability of the ex situ giant panda breeding program in China. Firstly, the limitations to successful reproduction have been identified through a cooperative, multi-disciplinary biomedical survey conducted from 1998 through 2000 by Chinese and USA partners [10]. Vast amounts of fundamental data were used to adjust animal husbandry and

veterinary health protocols and to enhance the efficiency of assisted reproductive technologies, especially artificial insemination [13, 105]. As a result, the collective ex situ population of giant pandas has nearly tripled in 11 years from ~120 to > 300 individuals [10, 79]. One major outcome of this success has been the availability of more animals for understanding and characterizing the unique reproductive biology of the species [104]. For example, it is well established that the female giant panda is seasonally reproductive, being sexually receptive from February through May [37, 54]. What is particularly unusual is that there is only one estrus per year occurring 24 to 72 h in duration [4, 80]. Thus, the female of this species devotes less than 1% of its annual lifespan to sexual activity [79]. Most reproductive research in the giant panda has focused on the female, especially inter-relating a complicated repertoire of behaviors to temporal fluctuations in gonadal hormones during the peri-estrual and post-ovulatory intervals, through a pseudopregnancy or parturition [101, 106].

Studies of male giant pandas largely have centered on ejaculate characteristics measured during the breeding season and the sensitivity of sperm to rates of cooling/freezing [7, 13, 25]. Otherwise, there is a paucity of physiological information on the male, especially on the existence of reproductive seasonality [13, 85]. Observations of a few zoo held individuals suggest that there are changes in male behaviors [29, 40, 107] and alterations in testes size and testosterone over time [76, 108, 109]. But clearly documenting the existence of a seasonality phenomenon in the male giant panda has proven difficult due to too few available research specimens managed under the same environmental conditions in one location [54]. However, recent advances in propagation and the construction of a state-of-the-art

center at the Chengdu Research Base of Giant Panda Breeding have eliminated this impediment. Furthermore, improved anesthetic protocols [105] to allow safe, hands-on assessment of body and testes morphometry [110] and semen collection [13] as well as the emergence of fecal hormone metabolite monitoring [76] also have allowed for quantifying and inter-relating multiple metrics at one time.

Our hypothesis was that reproduction in the male giant panda is obligately seasonal. However, unlike the female where gonadal function onset is abrupt and sexual interest brief, reproductive capacity in the male changes gradually over time and in a protracted fashion that is reflected in variations in testis size, androgen production, semen quality and overt behaviors. Further, we predicted that prolonged seasonality in the male giant panda provides a mechanism evolved to accommodate the short and unpredictable estrus of multiple female conspecifics. This study was important for filling a major knowledge gap in the life history and adaptations of a unique and endangered species [10, 54]. Furthermore, understanding the regulators of male (and female) reproductive function will allow continued enhancement of the ex situ management program to achieve demographic and genetic stability, including allowing reintroduction of this species into remaining viable habitat in China [5, 6, 10].

Materials and Methods

Animals and approvals

Eight adult male giant pandas (5 – 21 yr of age) were maintained at the Chengdu Research Base of Giant Panda Breeding and the Chengdu Zoo (related and nearby institutions) in Sichuan Province, People's Republic of China (30°N, 104°E). All

individuals were born in captivity, with four males being proven breeders as demonstrated by the production of living young. Each giant panda was housed individually in enclosures with combined indoor (3.0 m x 3.0 m to 10 m x 20 m) and outdoor (12 m x 12 m to 20 m x 35 m) areas, all of which were illuminated by natural lighting. Water was available ad libitum, and freshly-cut seasonally available wild bamboo (10 – 20 kg daily) was provided to each male throughout the day along with a high fiber biscuit supplement (~1,000 gm/day/male; range, 700 – 1,500 gm/day/male) for additional fiber, vitamins and minerals (proprietary recipe of the Chengdu Research Base). All males were housed individually and in 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 (Feb –May) and for brief (2 to 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, University of Maryland, Chinese Association of Zoological Gardens, and Chengdu Research Base of Giant Panda Breeding. Transport of fecal samples (collection protocol below) from China to the USA was approved and monitored by the Convention on the International Trade of Endangered Species, United States Department of Agriculture and the United States Fish and Wildlife Service.

Characterization of seasonality in the female giant panda

To identify potential intervals of male reproductive seasonality, we first re-examined the phenomenon of fluctuating reproductive activity in the female giant panda.

Seasonality has been well-described for females, usually using a combination of changes in behavior [23, 40, 46] and hormonal patterns determined by plotting steroidal metabolites voided in urine [76, 80]. We used these combined and highly reliable approaches to measure the prevalence of female sexual activity, specifically peak estrus, during different times of the year. This was accomplished by monitoring behavioral fluctuations and estrogen profiles in 16 adult female pandas housed in these same two locations and over the course of 4 yr (n = 46 total 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). This interval was designated as 'peak breeding season'. However, intervals of peak estrus (14 of 46; 30%) also occurred from February 1 through March 21, an interval we labeled as 'early breeding season' (Fig. 1). There also were some instances (9 of 46; 20%) when peak sexual behavior plus estrogen excretion occurred 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 we labeled as the female non-breeding season. Therefore, based on these data and criteria, we were able to objectively identify four distinctive reproductive periods for female giant pandas: 1) non-breeding; 2) early; 3) peak and 4) late breeding season (Fig. 1). These distribution findings were used as a baseline for relating changes in reproductive activity in conspecific males (see below).

Using the National Oceanic and Atmospheric Administration online database for Chengdu, China [online: http://weather.noaa.gov], we also collected data on

seasonal fluctuations in daylight (h), humidity (%) and temperature (°C). Data over the course of the 3 yr study were used to generate weekly mean values for each of these metrics.

Testicular and body morphometry and ejaculate characteristics

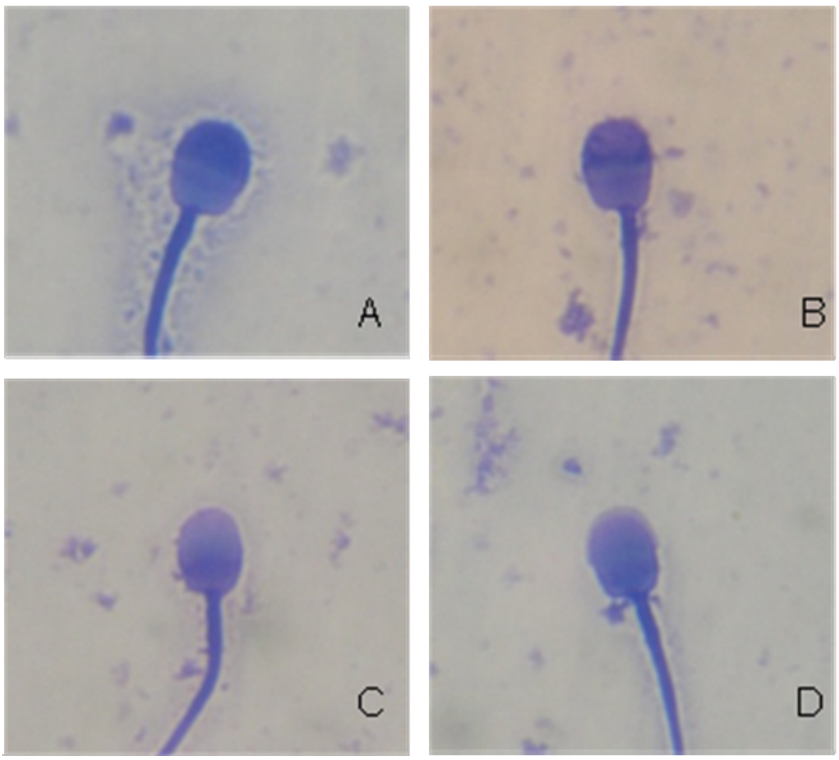
Each male was conditioned to walk across an electronic scale (Shanghai Yitai Electromechanical Equipment Co., Ltd. Shanghai, China) as needed with a minimum of once monthly to produce data on body mass. Each of the study males was anesthetized with the use of 10 to 12 mg/kg ketamine hydrochloride (Sinceta International Trading Co., Ltd., Shanghai, China) delivered by intramuscular injection [105] and, occasionally, isoflurane gas anesthesia was used (Ningbo Samreal Import and Export Co., Ltd., Shanghai, China) via a face mask or intubation to increase depth and/or duration of anesthesia. While under anesthesia, testicular and body morphometry measurements were collected for each panda [105, 110]. Specifically, the length and width of each testicle were measured using a digital caliper (Model #500-196-20 Absolute Digimatic Calipers, Mitutoyo Corp., Aurora, IL, USA), and volume of each testis was calculated using the formula: volume = $4/3\ \pi\ ab^2$ ('a' is 1/2 length; 'b' is 1/2 width) [13, 85]. Total testicular volume per male was determined by combining the right and left testis volumes. Calipers and/or a flexible tape were used to ascertain chest girth, abdominal girth, contour length of body from tip of nose to base of tail and tip of tail, right foreleg circumference at elbow and axial skin fold thickness, all of which have been used previously for assessing giant panda morphometry [110].

While each male was in a surgical plane of anesthesia, electroejaculation was conducted using a well-described approach [7, 13, 84, 85]. In brief, this involved the 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, Boring, Oregon, USA). Standardized sets of low voltage stimulations (2 – 5 V) over three series of 30 stimuli each were delivered over about 20 min to elicit an erection with ejaculation into a collection container with temperature-controlled (23°C) water jacket [13]. Seminal volume was measured and pH determined using pH indicator strips (Colorphast®, EM Science, Gibbstown, NJ, USA). A 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 – 400x) [13]. Sperm concentration was calculated using a standard hemocytometer method [13]. Total sperm per ejaculate was calculated (sperm concentration/ml x total volume of ejaculate) and recorded.

Sperm morphology was assessed after fixing a 10 µl sample of raw semen in 100 µl of fixative (0.3% glutaraldehyde in phosphate buffered saline) and using phase contrast microscopy (1,000x) to evaluate 200 sperm per sample [13]. In cases of multiple defects for a given spermatozoon, each was classified according to the most serious malformation [13]. For example, if a cell was observed to contain both a bent flagellum and an abnormal acrosome, it was categorized as having the latter abnormality [7]. Individual defects then were compiled into three general categories of sperm pleomorphisms (head, midpiece or flagellar) with deformities related to the head region being considered the most serious [13].

Sperm head morphology was further delineated by detailed evaluations of acrosomal integrity using rose bengal/fast green stain [13]. Briefly, an aliquot of raw, unfixed semen was diluted in Ham's F10 medium (Irvine Scientific, Santa Anna, CA; 1:10 semen-to-medium) and then 1 μl of this mixture added to 9 μl of rose bengal (High Purity Biological Stain, Cole-Parmer, Vernon Hills, IL) and fast green stain (Certified Biological Stain, Sigma-Aldrich, St. Louis, MO), incubated for 90 sec, and smeared on a glass slide. A minimum of 100 sperm acrosomes per sample were assessed by bright-field microscopy (1,000x) for either a: 1) normal intact apical ridge (uniform staining of the acrosome over the anterior half of sperm head); 2) damaged apical ridge (non-uniform 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) (Fig. 1). Depictions of these morphological defects and acrosomal types for the giant panda are provided in Howard et al [13] and Fig. 1.

Fig. 1



Acrosomal morphology of the giant panda spermatozoon with a (A) normal apical ridge (uniform staining of acrosome over the anterior half of the sperm head), (B) damaged apical ridge (non-uniform staining of acrosome), (C) missing apical ridge (lack of staining due to absence of the acrosome) or (D) loose acrosomal cap (loose membrane protruding above the level of the sperm head).

Fecal androgen assessment

Enzyme-immunoassays (EIA) recently have been validated for monitoring marked and subtle alternations in fecal gonadal steroid content in giant pandas (estrogen and progesterone in females [33, 101], androgens in males [76]). We took advantage of these advancements by collecting a fresh (< 1 h post-excretion) fecal sample from each of the eight male giant pandas every 48 to 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 was then 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 hormone analysis.

Prior to hormone analysis, samples were processed by freeze-drying (Lyophilizer, Labconco, Kansas City, MO), crushed and hormone extracted using previous methods [76]. For hormone extraction, 0.1 g of fecal powder was extracted using 90% ethanol. The extracts were vortexed and centrifuged to remove particulate, sonicated with glass beads and then dried under air and resuspended in 1 ml of BSA-free phosphate buffer before freezing for storage [33, 76]. The recently validated EIA [76] then was used, which relies 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, Pittsburgh, PA) and allowed to equilibrate for 12 to 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, Newport, RI) were added to the EIA microtiter plate.

An 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 inter-assay correlations of variation was < 10% and 10%, respectively [76].

Baseline (harmonic mean) concentrations of fecal androgen metabolites were determined through an iterative process [76]. In brief, baseline values were assessed yearly in each male, then for each male, then overall. 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 harmonic 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 [40] (Table 1). The behavior of each male was evaluated in detail during two consecutive 30 min focal observation periods conducted twice weekly continuously throughout the 3 yr study. Data were collected by a total of 11 observers who were intensively trained in behavioral identification and recording for 2 wk. Consistency among observers was monitored by comparing responses for the same observation period and found to be ≥80% over the study interval. Each animal was examined with a balanced number of morning (0800 – 1130 h) and afternoon (1300 – 1700 h) observations twice per wk. On the rare occasion that observations were perturbed by

an animal-keeper interaction (e.g., unscheduled shifting to an adjacent enclosure or breeding event), the data associated with that episode were deleted, and a replacement observation period conducted within 24 – 48 h.

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

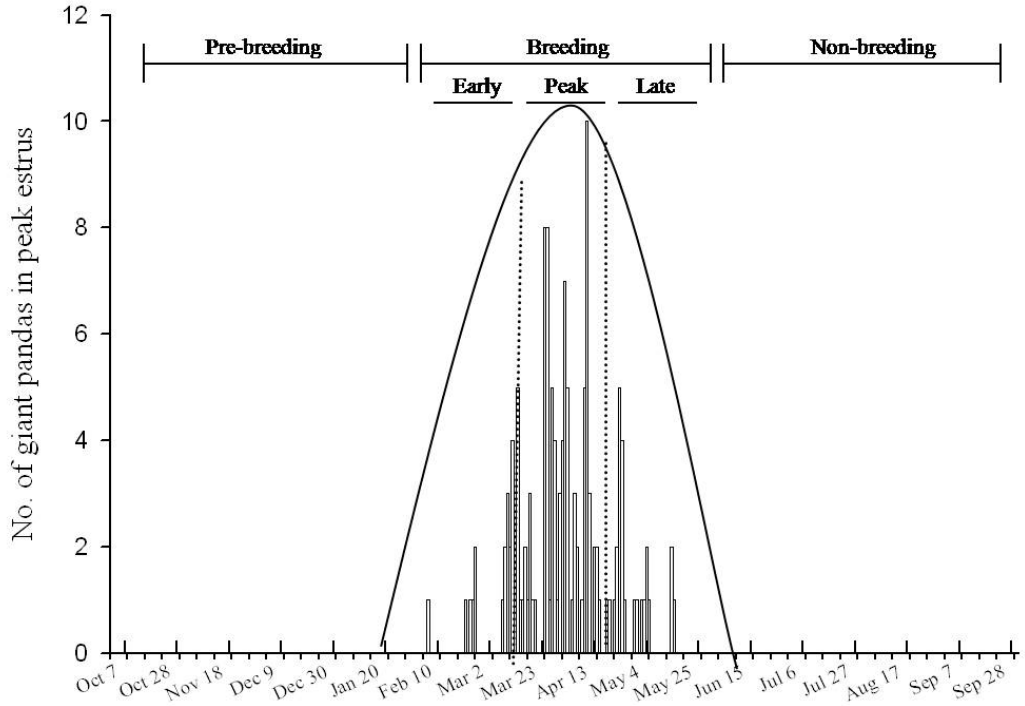
TABLE 1. Ethogram for male giant panda behaviors assessed during behavioral observation periods.

Behavior	Description of Behavior
Anogenital scent mark (SM)	Animal rubs anogenital region (tail up) against object or substrate with back and forth circular motion.
Handstand mark (SM-HM)	Animal raises body and hind limbs leave the ground; anogenital area may make contact with substrate.
Leg cock mark (SM-LM)	Animal is on three limbs with one hind leg raised and rubs anogenital area against object or substrate.
Reverse mark (SM-RM)	Animal backs to a vertical surface before marking in a circular motion, as opposed to rubbing back and forth.
Squat mark (SM-SQ)	Animal lower hindquarters to substrate/object and rubs anogenital area against object or substrate.
Handstand urine mark (UR-HM)	Like handstand mark, but instead of rubbing anogenital area on substrate, animal also urinates on substrate.
Leg cock urine mark (UR-LM)	Like leg cock mark, but instead of rubbing anogenital region, animal also urinates on substrate.
Affiliative interaction (AF)	Animals touches or sniffs conspecific; may occur with bites, paw swatting, wrestling, grappling while standing.
Barrier interact (BI)	Animal interacts with conspecific through or at a barrier; includes sniffing, pawing, vocalizing and rolling.
Olfactory behaviors	Animal investigates scent of an object, includes scent anoint, open mouth olfactory, lick olfactory (see below).
Scent anoint (SN)	Animal picks up object and rubs object over body includes rolling on, rubbing against objects or substrates.
Open mouth olfactory (OM)	Animal raises lips, exposing teeth and/or opens mouth while investigating scent.
Lick olfactory (OL)	Animal licks area while investigating scent.
Vocalization (VC)	Animal vocalizes by making audible sound; includes chirp, bleat, grunt, and squeal.
Investigate/explore (IX)	Animal's body is stationary or moving while engaged in investigation of object or substrate in enclosure.
Locomotor (LC)	Any traveling in the exhibit, including short or sustained directional travel between two points.
Stereotypy behaviors	Repetitive behaviors including non-motile stereotypy, locomotor stereotypy, pacing (see below).
Non-motile stereotypy (ST)	Range of repetitive behaviors including paw sucking, paw licking, pirouette, tongue flicking, vomiting, head swaying, body swaying. Body is generally stationary, i.e. not walking while performing stereotypy.
Locomotor stereotypy (LM-ST)	Repetitive motile behaviors in the same location as part of a travel pattern that is repeated more than three times. May include rolling, somersaults, and pirouettes while walking.
Pace (PC)	Animal travels same repetitive path at least three times, typically a short and uncomplicated pattern.
Rest/sleep (RS)	Animal is recumbent, seated or leaning against substrate, not moving except for intermittent shifts in position.
Stationary alert (SA)	Animal is standing, sitting or lying but remaining attentive, moving head from side to side or sniffing air.
Not visible (NV)	View of animal is obstructed.

Statistical analysis

Data for each male and each year of the 3 yr study interval was assessed for influence within the model and no effect was seen using fixed effects influence diagnostic (SAS©, 9.1.3, SAS 2003 Cary, NC) and 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 (% normal morphology, % abnormal acrosomes) were arc sin transformed. Morphometric, androgen and semen traits were summarized for each seasonal period and analyzed using ANOVA followed by Tukey-Kramer test of multiple comparison to determine differences among assessed metrics and time intervals (SAS©, 9.1.3, SAS 2003). Behavioral data were compared using GLMIX® (SAS 9.1.3, SAS 2003). Based on the preliminary 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., non-breeding, 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 'pre-breeding' season that was factored into statistical analyses. Differences were considered significant at $P < 0.05$. Data are presented as means ± SEM.

Fig 2.



Distribution of ex situ female giant pandas (n = 16) in peak estrus during breeding seasons in China. Peak estrus was 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 (Feb 1 - May 30) over 3 consecutive years. Breeding season was divided into three categories based on the number of females in peak estrus during the early (Feb 1 - Mar 21), peak (Mar 22 – Apr 15), and late (Apr 16 - May 31) breeding season.

Results

Body and testicular morphometry

There were no differences ($P > 0.05$) across reproductive seasons for any of the general body morphometrics described in the Materials and Methods. Data from example traits, including body mass, chest girth and abdominal girth, are presented in Table 2. Mean male body morphometrics including contour length of body from tip of nose to base of tail (170.0 ± 3.9, 131.4 ± 22.4, 184.7 ± 5.0 cm), right foreleg circumference at elbow (25.9 ± 1.0, 27.8 ± 1.1, 29.0 ±2.0 cm), and axial skin fold thickness (2.6 ± 0.3, 2.6 ± 0.6, 3.0 ± 0.3 cm) did not change ($P > 0.05$) between pre-breeding, breeding, and non-breeding seasons, respectively.

In contrast, there were changes ($P < 0.05$) in testis size between our designated reproductive seasons. The result was almost a 2-fold increase ($P < 0.05$) in testicular volume from the non-breeding (onset in Jun) to pre-breeding (onset in Oct) season, followed by another 50% increase at the early breeding period that was sustained through the late interval (Table 2). There were no differences ($P > 0.05$) in the size of the right versus left testis, and changes in size over time reflected both increased gonadal length and width (Table 2).

TABLE 2. Seasonal body and testicular morphometry in giant pandas in China during a 3 yr study.

	Pre-breeding Oct 1 – Jan 31	Early breeding Feb 1 – Mar 21	Peak breeding Mar 22 – Apr 15	Late breeding Apr 16 – May 31	Non-breeding 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.

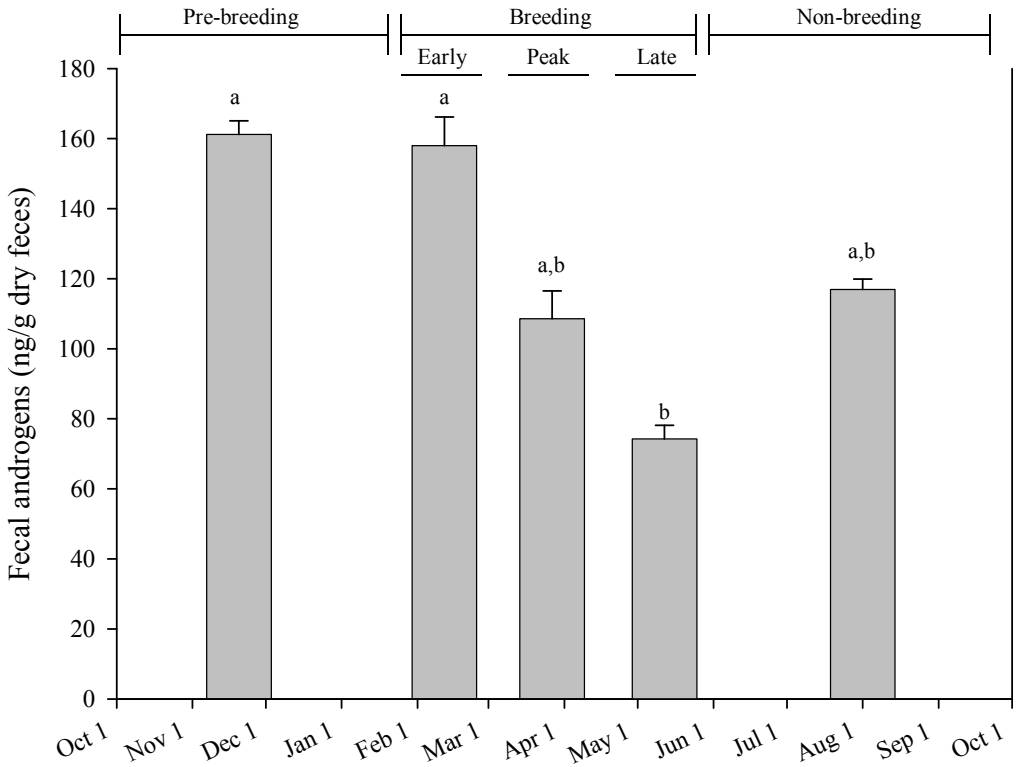
[a,b,c]Within a row, values with different superscripts denote differences among seasons ($P < 0.05$).

Androgen concentrations and patterns

There were differences ($P < 0.05$) in fecal androgen concentrations in male giant pandas over time, with basal levels (74.2 ± 3.9 ng/g dry feces; n = 414 samples) measured during the late breeding season (Fig. 3). Absolute maximal values detected during the pre-breeding (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 were higher ($P < 0.05$) than maximal values during the late breeding season (Fig. 3). Indicating the only low period of androgen production occurred during the late breeding season. 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) that continued to the late period mean nadir (Fig. 3).

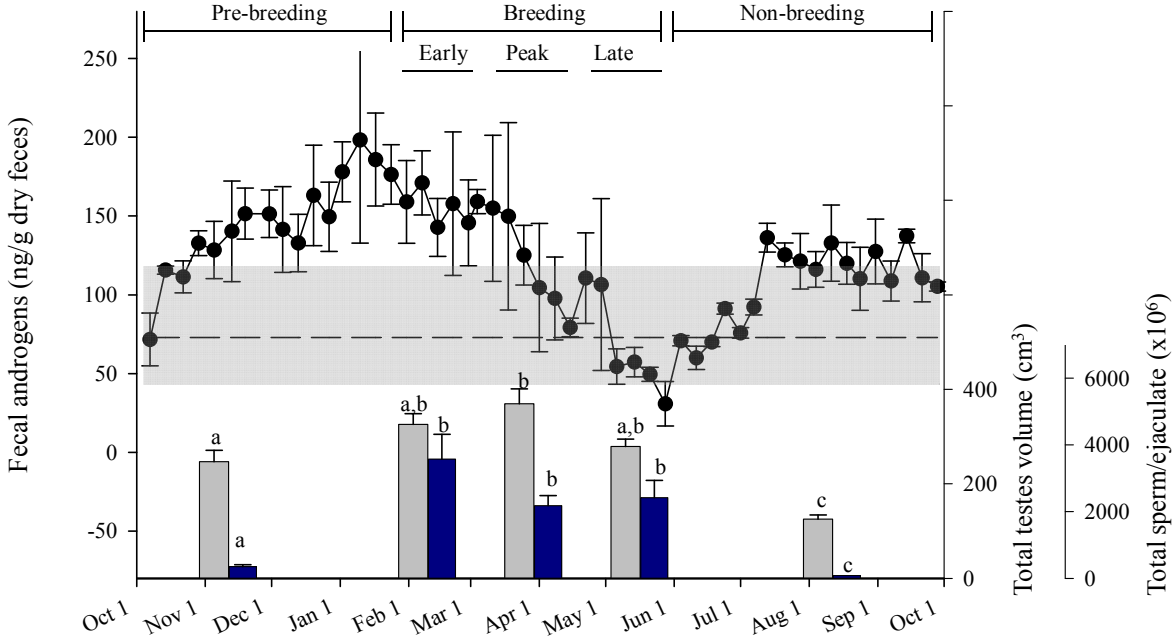
Temporal fluctuations in fecal androgen concentrations were best reflected in weekly means for the entire male cohort (Fig. 4) and by representative individuals (Fig. 5). The harmonic mean showing an androgenic baseline was calculated within two standard deviations (indicated by the shaded area). In all cases, nadir androgen excretion was observed in the late breeding season (mid-April through May) with variations until October remaining within (or near) baseline (Fig. 4). However, October through January (pre-breeding interval) was associated with distinct increases in androgen production that were sustained from February through mid-April (early and peak breeding periods; Fig. 4), intervals when most females were in estrus (Fig. 2). Interestingly, by April 15 (and despite 19% of females still not displaying an annual estrus), androgen production was declining towards the nadir zone (Fig. 4) about 6 wk before onset of the female non-breeding season (June 1).

Fig. 3



Total androgen concentrations in adult male giant pandas (n = 8) in China during a 3 yr period. Bars represent mean (± S.E.M.) total androgens within a season during the pre-breeding (Oct 1 through Jan 31), early breeding (Feb 1 - Mar 21), peak breeding (Mar 22 - Apr 15), late breeding (Apr 16 - May 31) and non-breeding (Jun 1 - Sept 30) season. Means with different superscripts represent differences among seasons ($P < 0.05$).

Fig. 4



Fecal androgens, testicular volume and total sperm per ejaculate in male giant pandas (n = 8) in China during 3 yr period. Mean (± S.E.M.) fecal androgens (closed circles) during pre-breeding (Oct 1 - Jan 31), early breeding (Feb 1 - Mar 21), peak breeding (Mar 22 – Apr 15), late breeding (Apr 16 - May 31) and non-breeding (Jun 1 – Sep 30) seasons. Baseline androgen (dashed line) ± S.E.M. (shaded area). Mean (± S.E.M.) testicular volume (gray bar) and total sperm per ejaculate (black bar) are shown. Means with different letters within each trait represent differences among seasons ($P < 0.05$).

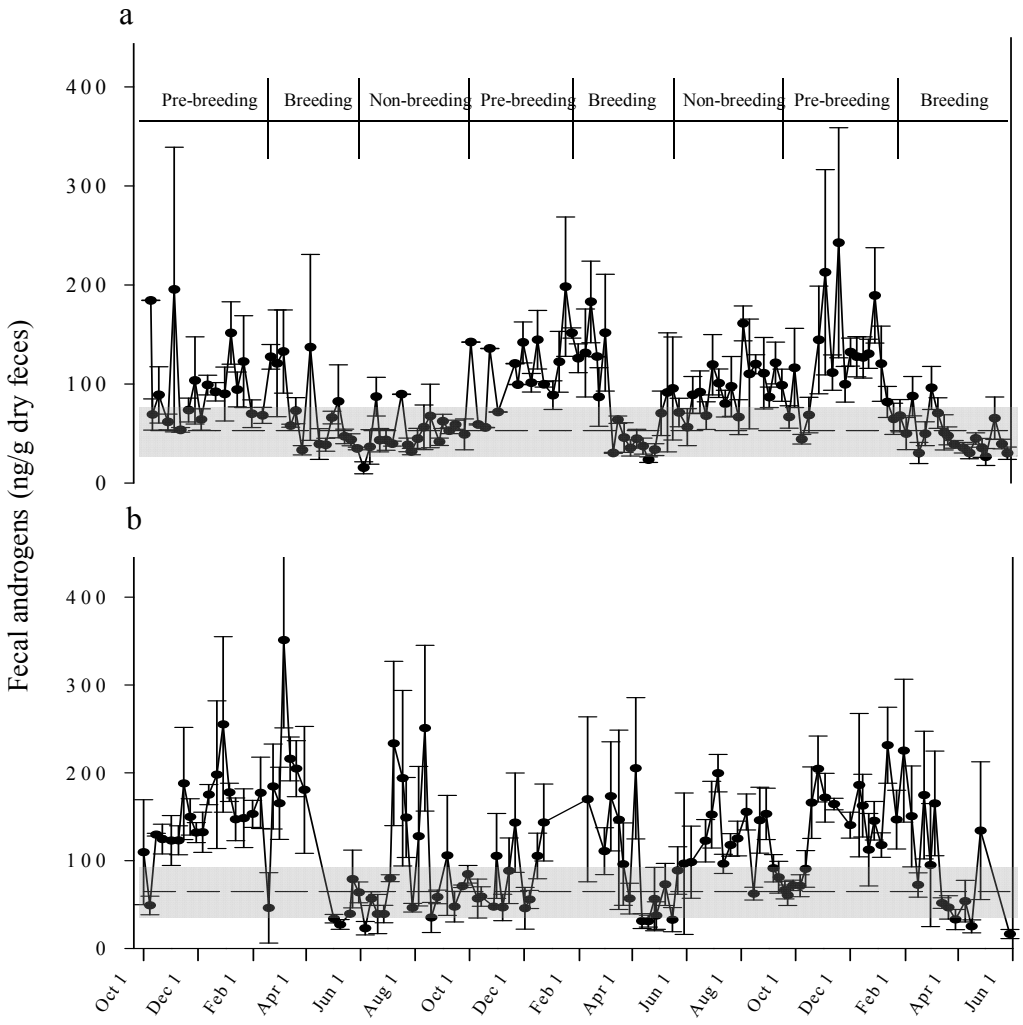
When applying our statistical model to evaluate androgen patterns among males over time, there were subtle but insignificant ($P > 0.05$) variations among males (Fig. 5 a, b). There were no androgen pattern differences ($P > 0.05$) within given males over time, with onset of the rise and fall in this metabolite consistent ($P > 0.05$) within an individual from year to year (data not shown).

Ejaculate characteristics

Ejaculate traits followed seasonal patterns in testis size and androgen excretion (Table 3). Only 50% of males (three of six individuals) produced spermic ejaculates during electroejaculation episodes conducted during the pre-breeding season. In contrast, 100% of males produced spermic ejaculate during the early, peak and late seasons compared to 0% (0 of 6) during the non-breeding period (all of the latter also failing to produce seminal fluid during stimulation).

Ejaculate volume, sperm concentration and total sperm per ejaculate were lowest during the non-breeding season and highest during the early, peak and late breeding season compared to the pre-breeding period (Table 3; $P < 0.05$). Time of year had no influence ($P > 0.05$) on ejaculate pH or sperm motility traits (Table 3). The inter-relationship of total sperm per ejaculate with total testes volume and mean weekly androgen excretion throughout the year is depicted in Figure 4. Increased number of ejaculated spermatozoa followed a protracted interval of larger testes size and more androgen excretion.

Fig. 5



Representative weekly androgen profiles of two individual male giant pandas during the pre-breeding (Oct1 - Jan 31), early breeding (Feb 1 - Mar 21), peak breeding (Mar 22 - Apr 15), late breeding (Apr 16 - May 31) and non-breeding (Jun 1 - Sep 30) season over 3 consecutive years. 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.

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

	Pre-breeding Oct 1 – Jan 31	Early breeding Feb 1 – Mar 21	Peak breeding Mar 22 – Apr 15	Late breeding Apr 16 – May 31	Non-breeding 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 ($x10^6$)*	601.3 ± 586.4[a]	1,955.3 ± 371.8[b]	1,669.7 ± 508.8[b]	1,780.4 ± 241.0[b]	0.0[c]
Total sperm/ejaculate ($x10^6$)*	61.9 ± 58.5[a]	3,571.2 ± 744.6[b]	2,178.2 ± 299.5[b]	2,414.9 ± 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*[1]	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 represent means ± SEM. No available ejaculate is represented as n/a.

[1]Scale 0 to 5; 5 = best.

[a,b,c]Within a row, values with different superscripts denote differences among seasons ($P < 0.05$).

Giant pandas generally produced more than 50% structurally normal spermatozoa per ejaculate, with most malformations being related to midpiece or flagellar defects (Table 3). Head pleiomorphisms always comprised 5% or less of total forms analyzed. On the basis of our circannual evaluation, flagellar deformities were more prevalent ($P < 0.05$) during the pre-breeding than breeding season (Table 3). Virtually all of the structural abnormalities observed from October through January were associated with proximal cytoplasmic droplets (35.5 ± 12.4 % of all recovered spermatozoa). Likewise, giant pandas produced high proportions of spermatozoa with intact and normal acrosomes (Table 3). Even during the pre-breeding 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 3). The fewest sperm with a damaged apical ridge were measured during the early and peak breeding season ($P < 0.05$); otherwise, there was no variation in acrosomal integrity (Table 3).

Behaviors

There were changes in certain male giant panda behaviors over time with scent marking, vocalization and pacing clearly influenced ($P < 0.05$) by the seasonal periods (Table 4). For example, there was an increased incidence ($P < 0.05$) of males demonstrating handstand, squat and handstand urine markings with transition from non-breeding to pre-breeding/breeding season (Table 4). Although there was a tendency for a decline in all of these behaviors over three phases of the breeding season, variation among type of scent marking performed by individual males prevented differences from being significant ($P > 0.05$). Likewise, male vocalizations and pacing increased from the lowest incidence during the non-breeding period to become maximal

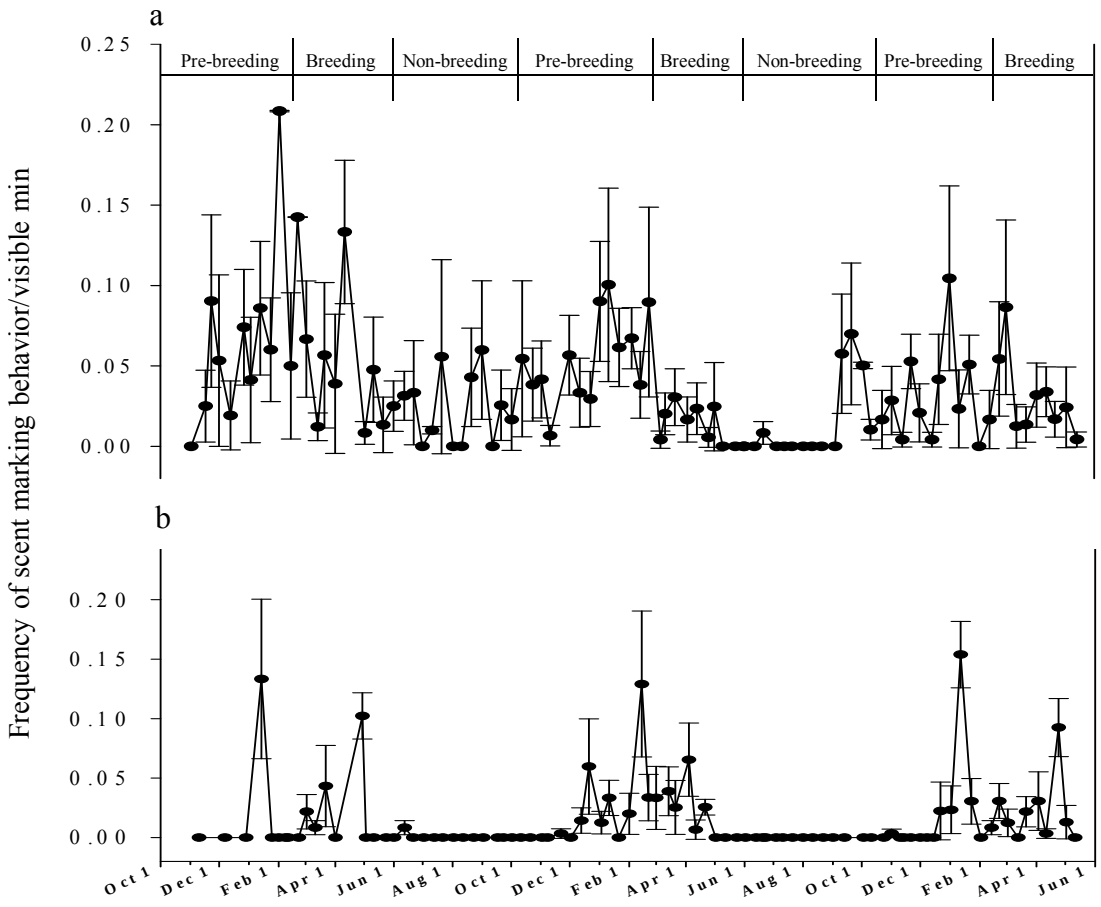
throughout the reproductive season, especially during the interval when most females were in estrus (Table 4). Changing patterns of scent marking, vocalization and pacing also were quantifiable within individual animals. To illustrate, Figure 6 depicts frequency of total scent marking in two representative males over the course of three breeding seasons. The individual in Figure 6a is a male that never successfully mated, but rather expressed aggression towards females. This animal displayed modest marking behavior throughout most of the year, including during non-breeding/pre-breeding intervals. In contrast, the results for the male giant panda depicted in Figure 6b, had sired multiple offspring with an array of females and in the absence of aggression, largely scent-marked only during late pre-breeding and throughout most of the breeding interval.

TABLE 4. Seasonal frequency of behaviors in male giant pandas in China during a 3 yr study.

	Pre-breeding Oct 1 – Jan 31	Early breeding Feb 1 – Mar 21	Peark breeding Mar 22 – Apr 15	Lat breeding Apr 16 – May 31	Non-breeding Jun 1 – Sep 30
No. males observed	8	8	8	8	8
Total scent marking ($x10^{-3}$)*	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 ($x10^{-3}$)	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 ($x10^{-3}$)	2.4 ± 0.4	2.0 ± 0.5	1.2 ± 0.5	0.4 ± 0.2	1.8 ± 0.5
Reverse mark ($x10^{-3}$)	4.2 ± 0.7	3.6 ± 0.8	5.2 ± 1.5	2.1 ± 0.6	1.1 ± 0.3
Squat mark ($x10^{-3}$)	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 ($x10^{-3}$)	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 ($x10^{-3}$)	1.4 ± 0.3	1.0 ± 0.3	1.1 ± 0.4	1.7 ± 0.5	1.0 ± 0.2
Affiliative interaction ($x10^{-3}$)*	0.1 ± 0.0	2.8 ± 1.2	2.3 ± 1.1	0.1 ± 0.1	0.5 ± 0.3
Barrier interaction ($x10^{-3}$)*	9.3 ± 1.0	25.5 ± 2.2	33.0 ± 4.6	14.8 ± 1.8	4.7 ± 0.7
Total olfactory behaviors ($x10^{-3}$)*	15.5 ± 1.8	15.6 ± 2.9	41.4 ± 10.1	27.1 ± 4.8	9.4 ± 1.2
Scent anoint ($x10^{-3}$)	3.6 ± 1.1	2.4 ± 0.8	4.7 ± 1.8	3.2 ± 1.4	1.4 ± 4.9
Open mouth olfactory ($x10^{-3}$)	6.1 ± 0.9	6.1 ± 1.0	21.3 ± 4.6	16.3 ± 2.7	6.0 ± 0.8
Lick olfactory ($x10^{-3}$)	5.6 ± 0.7	7.1 ± 2.2	15.4 ± 6.2	7.6 ± 1.8	1.8 ± 0.5
Vocalization ($x10^{-3}$)*	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($x10^{-3}$)*	42.5 ± 2.2	42.5 ± 4.4	57.9 ± 7.3	50.6 ± 4.4	22.2 ± 1.3
Non-motile stereotypy ($x10^{-3}$)*	44.7 ± 10.0	23.7 ± 6.5	10.6 ± 2.4	17.9 ± 8.3	36.7 ± 10.4
Total motile stereotypy ($x10^{-3}$)*	40.2 ± 3.0	49.2 ± 3.8	44.1 ± 5.2	31.2 ± 3.7	20.1 ± 2.5
Locomotor stereotypy ($x10^{-3}$)	19.2 ± 6.9	16.3 ± 2.0	7.9 ± 1.8	4.8 ± 1.1	5.9 ± 1.3
Pace ($x10^{-3}$)	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 min.
[a,b,c]Within a row, values with different superscripts denote differences among seasons ($P < 0.05$).

Fig. 6



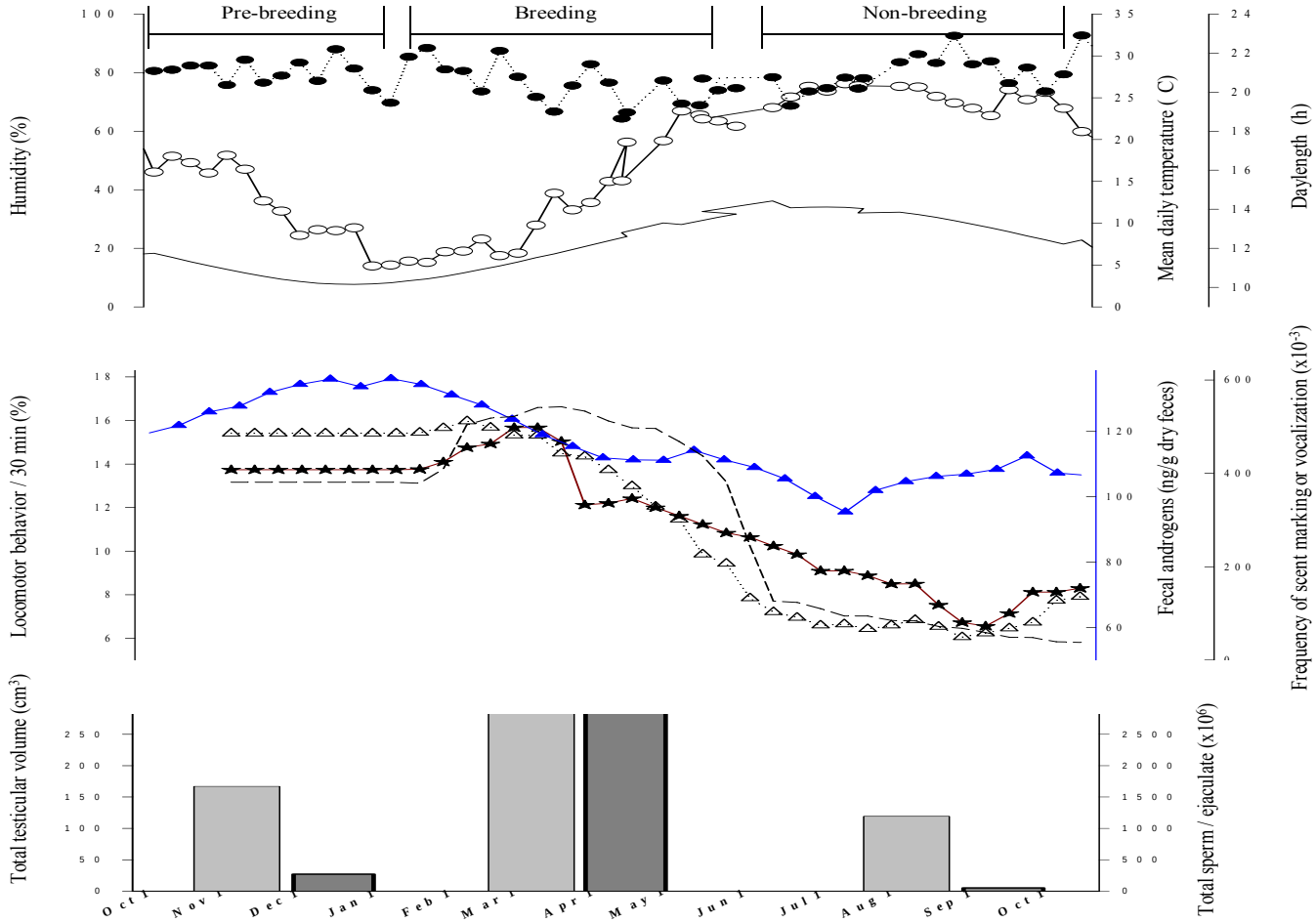
Representative 10 day profiles of frequency of total scent marking behavior (mean ± S.E.M.) occurring per visible minute in two male giant pandas during pre-breeding (Oct 1 - Jan 31), early breeding (Feb 1 - Mar 21), peak breeding (Mar 22 - Apr 15), late breeding (Apr 16 - May 31) and non-breeding (Jun 1 - Sept 30) season over a consecutive 3 yr period.

Inter-relationships of combined metrics

When collective data on testes volume, androgen patterns, sperm concentration and key behaviors were integrated with mean fluctuations in humidity, ambient temperature and day length, we were able to construct a model of inter-relationships occurring during annual reproduction in the male giant panda (Fig. 7). Temperature fluctuated with changes in day length, but not over differing years, and humidity was invariant. As day length waned in late summer and autumn, the amount of excreted androgen increased in the absence of detectable increases in testes mass. Maximal androgen concentrations coincided with shortest day length (<10 h) and the coolest ambient temperatures. Environmental humidity was irrelevant to male patterns.

Within 30 to 45 days of elevated androgens, there were increased activities related to scent marking, vocalization and locomotion and all occurring maximally during periods of shortest day length. Furthermore, during the last quarter of the year (and at least 60 days prior to the first female demonstrating estrus), testes volume and corresponding sperm production were markedly elevated ($P < 0.05$). Both continued to rise, with highest sperm output reached in April about 3 mo after maximal androgen production in January. The periods of longest day length (> 12 h) in Chengdu occurred during June through August. During these months, testes mass, fecal androgen concentration, sperm concentration and reproductive behaviors (Fig. 7) were at nadir. The incidence of scent marking appeared ubiquitous after October 1 through the peak breeding period as day length decreased, but both the frequency of vocalization and pacing were maximal at least 30 days after measuring maximal androgen excretion.

Fig. 7


Pre-breeding
Breeding
Non-breeding
Humidity (%)
Mean daily temperature (C)
Daylength (h)
Locomotor behavior / 30 min (%)
Fecal androgens (ng/g dry feces)
Frequency of scent marking or vocalization (x10-3)
Total testicular volume (cm3)
Total sperm / ejaculate (x106)
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct

Seasonal reproduction in the male giant panda depicting the interrelatedness of daylength (closed circles), fecal androgen concentrations (triangles), vocalizations (squares), total scent marking (solid line), total locomotor behaviors (stars), total testicular volume (gray bars), total sperm per ejaculate (black bars) over consecutive yr during the pre-breeding (Oct 1 - Jan 31), breeding (Feb 1 - May 31) and non-breeding (Jun 1 – Sep 30) season base on evaluation of eight captive males over 3 consecutive years..

Discussion

The female giant panda is renowned as an example of a mammal that devotes little time (only 24-72 h annually) to sexual interaction with a male [79]. Significant research has been conducted to characterize and understand periestrual events in this species [32, 33, 54, 101], with results then used to help create a self-sustaining ex situ collection of this endangered species by both natural copulation and artificial insemination [9, 13, 79].

The female giant panda is clearly an obligate seasonal breeder [4, 14, 54], which was confirmed in our preliminary evaluations at our research site; 100% of estrous periods were detected from February through May. Although there are no systemic cause and effect data, there is the perception that the onset of female sexual receptivity in this species is driven by increasing day length [104]. Nonetheless, the female enters estrus somewhat unpredictably, experiencing a 4 to 15 day proestrus characterized by gradually rising estrogen (detected in urine [33] and feces [101]) and behavioral shifts (eventually 'bleating', tail-up, backward walking) that culminate in brief (2-15 min) copulatory periods [30, 34]. Otherwise, the consistently baseline, flat estrogen profiles and lack of expressive sexual behaviors for ~350 days per year indicate that gonadal function is normally suppressed before reinitiated quickly near breeding period onset. In contrast, the male of this species has evolved a markedly different strategy. From the present study monitoring morphological, physiological and behavioral traits simultaneously in multiple individuals, it was clear that the male giant panda experienced reproductive activation over a protracted interval. In fact, when all data were inter-related, it appeared that male gonadal function was only

briefly in quiescent abeyance. Rather, there were subtle increases in androgen excretion beginning within 6 wk of the end of the active female breeding season. This androgen production remained modest until late October when a more dramatic rise coincided with increased sperm production capacity and male sexual behaviors reaching peaks during periods of short day length.

The giant panda is one of eight Ursidae species [111], being the most phylogenetically distant from other members of this family [20]. Although reproductively unique for its brief and singleton estrus per annum [54], the giant panda shares a seasonal breeding trait with most other temperate living ursids, including the brown bear (*Ursus arctos*) and American black bear *(U. americanus),* exposed to fluctuating photoperiods [54]. These examples help support what appears to be a growing consensus that increasing day length is a regulator for provoking reproduction in bears in general [54]. But the specific mechanism neither is well understood nor consistent, being somewhat confounded by ursids that live in tropical environments like the Andean (*Tremarctos ornatus,* also known as the Spectacled bear [112]), sloth (*Ursus ursinus* [113]) and Malayan sun (*Helarctos malaynus* [57]) bear. For these animals, photoperiod does not waver significantly from a 12 h light: dark cycle, and, while capable of reproducing circannually, these species experience peak breeding events, typically multiple, during certain times of the year [54]. For example, the male sun bear undergoes a 3 to 4 mo testicular regression, but without any detectable seasonal variation in circulating testosterone patterns [58]. Female conspecifics appear to breed throughout the year, but with a greater incidence of reproductive activity from November through January [57]. Thus, in tropical bears,

other reproductive cues may be at play such as rainfall and levels of nutrition. Although Spady et al. [54] found some correlation between geographic location and timing of seasonal estrus in *U. arctos, U. maritimus, U. americanus, A. melanoleuca*, there has been no consistent impact of rainfall, temperature or growing season [54]. High nutrition was seasonally constant in our study population of male giant pandas and, thus, was not a factor in our observation of male reproductive seasonality. This high nutritional plane probably also explains the lack of any significant changes in non-reproductive morphometrics, for example, body mass. Although empirical data is unavailable for free-living giant pandas they may display similar patterns to black bears that increase in body size, largely to accommodate energy storage to allow males to roam further and longer in search of females [114, 115].

Regardless of the primary stimulus, the male giant panda experienced waves in reproductive activity, including seasonal differences in testes volume, androgen patterns, sperm density and sexual behaviors. Many of these changes occurred 3 to 5 months before the interval when most females displayed their brief estrus. This leisurely change in male reproductive activity was consistent with previous findings in the brown bear [42], Asiatic black bear (*Ursus thibetanus*; [51]), polar bear (*Ursus maritimus;* [56, 116] and American black bear [53, 117]. There is evidence that each of these species experience gradually rising levels of androgens, sperm production and/or certain behaviors 1 to 5 mo before peak female reproductive activity. However, the shift in timing and temporal patterns appears different among species. For example, reproductive recrudescence in the female American black bear occurs in January while the male is still hibernating [56] but experiencing increasing

testosterone that peaks 3 mo later in April coincident with departing the den [56]. Elevated androgen occur long before the female achieves peak estrual activity (June through July) in this species, no doubt needed for facilitating maximal sperm production [56]. But, like the giant panda, sustained androgen production appears unnecessary for the entire breeding season of the black bear. For example, testosterone already is declining in males by May and 1 to 2 mo prior to peaks in the incidence of female estrus, indicating that elevated androgens pre-set the behavioral repertoire associated with later mating activities. Interestingly, seasonal gonadal change in the black bear is even more gradual and prolonged than we found in the giant panda. The difference likely is due to the former (and not the latter) being a hibernator as rising testosterone appears critically involved in stimulating the black bear's metabolism to stop hibernation and leave the den [54, 56]. Like the giant panda, the polar bear does not hibernate, and the male begins recrudescence by producing more testosterone during March and only 1 mo before the female's peak sexual activity (Apr) that can extend through May [56]. Thus, the male polar bear appears to require less time to prepare for the breeding season and then experiences a more gradual fall in androgen activity with nadir sometimes not being reached until November [53]. Thus, while the female giant panda and polar bear both express peaks in estrus at similar times of the year, the temporal patterns in male androgen profiles both pre- and post-peak breeding activity are significantly different.

For the male giant panda, there was a logical progression in male reproductive events overtime. We detected elevated excreted androgen before measuring volumetric increases in testes size, but this observation confirmed other recent

findings from our laboratory demonstrating the utility of monitoring gonadal and adrenal steroidal activity in male giant panda feces [76]. The latter study [76] involved only five males (in three locations) and generally a less frequent 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. [76] 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 to prepare males for the rigorous battles to gain access to estrual females in nature. Regardless, steroidal metabolites can clearly be monitored even in the highly fibrous feces [76] of the bamboo-eating giant panda, thereby offering many research opportunities [76], including inter-relating hormone patterns and sperm production.

For example, our findings revealed a 3 mo interval between the peaks in androgen (Jan) and sperm (Apr) production, suggesting that reproductive recrudescence requires approximately 90 days for spermatogenesis to fully reinitiate. Similar data are lacking for most ursids, although the Japanese black bear (*Ursus thibetanus japonicas)* that lives in a temperate zone produces increasing numbers of sperm from March/April that precedes the May/June peak of female receptivity for that species [51, 52]. Our findings were unequivocal in revealing cessation of spermatogenesis in August, a phenomenon that has not been documented in other ursids other than the Japanese Black Bear (*Ursus thibetanus japonicus*) [117]. In the Japanese black bear, five periods have been identified in the reproductive cycle associated with sperm production [51, 52]. Within these periods, a period of

quiescence was identified in spermatogenesis degeneration during September [118] through January [51, 52]. The onset of seasonal sperm production in giant pandas was detected in November, closely tracking the gradual autumnal rise in excreted androgen. Prodigious sperm densities were apparent in November and January, at least 4 mo before the highest incidence of estrous activity, which coincided with peak sperm concentrations that then were sustained throughout the remainder of the female panda breeding season. From a fitness perspective, this reproductive strategy optimizes the opportunity to fertilize female giant panda during an extremely brief and singular estrus that can occur unpredictably from Feb through May.

Beyond having a sperm dense character, giant panda ejaculate also was notable for high cellular motility and proportions of structural normal spermatozoa, including acrosomal integrity. Among bears, the giant panda appears to produce far greater total sperm counts (by 20-fold) compared to, for example, the Japanese black bear ($84.0 \pm 32.2 \times 10^6$ motile sperm/ejaculate) [119], but similar to the Hokkaido brown bear (*Ursus arctos yesoensis*) ($1{,}387 \pm 2160 \times 10^6$ motile sperm/ejaculate) [120]. From a practical perspective this sperm-rich ejaculate is advantageous, especially as artificial insemination (AI) is used commonly among panda in breeding centers. As AI pregnancies have been achieved with as few as 100 million motile spermatozoa [13], a single ejaculate can be used to inseminate multiple females and remaining fractions cryopreserved for later use.

Many of the measured behaviors for the male giant panda are reproductively related, especially those associated with scent-marking [30, 40], locomotor activities [40] and vocalization [36]. Because these behaviors are tied to reproductive fitness,

they are likely driven by changes in circulating androgens. Behavior has been the most studied of all giant panda biological functions, primarily in animals in ex situ collections and during the peri-estrus interval. In the female, it is well known that vocal behavior and scent marking increases during the breeding season [40]; more recent data indicates that the vocalization conveys critical information about sex, age, receptivity and even genetic relatedness [36].

Behavioral studies of captive male giant pandas indicated that mating failure were associated with decreased libido and increased aggression [121]. In the wild, male giant pandas rely on hand stand scent marking to deposit scent from the anogenital scent gland as an essential communication between giant pandas. The elevation in the scent deposited, by hand stand marking, provides information to other pandas about size and perhaps dominance [122, 123]. In this context, we monitored this behavior relative to season.

The present study found that one of the most significant male behavioral changes over time, was the incidence of scent-marking, normally used to indicate and protect territory from other male conspecifics or to communicate hormonal information to the female [122, 123]. Our findings not only confirmed earlier observations that this behavior was retained in captivity by male pandas [40, 123], but that certain activities, especially associated with handstand marking increased leading up to the breeding period, within the prebreeding season. This increased occurrence of handstand marking is perhaps to improve reproductive success and increase the chance of obtaining a mate. The prevalence of handstand and squat marking was

sustained through the breeding season, although total scent-marking declined by late season and in parallel with the similar decline in androgen excretion.

A comparable pattern was also observed in frequency of vocalizations with an increasing trend during prebreeding followed by a gradual and then significant rise during the peak breeding period. This was not surprising given recent, important findings that female giant pandas have the capacity to perceive vocal signatures of different conspecifics that, in turn, appear related to variations in male body size [124]. In those studies, females were also found to identify novel males by vocalizations [124] perhaps allowing them to identify potential mates. Further, fluctuations in vocal signature appear to be a heritable trait, indicating relatedness can be conveyed by vocalizations [36, 124]. These data combined with our data demonstrating occurrence of vocalization increases during breeding versus non-breeding seasons, are evidence that vocalizations are a crucial element of mate choice. Since the giant panda only encounters conspecifics in the wild near and during the breeding season, the occurrence of vocalization behaviors should be highest during the breeding season as was seen in the captive males studied here. Additionally because the panda lives in dense forestation, vocalizations would convey important aspects for mate selection during the breeding season. These vocalizations communicate reproductive status to conspecific females, and also to competing males.

A related critical behavior was prevalence of locomotion, with a change in incidence of pacing changing most over time. This locomotor activity began trending upward within the prebreeding period and was maximal during the period of peak

sexual activity. There was a similar trend in total motile stereotypy and locomotor stereotypy, although variation among males prevented statistical differences. However, our findings revealed that even onset of early breeding season, was associated with greater pacing activity, no doubt an innate behavior associated with pursuit of a mate. Similar activities are well-established for the free-living brown [125, 126] where males routinely expand beyond the home range to explore habitats of often multiple females in adjacent ranges. There are indications that this same behavior occurs in giant pandas in nature [4] and we now have confirmed that even males confined to captivity expressed circannual variations in locomotor activity such as pacing.

Besides providing insight into 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, inter-disciplinary studies, led by new data in the reproductive sciences combined with improvements in animal husbandry and preventative medicine, have been used to convert a stagnant captive population of giant pandas into one that is self-sustaining with a population of more than 300 individuals. This has included increased use of artificial insemination to ensure that all females (including those that fail to breed naturally) have the opportunity to reproduce [13, 76]. In this context, the systematic banking of spermatozoa has become common [13]. 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 a management perspective then, there appears to be

little point to attempting to collect and cryopreserve spermatozoa before November and after June. Nonetheless, testes function in the male is prolonged allowing an interval of at least 6 months (Jan to Jun) to collect, and then store spermatozoa useful to the genetic and demographic management of this endangered species.

Chapter 3:

The peripubertal male giant panda produces sperm and elevated androgens before achieving behavioral maturity

Abstract

The onset of sexual male maturity is anecdotally identified as 5.5 y in the male giant panda because of a few successfully breeding individuals, but the physiology surrounding this developmental stage is poorly understood. This study examined sexual maturation in pre-pubertal (4.5 yr, n = 3), peri-pubertal (5.5 yr, n = 4), pubertal (6.5 yr, n = 5), young mature (7.5 – 8.5 yr, n = 3), mature (7.5 – 15.5 yr, n = 7) and aging mature (>15.5 yr, n =4) male giant pandas in a captive Chinese population over a 3 yr period during January through May of each year by evaluation of: 1) increasing fecal androgen concentration, 2) production of high quality sperm, 3) changes in body morphometrics, and 4) development male characteristic behaviors, such as scent marking. Sperm was produced by all males and most consistently in males 5.5 yr and older. Fecal androgens and body weight increased in males 6.5 yr of age and older, followed by a decline in males >15.5 yr. Social and energy expending behaviors were highest among males 4.5 yr of age, whereas reproductive related behaviors (scent marking) and energy expending pacing behaviors were highest among males 6.5 to 15 yr of age. Handstand urine marking was highest in mature males (9.5-15.5 yr). These data provide evidence that while sperm production, androgen production and body size have clear differences among age groups and appear to indicate the initiation of puberty in the pandas begins around 5.5 yr. Behavioral changes in the giant panda is less defined, and become more dynamic between 6.5 – 15.5 yr in the

male pandas culminating with development of hand stand scent marking at 9.5 yr. These data suggest that behavioral maturity is the last to change during the process of sexual maturation with complete sexual maturation (body morphometry, androgens, sperm traits, and sexual behaviors) attained between 7.5 to 9.5 yr of age in the male giant panda.

Introduction

The female giant panda is highly seasonal, with brief period of estrus briefly occurring for about 24 h between February and May of each year [4]. Studies have suggested reproductive potential in the male of the species is also seasonal [13, Aitken-Palmer chapter 1], but is less abbreviated allowing breeding to occur with multiple females throughout the breeding season. The giant panda and its exquisitely short breeding interval have been the focus of much study, however until this point there has been little study on puberty of this species. Specifically, the process of maturation leading up to and resulting in reproductive success is not understood for the male giant panda. There are reports of female giant pandas giving birth at the young age of 4.5 yr in China [Howard, personal communication], but the majority of young females in the Giant Panda International Studbook have given birth at 5.5 yr and 6.5 yr. It is possible that the males and females go through puberty at a similar age, but there is little documentation for this.

Anecdotal evidence from Chinese breeding centers indicate males aged 6.5 yr are capable of successful natural breeding, however this has not been the case for all males housed in Chinese breeding centers or zoos within the United States. In China,

some captive males at 5.5 yr of age have been shown to produce sperm of normal appearance and good motility [Howard, personal communication], and one male in captivity has reproduced successfully at this age. Young males (less than 6 yr of age) that produce sperm or even sire offspring seem to be the exception, while many male giant pandas do not produce viable sperm until after 6 yr of age [Howard, personal communication, 40, 121].

Although puberty is poorly understood, problems associated with reproductive failure occur in the captive male giant pandas [121]. The bases of these problems appear to range from decreased sexual motivation for copulation to severe aggression directed toward potential mates [121]. Further, individual males with abnormal breeding behavior may have had abnormal early development, indicating early behavioral development is important for reproductive success in the adult giant panda [121]. A better understanding of these early events will provide the basis for interventions and improved reproductive success.

Little is known about sexual maturation in the wild giant panda, but it is generally accepted that in the wild, cubs remain with their mothers for the first 1-2.5 yr [127] and then disperse [128]. The age for male dispersal versus female dispersal in the wild is not known; but dispersal and long distance migration is recognized as an important mechanism for inbreeding avoidance in wild populations [128]. Because the giant panda is polygynous, mate selection is dynamic requiring individuals to travel long periods to find mates [4, 128]. Because of the importance of dispersal and finding mates, locomotor activity is necessary for reproductive success by the adult male giant panda. Unfortunately, new pressures such as habitat

fragmentation and space restriction in captivity, cause constraints on the amount of space available for the giant panda's home range and corresponding locomotor activity.

Improved understanding of the physiological changes associated with sexual maturation in the giant panda will improve selection of mature males for captive breeding programs, and avoid serial semen collection as a marker of breeding capability. Evaluation of behavior, androgens and other indicators beyond semen quality will allow selection of males most likely to be successful during natural breeding. If we understand male giant panda sexual maturity, male pandas with delayed sexual maturity could be identified and interventions implemented to effectively manage these individuals.

The purpose of this study was to examine sexual maturation in the male giant panda to ultimately develop a time line for the initiation of puberty and attainment of full sexual maturity. Both endocrine and behavioral markers of reproductive function were monitored including: 1) increasing fecal androgen concentration, 2) production of high quality sperm, 3) changes in body morphometrics, and 4) male characteristic behaviors, such as scent marking.

Materials and Methods

Animals and approvals

Nine adult male giant pandas (4 – 21 yr) were maintained at two nearby locations, the Chengdu Zoo and the Chengdu Research Base of Giant Panda Breeding in Sichuan Province, People's Republic of China (30°N, 104°E). All individuals were captive

born, with four proven capable of successful breeding as demonstrated by the production of living young. Each giant panda was housed individually in enclosures with combined indoor (3.0 m x 3.0 m to 10 m x 20 m) and outdoor (12 m x 12 m to 20 m x 35 m) areas, all of which were illuminated by natural lighting. Water was available ad libitum, and freshly-cut seasonally available wild bamboo (10 – 20 kg daily) was provided to each male along with a high fiber biscuit supplement (~1,000 gm/day/male; range, 700 – 1,500 gm/day/male) for additional fiber, vitamins and minerals (proprietary recipe of the Chengdu Research Base). All males were housed in olfactory, auditory and visual proximity to adult and juvenile conspecific male and females. A male and female were allowed to physically interact within the same enclosure only during the periestrual interval of the breeding season (Feb –May) for brief (2 – 15 min) periods. Data were collected on nine individuals during an interval from Jan 1 – May 31 over 3 years consecutively.

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

Testicular and body morphometry and ejaculate characteristics

Each male was conditioned to walk across an electronic scale (Shanghai Yitai Electromechanical Equipment Co., Ltd. Shanghai, China) as needed with a minimum

of once monthly to collect data on body mass. Each male within the study was anesthetized using 10 to 12 mg/kg ketamine hydrochloride (Sinceta International Trading Co., Ltd., Shanghai, China) delivered by intramuscular injection [Janssen 2006 a] and, as necessary isoflurane gas anesthesia (Ningbo Samreal Import and Export Co., Ltd., Shanghai, China) was used via a face mask or intubation to increase depth or duration of anesthesia. Under general anesthesia, testicular and body morphometry measurements were collected for each giant panda [105, 110]. Specifically, the length and width of each testicle were measured using a digital caliper (model #500-196-20 Absolute Digimatic Calipers, Mitutoyo Corp., Aurora, IL, USA), and volume of each testis was calculated using the formula: volume = $4/3\ \pi\ ab^2$ ('a' is 1/2 length; 'b' is 1/2 width) [13]. Total testicular volume per male was determined by combining the right and left testis volumes. Calipers and/or a flexible tape were used to ascertain chest girth, abdominal girth, contour length of body from tip of nose to base of tail and tip of tail, right foreleg circumference at elbow and axial skin fold thickness, all of which have been used previously for assessing giant panda morphometry [110].

While each male was in a surgical plane of anesthesia, electroejaculation was conducted using a well-described approach [7, 13, 85]. In brief, this involved the 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, Boring, Oregon, USA). Standardized sets of low voltage stimulations (2 – 5 V) over three series of 30 stimuli each were delivered over about 20 min to elicit an erection with ejaculation into a collection container with temperature-controlled (23°C) water jacket [13, 85].

Seminal volume was measured then seminal pH determined using pH indicator strips (Colorphast®, EM Science, Gibbstown, NJ, USA). A 5 µl aliquot of ejaculate was examined for a subjective estimate of sperm motility (0 – 100%) and forward progressive motility (scale, 0 – 5; 5 = best, fast forward cellular trajectory) under phase contrast microscopy (200 – 400x) [13]. Sperm concentration was calculated using a standard hemocytometer method [13]. Total sperm per ejaculate was calculated (sperm concentration/ml x total volume of ejaculate) and recorded.

Sperm morphology was assessed after fixing a 10 µl sample of raw semen in 100 µl of fixative (0.3% glutaraldehyde in phosphate buffered saline) and using phase contrast microscopy (1,000x) to evaluate 200 sperm per sample [13, 84]. In cases of multiple defects for a given spermatozoon, each was classified according to the most serious malformation [13, 84]. For example, if a cell was observed to contain both a bent flagellum and an abnormal acrosome, it was categorized as having the latter abnormality [7]. Individual defects then were compiled into three general categories of sperm pleomorphisms (head, midpiece or flagellar) with deformities related to the head region being considered the most serious [13, 85]. Sperm head morphology was further delineated by detailed evaluations of acrosomal integrity using rose bengal/fast green stain [13]. Briefly, an aliquot of raw, unfixed semen was diluted in Ham's F10 medium (Irvine Scientific, Santa Anna, CA; 1:10 semen-to-medium) and then 1 µl of this mixture added to 9 µl of rose bengal (High Purity Biological Stain, Cole-Parmer, Vernon Hills, IL) and fast green stain (Certified Biological Stain, Sigma-Aldrich, St. Louis, MO) incubated for 90 sec and smeared on a glass slide. A minimum of 100 sperm acrosomes per sample were assessed by bright-field

microscopy (1,000x) for either a: 1) normal intact apical ridge (uniform staining of the acrosome over the anterior half of sperm head); 2) damaged apical ridge (non-uniform 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) (Fig. 1). Depictions of these morphological defects [13] and acrosomal types (Fig. 1) for the giant panda are provided.

Fecal Androgen assessment

Enzyme-immunoassays (EIA) recently have been validated for monitoring marked and subtle alternations in fecal gonadal steroid content in giant pandas (estrogen and progesterone in females [101], androgens in males [76]). This study took advantage of these advancements by collecting a fresh (< 1 h post-excretion) fecal sample from each of the eight male giant pandas every 48 to 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 was then sealed and stored frozen (-20°C) until processing. Samples were batch-shipped frozen to the Smithsonian Conservation Biology Institute (Front Royal, VA) for processing and hormone analysis.

Prior to hormone analysis, samples were processed by freeze-drying (Lyophilizer, Labconco, Kansas City, MO), crushed and hormone extracted using validated methods [76]. For hormone extraction, 0.1 g of fecal powder was extracted using 90% ethanol. The extracts were vortexed and centrifuged to remove particulate, sonicated with glass beads and then dried under air and resuspended in 1 ml of BSA-free phosphate buffer before freezing for storage [76]. The EIA [76]

relies on a polyclonal anti-androgen R156/7 antibody (C. Munro, University of California, Davis, CA) in a 96-well microtiter plates assay (Nunc-Immuno, Maxisorp; Fisher Scientific, Pittsburgh, PA). Briefly, the antibody was allowed to equilibrate for 12 to 18 h (4°C); unadsorbed anti-serum 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, Newport, RI) were added to the EIA microtiter plate. A peroxidase 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 chomagen 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 inter-assay correlations of variation was < 10% and 10%, respectively [76].

Baseline (harmonic mean) concentrations of fecal androgen metabolites were determined through an iterative process [33, 76]. In brief, baseline values were assessed yearly in each male, then for each male, then overall. 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 harmonic mean was considered androgen baseline and was expressed as mean ± SEM.

Behavioral assessment

Male behavioral data were collected based on a modified ethogram for this species [40] (Table 1). The behavior of each male was evaluated in detail during two consecutive 30 min focal observation periods conducted twice weekly continuously

throughout the 3 yr study. Data were collected by a total of 11 observers who were intensively trained in behavioral identification and recording for 2 wk. Consistency among observers was monitored by comparing responses for the same observation period and found to be ≥80% over the study interval. Each animal was examined with a balanced number of morning (0800 – 1130 h) and afternoon (1300 – 1700 h) observations twice per wk. On the rare occasion that observations were perturbed by an animal-keeper interaction (e.g., unscheduled shifting to an adjacent enclosure or breeding event), the data associated with that episode were deleted, and a replacement observation period conducted within 24 – 48 h.

Behaviors were summarized in two ways: 1) all-occurrence (number of behavior occurrences/visible min); and 2) instantaneous sampling (number of minutes behavior occurred/60 min observation period). The rate of occurrence for a behavior was determined by dividing the frequency of the behavior by the number of min 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 min during the focal period. Percentage of time engaged in a particular behavior then was determined for each animal.

Statistical analysis

Data for each male were pooled across the three years of study. Data were checked for influence and effect of individual male and year within the model and no effect was seen using fixed effects influence diagnostic (SAS© , 9.1.3, SAS 2003 Cary, NC, USA). Each reproductive characteristic (body morphometric, fecal androgen concentration, sperm traits, and behavior) were tested for normality. Body

morphometrics were non-parametric and were log transformed. Non-parametric spermic trait percentiles (% normal morphology, % abnormal acrosomes) were transformed using arc sin. Tukey-Kramer test of multiple comparison were used to determine differences between reproductive characteristics and age groups (young, prepubertal, peripubertal, pubertal, mature and geriatric) (SAS©, 9.1.3, SAS 2003 Cary, NC, USA). Behavioral data were compared using GLMIX® (SAS 9.1.3, SAS 2003 Cary, NC, USA).

Results

Male age groups

Males in the study were separated into six groups during the study: 1) pre-pubertal (4.5 yr), 2) peri-pubertal (5.5 yr), 3) pubertal (6.5 yr), 4) young mature (7.5 – 8.5 yr), 5) mature (9.5 – 15.5 yr), and 6) aging mature (> 15.5 yr). As the individuals aged during the 3 yr study, they were redistributed in other age groups as indicated. As a result, some individuals were included in multiple age groups. None of the males in the pre-pubertal group (4.5 yr) sired offspring during the study. One male in the 5.5 yr group sired one healthy cub when he was 5.5 and 6.5 yr during the study. He was the only proven male less than 7.5 yr of age in the study. There were proven males in each of the older age groups (7.5 – 8.5, 9.5- 15.5 and >15.5 yr), totally about 50% of males in these groups. One of the individuals included in the study at 4.5, 5.5 and 6.5 yr sired his first offspring when he was 9.5 yr of age, after this study was completed.

The males in this study were somewhat related. The exact relatedness is unclear, but two males in the 4.5 yr age group share the same dam and one male in

the aging mature group sired to two other males used in the study. Although these male may share similar familial characteristics, this group of males is the second largest male group in the global captive giant pandas population.

TABLE 5. Body and testicular morphometry of captive giant pandas in China at different age groups during a 3 year study (January – May).

Age (years)	4.5	5.5	6.5	7.5 to 8.5	9.5 to 15.5	>15.5
No. of males	3	4	5	3	7	4
Body morphometry*						
Body weight (kg)	103.4 ± 13.7[a,b]	119.0 ± 9.0[b]	124.7 ± 8.0[b]	121.3 ± 8.2[b]	125 ± 4.6[b]	102.3 ± 2.4[a]
Chest girth (cm)	112.2 ± 3.9	111.7 ± 1.5	93.8 ± 17.7	111.3 ± 3.8	102.8 ± 10.4	86.4 ± 10.9
Abdominal girth (cm)	75.5 ± 34.5	116.8 ± 3.2	97.1 ± 18.2	112.8 ± 6.4	113.8 ± 2.7	103.0 ± 12.5
Right tibial length (cm)	29.0 ± 1.0	28.2 ± 1.6	30.7 ± 2.3	29.5 ± 0.9	30.1 ± 0.9	28.0 ± 2.5
Right foreleg circ (cm)	46.0 ± 6.0	54.8 ± 6.2	49.5 ± 2.1	47.2 ± 3.4	50.1 ± 2.4	45.5 ± 1.2
Right foreleg length (cm)	26.0 ± 0.0	33.7 ± 7.2	26.7 ± 0.6	28.5 ± 0.9	26.9 ± 1.4	27.3 ± 1.8
Right hind foot length (cm)	17.7 ± 2.3	20.2 ± 0.9	20.7 ± 1.5	18.8 ± 2.2	22.9 ± 0.4	23.0 ± 0.7
Right axial skin fold (cm)	2.1 ± 0.2	2.9 ± 0.3	2.4 ± 0.3	2.1 ± 0.3	2.3 ± 0.3	2.8 ± 0.8
Testicular morphometry*						
Total testicular volume (cm^3)	299.5 ± 56.1	360.0 ± 16.1	351.8 ± 29.7	350.0 ± 0.2	338.8 ± 14.6	350.2 ± 9.9
Right testis volume (cm^3)	138.2 ± 1.9	157.9 ± 7.0	148.2 ± 7.8	162.3 ± 4.5	145.2 ± 9.1	152.1 ± 8.6
Right testis length (cm)	7.7 ± 0.2	8.2 ± 0.1	8.2 ± 0.1	8.3 ± 0.1	7.9 ± 0.2	8.1 ± 0.2
Right testis width (cm)	5.9 ± 0.1	6.0 ± 0.1	5.8 ± 0.1	6.1 ± 0.0	5.9 ± 0.1	6.0 ± 0.1
Left testis volume (cm^3)	161.2 ± 55.6	202.1 ± 22.1	203.7 ± 29.5	187.5 ± 4.3	193.6 ± 11.7	198.2 ± 13.3
Left testis length (cm)	7.7 ± 0.3	8.5 ± 0.2	8.3 ± 0.2	8.7 ± 0.1	8.3 ± 0.2	8.5 ± 0.2
Left testis width (cm)	6.1 ± 1.0	6.7 ± 0.4	6.8 ± 0.5	6.4 ± 0.1	6.6 ± 0.2	6.6 ± 0.3

*Values are means ± S.E.M.

[a,b,c]Values with different superscripts within a row denote differences among age groups ($P < 0.05$).

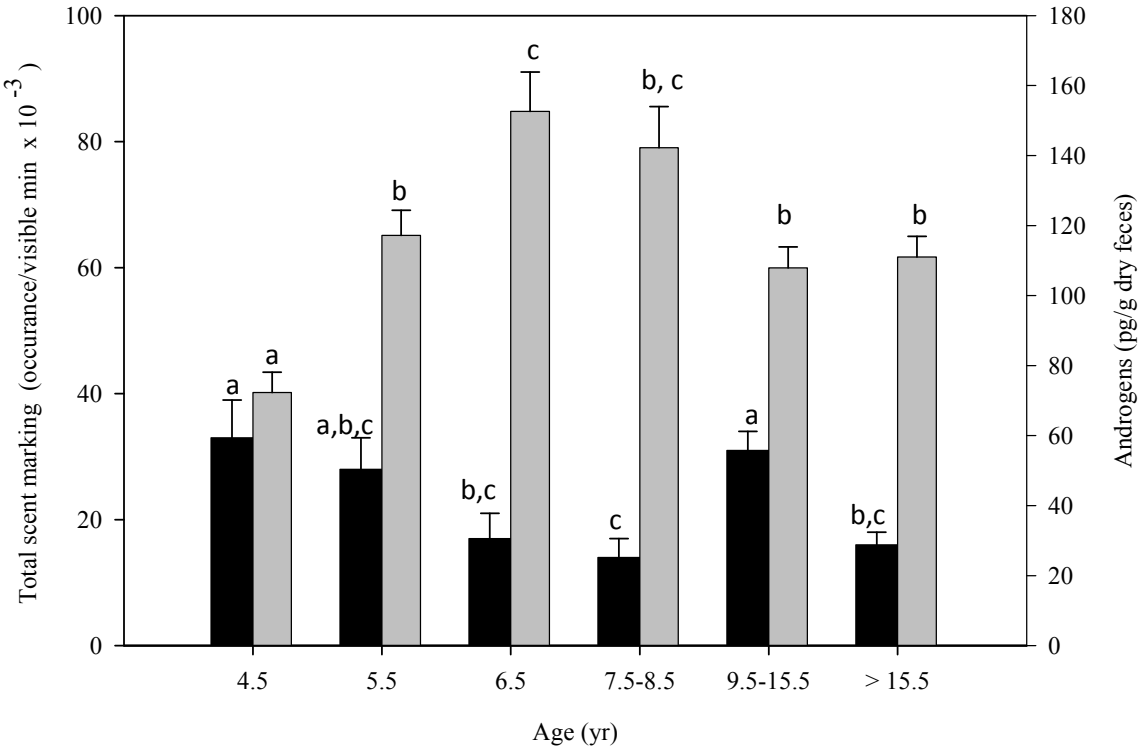
Body morphometry

Body morphometrics were measured for each age group in the study (Table 5). When body morphometrics were compared among age groups, differences between body weights were found (Table 5). The aging mature (>15.5 yr) males were smallest in weight compared to pubertal 6.5 yr, young mature 7.5-85 yr and mature males 9.5-14.5 yr (Table 5). The aging mature males were similar in body weight as the pre-pubertal 4.5 yr males (Table 5). No other body morphometric measured was different between age groups. Even testicular volume did not differ between age groups ($P < 0.05$, Table 5).

Fecal androgens

Fecal androgen concentration was measured for all males in the study and differed among age groups (Figure 8). The pre-pubertal males (4.5 yr) had the lowest androgen concentration (72.3 ± 5.8 ng/g dry feces) of any male group. Androgens showed increasing concentrations in peri-pubertal males (5.5 yr; 117.2 ± 7.2 ng/g dry feces) rising maximally in pubertal males (6.5 yr; 152.6 ± 11.3 ng/g dry feces) before decreasing with young mature males (7.5-8.5 yr; 142.3 ± 11.7 ng/g dry feces). Fecal androgen concentrations reached a basal concentration in the older age groups including both the mature (9.5 – 15.5 yr; 107.9 ± 6.0 ng/g dry feces) and aging mature males (>15.5 yr; 111.0 ± 5.9 ng/g dry feces) (Figure 8).

Fig. 8



Total fecal androgen concentrations (gray bars) and total frequency of scent marking (black bars) of prepubertal (4.5 yr, n = 3), peri-pubertal (5.5 yr, n = 4), pubertal (6.5 yr, n = 5), young mature (7.5 – 8.5 yr, n = 3), mature (7.5 – 15.5 yr, n = 7) and aging mature (>15.5 yr, n =4) male giant pandas in China during Jan through May over a 3 year period. Bars represent mean (± S.E.M.). Means with different superscripts represent differences among age groups ($P < 0.05$).

Semen collection and ejaculate traits

An erection with ejaculate was always obtained during electroejaculation (100%) of the peri-pubertal, pubertal, young mature, mature, and aging mature males, but not the pre-pubertal (4.5 yr) males (75%, $P < 0.05$). During electroejaculation, rectal probe size used varied dependant on overall body size of the male (5.5 yr: 2.6, 3.9 cm; 6.5 yr: 2.6, 3.9, 4.3 cm; 7-8 yr: 2.6, 3.9 cm; 9.5 – 14.5 yr: 2.6, 3.2, 3.9 cm; >15.5 yr: 2.6, 3.9 cm). Ejaculate was usually obtained during series 1 or 2 of the procedure, but could be collected during subsequent series of stimulations. The pH of the ejaculate was highest in the peri-pubertal (5.5 yr) males compared to pubertal (6.5 yr) males ($P < 0.05$), but did not differ from other age groups (Table 6).

Spermic ejaculates were obtained from males in each age group including 50% of the pre-pubertal (4.5 yr) males (Table 6). The volume of ejaculate obtained from the pre-pubertal (4.5 yr) males was small (<1 ml) but did not differ from sample volume obtained from males in the other age groups (Table 6). There was no difference in sperm concentration, total sperm count, initial motility, forward progressive status, intact acrosomes, or normal sperm morphology among fresh ejaculates for all age groups ($P > 0.05$; Table 6).

There were differences in the types of sperm morphological abnormalities observed (Table 6). Macrocephalic sperm were most commonly produced by aging mature males versus other age groups (>15.5 yr; Table 6). The pubertal (6.5 yr) males had the lowest incidence of bent midpiece with cytoplasmic droplets compared to the pre and peripubertal males ($P < 0.05$). Bent necks were more frequently ($P < 0.05$) seen in sperm from males 5.5 – 7.5 yr of age.

TABLE 6. Ejaculate and sperm traits of giant pandas in different age groups during 3 years of study (January – May).

Age (year)	4.5	5.5	6.5	7.5 to 8.5	9.5 to 15.5	>15.5
No. of males	3	4	5	3	7	4
No. of semen collections	4	9	11	7	18	11
No. of spermic ejaculates	2	8	11	6	18	11
Ejaculate volume (ml)*	0.15 ± 0.12	2.7 ± 0.5	1.6 ± 0.5	1.1 ± 0.5	1.9 ± 0.4	1.9 ± 0.4
Ejaculate pH*	8.2 ± 0.1[a,b]	8.8 ± 0.1[a]	8.3 ± 0.1[b]	8.6 ± 0.1[a,b]	8.5 ± 0.1[a,b]	8.6 ± 0.1[a,b]
Sperm concentration/ml ($x10^6$)*	3049.0 ± 549.0	1438.0 ± 225.5	2245.5 ± 557.7	1656.1 ± 306.7	2038.2 ± 292.3	1335.8 ± 267.1
Total sperm/ejaculate ($x10^6$)*	191.2 ± 162.6	3085.3 ± 838.0	4160 ± 2422.4	1699.4 ± 720.33	3878.3 ± 1015.4	2002.2 ± 482.1
Sperm motility (%)*	80.0 ± 10.0	83.6 ± 2.6	88.6 ± 1.2	80.0 ± 5.0	75.3 ± 5.0	73.6 ± 3.0
Sperm forward progression[1]*	3.5 ± 1.5	4.1 ± 0.6	4.0 ± 0.2	3.7 ± 1.7	3.8 ± 4.4	3.7 ± 0.2
Sperm morphology (%)*						
Normal sperm	55 ± 13	55 ± 6	67 ± 6	58 ± 8	63 ± 5	52.9 ± 7.1
Abnormal sperm						
Macrocephalic	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.3 ± 0.1[b]
Microcephalic	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.2
Abnormal acrosome	1.0 ± 1.0	1.5 ± 0.8	0.3 ± 0.1	0.4 ± 0.3	1.8 ± 0.6	1.5 ± 0.6
Abnormal midpiece	3.5 ± 0.5	0.6 ± 0.3	2.2 ± 1.4	0.7 ± 0.3	0.6 ± 0.3	1.3 ± 0.4
No midpiece	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Bent midpiece with droplet	9.5 ± 4.5[a]	10.3 ± 2.9[a]	2.4 ± 0.7[b]	4.4 ± 1.3[a,b]	6.1 ± 1.8[a,b]	7.3 ± 1.8[a,b]
Bent midpiece without droplet	0.0 ± 0.0	10.6 ± 3.3	9.6 ± 3.4	9.4 ± 5.0	9.9 ± 2.5	12.8 ± 4.4
Proximal droplet	8.0 ± 2.0	6.4 ± 2.7	2.6 ± 1.2	11.7 ± 7.7	2.2 ± 0.7	1.8 ± 0.4
Distal droplet	8.5 ± 7.5	2.5 ± 1.3	2.7 ± 1.4	0.9 ± 0.9	6.4 ± 3.7	4.4 ± 2.1
Coiled tail	9.0 ± 3.0	6.3 ± 2.0	3.2 ± 1.5	3.7 ± 1.8	1.8 ± 0.6	3.0 ± 1.0
Biflagellete	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Bent tail with droplet	1.5 ± 0.5	1.0 ± 0.4	1.8 ± 1.3	3.4 ± 1.9	3.5 ± 1.0	3.3 ± 0.8
Bent tail without droplet	0.0 ± 0.0	0.6 ± 0.3	1.8 ± 0.7	3.1 ± 2.2	3.3 ± 1.3	2.7 ± 1.3
Bent neck	0.5 ± 0.5[a]	4.9 ± 1.6[b]	6.6 ± 2.6[b]	4.4 ± 2.6[b]	1.4 ± 0.4[a]	8.4 ± 2.6[b]
Acrosomal integrity (%)*						
Normal apical ridge	92.0 ± 0.0	87.6 ± 5.9	86.4 ± 5.7	67.0 ± 14.0	79.4 ± 3.6	84.6 ± 2.5
Damaged apical ridge	7.0 ± 0.0	12.0 ± 5.8	16.0 ± 5.4	29.0 ± 11.0	16.6 ± 3.2	12.6 ± 2.0
Missing apical ridge	1.0 ± 0.0	0.4 ± 1.1	1.8 ± 0.7	4.0 ± 3.0	3.9 ± 1.0	3.9 ± 2.5
Loose acrosomal cap	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1

*Values are means ± S.E.M.

[1]Scale 0 to 5; 5 = best.

[a,b,c]Values with different superscripts within a row denote differences among age groups ($P < 0.05$).

TABLE 7. Frequency of behaviors (% of behaviors/hr observed) in captive male giant pandas in China during 3 years of study (January – May).

Age (year)	4.5	5.5	6.5	7.5 to 8.5	9.5 to 14.5	>15.5
No. male observed	3	5	5	4	6	7
Behaviors*						
Scent marking						
Handstand mark	$0.00 \pm 0.0^{a,b}$	$0.00 \pm 0.0^{a,b}$	0.00 ± 0.0^{a}	$0.03 \pm 0.0^{a,b}$	0.04 ± 0.0^{b}	0.00 ± 0.0^{a}
Leg cock mark	0.04 ± 0.0	0.01 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.02 ± 0.0
Reverse mark	$0.01 \pm 0.0^{a,}$	0.11 ± 0.0^{b}	$0.06 \pm 0.0^{a,b}$	$0.05 \pm 0.0^{a,b}$	0.02 ± 0.0^{a}	0.03 ± 0.1^{a}
Squat mark	$0.14 \pm 0.0^{a,b,c}$	0.21 ± 0.0^{b}	$0.14 \pm 0.0^{a,b}$	$0.04 \pm 0.0^{b,c}$	0.17 ± 0.0^{a}	0.00 ± 0.0^{c}
Handstand urine mark	0.01 ± 0.0^{a}	0.00 ± 0.0^{a}	0.01 ± 0.0^{a}	0.02 ± 0.0^{a}	0.09 ± 0.0^{b}	0.04 ± 0.0^{a}
Leg cock urine mark	0.00 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.00 ± 0.0
Social						
Affiliative interaction	0.73 ± 0.2^{a}	0.17 ± 0.1^{b}	0.06 ± 0.0^{b}	0.00 ± 0.0^{b}	0.03 ± 0.0^{b}	0.09 ± 0.1^{b}
Barrier interaction	0.50 ± 0.1	0.45 ± 0.1	0.73 ± 0.1	0.51 ± 0.1	0.74 ± 0.1	0.51 ± 0.1
Olfactory						
Body rub	$0.12 \pm 0.0^{a,b}$	0.06 ± 0.0^{a}	0.06 ± 0.0^{a}	$0.17 \pm 0.0^{a,b}$	0.25 ± 0.0^{b}	$0.12 \pm 0.0^{a,b}$
Scent anoint	0.03 ± 0.0	0.03 ± 0.0	0.05 ± 0.0	0.01 ± 0.0	0.02 ± 0.0	0.04 ± 0.0
Open mouth olfactory	0.02 ± 0.0	0.05 ± 0.0	0.07 ± 0.0	0.06 ± 0.0	0.10 ± 0.1	0.05 ± 0.0
Lick olfactory	0.00 ± 0.0	0.01 ± 0.0	0.03 ± 0.0	0.02 ± 0.0	0.03 ± 0.0	0.04 ± 0.0
Vocalization	0.08 ± 0.0	0.04 ± 0.0	0.03 ± 0.0	0.12 ± 0.0	0.07 ± 0.0	0.09 ± 0.0
Feeding	25.6 ± 1.5^{a}	19.1 ± 1.2^{b}	19.6 ± 1.1^{b}	$20.9 \pm 1.0^{a,b}$	20.0 ± 0.9^{b}	$22.2 \pm 1.0^{a,b}$
Stationary alert	$8.04 \pm 0.6^{a,b}$	$7.97 \pm 0.5^{a,b}$	9.50 ± 0.5^{a}	9.22 ± 0.4^{a}	7.24 ± 0.3^{b}	6.51 ± 0.3^{b}
Resting	$16.6 \pm 1.7^{a,b}$	21.6 ± 1.5^{a}	18.4 ± 1.3^{a}	$16.8 \pm 1.0^{a,b}$	13.7 ± 1.0^{b}	20.0 ± 1.0^{a}
Locomotor	$4.38 \pm 0.4^{a,b}$	$6.07 \pm 0.5^{b,c}$	$5.98 \pm 0.4^{b,c}$	$4.52 \pm 0.3^{a,b}$	6.65 ± 0.4^{a}	4.30 ± 0.3^{c}
Investigation/exploratory	$2.10 \pm 0.2^{a,b,c}$	$1.93 \pm 0.2^{a,b,c}$	2.18 ± 0.2^{a}	$2.00 \pm 0.2^{a,b}$	$1.48 \pm 0.1^{b,c}$	1.40 ± 0.1^{c}
Non-motile stereotypy	0.30 ± 0.1	0.04 ± 0.0	$0.0.7 \pm 0.0$	0.00 ± 0.0	0.08 ± 0.0	0.08 ± 0.0
Motile stereotypy						
Locomotor stereotypy	0.17 ± 0.1^{a}	0.72 ± 0.3^{a}	$1.05 \pm 0.3^{a,b}$	0.13 ± 0.0^{b}	1.96 ± 0.3^{b}	$1.17 \pm 0.2^{a,b}$
Pace	0.00 ± 0.0^{a}	0.21 ± 0.2^{a}	0.67 ± 0.2^{a}	$1.49 \pm 0.3^{a,b}$	6.29 ± 0.5^{c}	2.20 ± 0.3^{b}

*Values are means ± SEM.

[a,b,c]Values with different superscripts within a row denote differences among seasons ($P < 0.05$).

Behavioral observations

Behaviors noted during the observation periods (instantaneous behaviors) showed pacing occurred infrequently in pre-pubertal (4.5 yr) males (Table 7). In fact, pacing rarely occurred in pre-pubertal (4.5 yr) males (0.036 ± 0.0 occurrence of behavior/visible min). Pacing was seen most frequently in mature males (9.5 – 15.5 yr, $P < 0.05$) as both an all occurrence behavior (1.72 ± 0.1 occurrence of behavior/visible min; other age groups range 0.04 – 0.5) and instantaneous behavior (Table 7). Other activity, such as locomotor stereotypic behaviors occurred more frequently ($P < 0.05$) in the young mature (7.5 – 8.5 yr) and mature males (9.5 – 15.5 yr) than the pre-pubertal (4.5 yr) and peri-pubertal (5.5 yr) males (Table 7); whereas, non-stereotypic locomotor activity was lowest ($P < 0.05$) in aging males (>15.5 yr) compared to the mature males (9.5 – 15.5 yr) (Table 7).

Rates of non-reproductive related behaviors such as feeding, stationary alert and resting differed among age groups (Table 7). Social behaviors such as affiliative interactions were most frequent ($P < 0.05$) among the pre-pubertal (4.5 yr) males (Table 7) compared to the other age groups. Barrier interactions with other conspecifics was highest ($P <0.05$) in the mature males (9.5 – 15.5 yr, 0.68 ± 0.1 occurrence of behavior/visible min) compared to the peri-pubertal males (5.5 yr, 0.36 ± 0.1 occurrence of behavior/visible min), but did not differ ($P > 0.05$) among the other age groups (4.5 yr, 0.62 ± 0.1; 6.5 yr, 0.73 ± 0.1; 7.5 -8.5 yr, 0.51 ± 0.1 occurrence of behavior/visible min). Comparison of all olfactory behavior (body rub, olfactory lick, olfactory mouth open, scent anoint) revealed that body rubbing occurred more ($P < 0.05$) in mature (9.5 – 15.5 yr) compared to the 5.5 – 6.5 yr old

males. Differences among age groups in body rubbing behaviors are shown in Table 7.

Vocalization often occurred concurrently with other behaviors. During instantaneous behavior observation, other behaviors were recorded preferentially over vocalizations (Table 7). All vocalizations were recorded during the all occurrence behavior records and occurred more often in mature males (9.5 – 15.5 yr; 8.9 ± 1.2 occurrence/visible min) compared to pubertal males (6.5 yr; 2.5 ± 0.7 occurrence/visible min) but other age groups did not differ (range 5.4 – 9.0 occurrence/visible min).

Scent marking without urination occurred in all males of all age groups. Scent marking with urination (handstand and leg cock mark) was not observed in the peri-pubertal (5.5 yr) males and was rarely observed in pre-pubertal (4.5 yr) and pubertal (6.5 yr) males (range: 0.005 – 0.017 occurrence/visible min). Handstand scent marking with urination occurred most frequently ($P < 0.05$) in mature (9.5 – 15.5 yr) males when observed by instantaneous observation (Table 7) and all occurrence measurements (0.37 ± 0.0 occurrence/visible min). Other age groups were observed to have handstand scent marking with urination less frequently (Table 7; range: 0.0 – 0.2 occurrence/visible min). Squat marking was observed the least ($P < 0.05$) in mature and aging mature males (range 0.02 – 0.1 occurrence/visible min) compared to other age groups (0.4 – 0.7 occurrence/visible min). These results differed slightly from the data reported in Table 7 which shows the instantaneous observations. Reverse scent marking was observed most frequently in peri-pubertal (5.5 yr) and pubertal (6.5 yr) (range: 0.21 – 0.24 occurrence/visible min) compared to mature (9.5

– 15.5 yr) and pre-pubertal (4.5 yr) males (range: 0.03 – 0.05 occurrence/visible min). Other differences were observed for reverse scent marking during instantaneous behavior (Table 7).

The most common type of scent marking observed by the mature males was hand stand marking without urination. Mature (9.5 – 15.5 yr) and young mature (7.5 – 8.5 yr) males were observed scent marking by handstand (range: 0.03 – 0.06 occurrence/visible min) more than other age groups (range: 0.003 – 0.01 occurrence/visible min) ($P < 0.05$). Leg cock marking without urination was seen to occur more frequently in aging mature males (>15.5 yr, 0.12 ± 0.0 occurrence/visible min) compared to mature males (9.5 – 15.5 yr, 0.02 ± 0.0 occurrence/visible min) and pubertal males (7.5 – 8.5 yr, 0.01 ± 0.0 occurrence/visible min). There was no difference among age groups for the frequency of leg cock scent marking with urination (range: 0.00 – 0.06 occurrence/visible min).

When all scent marking behaviors were combined, the mature males (9.5 – 15.5 yr, 0.03 ± 0.0 occurrence/visible min) scent marked more frequently ($P < 0.05$) than the young mature (7.5-8.5 yr, 0.01 ± 0.0 occurrence/visible min) and aging mature males (>15.5 yr, 0.02 ± 0.0 occurrence/visible min), but not more frequently than the other age groups (4.5 – 6.5 yr, range: 0.02 – 0.03 occurrence/visible min) (Fig. 8).

During observational periods, there were minutes when the individual male pandas could not be seen (range: 0.8 – 2 % behavior/h observed). This occurred with highest incidence for young mature males (7.5 – 8.5 yr, 2.0 ± 0.2 % behavior/h

observed), but did not significantly affect interpretation of the results due to the relatively low time of obscured vision ($P < 0.05$).

Discussion

Studies in bear species have examined behavioral changes associated with reproductive fitness [42, 126, 128-130], but few studies relate body morphometrics, with gamete quality, steroid hormone concentrations, and reproductive behavior in the maturing male bear [41]. This is the first study to examine the interaction between body morphometrics, sperm quality, androgen concentration, and behavior for the maturing giant panda. The results from this study indicate that sexual maturity in the male giant panda is multi-faceted and occurs as a cascade of change.

In other bear species, reproductively related hormonal changes during sexual maturity are associated with changes in body morphometrics [115], changes in testicular size, alterations in sperm quality [50, 56] and ultimate behavioral dispersal from their siblings and mothers for inbreeding avoidance [114, 131].

The onset of puberty is associated with the ability to produce sperm, but sexual maturity to optimize reproductive fitness depends on the function of all facets of the reproductive axis, including spermatogenesis, steroidogenesis, androgen dependent secondary sex characteristics, increased body weight, and androgen dependent sexual behavior [59, 132]. The onset of puberty in mammals is preceded by increasing production and release of hypothalamic gonadotropin releasing hormone (GnRH) [60]. These events reflect changes in the synthesis and/or alteration in GnRH associated with the pubertal rise of the gonadotropins, luteinizing hormone

(LH) and follicle stimulating hormone (FSH). As the testes become more steroidogenic and sensitive to testosterone, there is a positive effect on the response of testicular cells to the actions of FSH and LH, which enhance spermatogenesis and steroidogenesis [60], respectively.

Testosterone is essential for spermatogenesis during and after the onset of puberty and circulating testosterone along with other androgens is critical for the development of accessory sex organs, hormone dependent behavior, and metabolic function [59- 61, 63]. Body size and weight appears to be a conserved change that occurs during sexual maturation across a number of species [64, 65]. Some speculate that it is body weight that triggers the onset of puberty [64, 65]. Other suggest that increased body mass is a result of pubertal increases in testosterone [64, 65]. While weight gain and changes in body morphology may be associated with puberty, these physical changes are only a portion of the changes occurring in the male during onset of puberty. In species that are known to be seasonally breeding, pubertal cues are associated with photoperiod [59]. The giant pandas in this study showed no differences in body morphometrics except body weight. This suggests that the primary skeletal growth occurs prior to 4.5 yr of age, the youngest age group in this study. Because body weight did increase between 4.5 yr to 6.5 yr, this indicates that muscle and fat deposition are changing in these individuals, perhaps due to changes in circulating androgen concentration and the deposition of lean muscle mass.

In black bears, different body morphometrics change throughout the lifetime of a male, with long bone physeal closure occurring between 2 – 7 yr [133]. Saggital crest, a secondary sex characteristic in black bears, reached maximal size by 8 yr of

age [133]. These changes in black bear reached a limit associated with sexual maturity [133]. Because no changes were seen in body size development other than weight change, further study of giant panda growth and skeletal formation in males younger than 4.5 yr, may provide additional information on the changes occurring in these young individuals. Although there were no changes in long bone measurements or body contour in these study males, body weight changed significantly from pre-pubertal males (4.5 yr) to pubertal males (6.5 yr) and mature males (9.5 – 15.5 yr).

Since the changes in body size trend up as androgens increase, body size appears to be a characteristic in the giant panda that may be driven by gonadotropin increase and the associated androgen surge occurring in males 6.5 yr. Male pandas 6.5 – 8.5 yr had the highest fecal androgen concentration suggesting these ages have the most reproductive steroid hormone activity indicating gonadostat upregulation occurs between 6.5 – 8.5 yr in the male giant panda.

It is interesting to note, aging mature males (>15.5 yr) were similar in body size to the youngest (4.5 yr) males in the study and significantly smaller than the mature males or males in all other age groups. This suggests that the older males are losing muscle mass or fat stores as they age. This body morphometric change in the aging male is not associated with decreased fecal androgens as they still have high concentrations of circulating androgens (Fig 8). Instead these changes may be associated with other physiologic changes occurring during aged life, such as decreased protein adsorption, increased protein loss (renal or enteric), or general muscle atrophy.

Although giant panda body weight and fecal androgen followed a similar increasing trend with age, testicular size did not. When looking at the testes volume data there was no difference among age groups (Table 5). This lack of difference may be attributed to the wide variation in testes size within age group. It is interesting to note, the standard error is the smallest in the young mature group (7.5 – 8.5 yr) males indicating the lowest variance between individuals, but the biological significance of this is unknown.

The initiation of sperm production is often recognized as the outcome of puberty and often recognized as the defining moment in puberty. In this study, sperm was produced early by giant pandas, and was even produced by individuals with low fecal androgen. Because there were no differences in sperm quality obtained by electroejaculation among age groups during the study, the production of sperm must actually begin before the age of 4.5 yr in giant pandas. Another explanation, since only 50% of the young (4.5 yr) males produced spermic ejaculate; it is possible that sperm production and subsequent collection via electroejaculation in the giant panda is an all or nothing event associated with the development of sex accessory glands. If these accessory glands are not fully developed, they cannot be stimulated by the probe during electroejaculation resulting in no semen collected. This is an area for further study.

Presence of sperm in the epididymis was used to indicate sexual maturity in the Hokkaido brown bear [41], Alaskan brown bear [42], grizzly bears in the Yukon [43] and grizzly bears in the continental United States [42]. Using epididymal sperm, it is accepted that the Hokkaido brown bear begins producing sperm at 2 to 5 yr old

[41], whereas the Alaskan brown bear is 4.5 yr [42], the Yukon grizzly bear is 5-7 yr [43] and grizzly bears in the continental United States at 3.5 to 5.5 yr of age [44]. The polar bear produces sperm at 5.8 yr, but does not sire young until after this age [116]. These data from other bear species demonstrate sperm production can occur relatively young, however additional information indicates age and production of sperm do not correlate with paternity [115]. Instead, body size and age are associated with reproductive success and resulting paternity [115]. Occasionally young male brown bears are believed to mate, but sperm competition within female bears ensures only a few mature males sire a high proportion of offspring [115]. This suggests other factors such as behavior are involved in male maturity and resulting reproductive success in bear species.

During sexual maturation, it is the increase steroidogenic capacity associated with increased production of testosterone and other androgens during the prepubertal period that initiate spermatogenesis to obtain adult testis function [59]. This increased androgen production, specifically testosterone leads to the development of secondary sexual characteristics and sexual behavior [59]. In giant pandas, behavior has been well studied and has been used to understand communication [36, 40], reproduction [29, 46], visual capability [45] and social interactions and is believed to convey important physical, age and reproductive information [23, 36, 134]. Behavioral research also has been used to identify areas of stress influencing health and reproductive success [40, 107]. Because reproductive behavior is hormone dependent [11, 49], it is important to examine behavior to understand reproductive success. More specifically, successful reproduction relies on coordinated function of

endocrine and behavioral components of reproduction. Circulating androgen concentrations are closely linked with reproductively related behaviors, such as mounting, intromission and ejaculation [49] many of these behavior are abnormal in captive male giant pandas [121] and require further study.

The behavioral changes occurring in the giant pandas study here interestingly fluctuation in the frequency and type of behaviors among the age groups. The youngest group (4.5 yr) was seen to have the highest numbers of social behaviors and even had as much locomotor behavior as older pandas (7.5 – 14.5 yr). Previous studies found similar patterns [40] indicating activity was inversely correlated with age and that tactile contact is more frequent during early social interactions.

Additionally, locomotor behavior has been related to the natural behavior of pandas searching for mates in the wild [40] and is necessary for normal dispersal from their mothers and siblings for inbreeding avoidance [129, 135]. Our data support initial research, and indicates young pandas participate in locomotor behaviors perhaps for developmental or dispersal reasons while mature males spend a lot of time on locomotor behaviors perhaps in part to search for a mate [4, 135]. Because the pandas in this study were captive, pacing was the most commonly observed locomotor behavior by mature males. This is likely due to the space constraints of captive enclosures, during expression of a hormonally driven behavior.

Scent marking is unique to pandas among ursids, as the only bear species to have paired scent glands [40]. Chemicals in this scent produced by these glands have been shown to differ with age [135]. Scent marking deposits this chemical on substrate within the environment and is believed to be a communication behavior of

mature breeding males. Scent marking of various types, was observed in all age groups studied. Interestingly, among types of scent marking, handstand scent marking with or without urination has been identified as a special behavior believed to indicate large body size and aggressive mate competition [122].

In our study, handstand marking was most common in breeding age (9.5 – 15.5 yr) mature males and rarely seen in pre or peri-pubertal males. Handstand marking is a relatively difficult behavior and may require a period of time to learn this behavior or develop the skill to hold the legs over the head. In the study by White et al [122], young males avoided scent deposited on an elevated structure mimicking scent marking by handstand scent marking. This particular behavior may be critical to convey large body size and reproductive fitness [122, 123]. Mature males performed this behavior more than any other age group in our study. Because handstand urine marking occurred less often in males less than 9.5 yr, this behavior, critical for successful reproductive communication, is the last change to occur in the maturing male panda.

In our study, hand stand marking was also less common in aging mature males. These males may no longer be able to do this behavior due to decreased agility and muscle mass, although specific orthopedic changes were not documented in our study population. Instead, the aging mature males more frequently performed leg cock marking, which is physically less demanding than handstand marking. Locomotor activity was also less frequent in geriatric males suggesting a preference for less energy expending behaviors. Other studies also [40] found older individuals were less active than younger males.

Vocalization in giant pandas carries unique information such as sex and age of individual pandas [36, 124] and is observed in wild pandas vocalizing from trees during breeding [40]. Although no difference was seen in the frequency of vocalization in the age groups studied, vocalizations may be equally important to all age groups, and not a behavior associated with sexual maturity. Charlton and colleagues [124] found that adult males showed interest in vocalizations of higher frequency, indicative of young males, whereas adult females showed more interest in lower frequency vocalizations, which are those typical of breeding males. Our results suggest vocalizations are important cues for social interaction among all male giant panda age groups.

In summary, sperm was produced by male giant pandas in all age groups, but most frequently in males 5.5 yr and older. Fecal androgen and body weight increased in males that were 6.5 yr and older. Declining body size was seen with increased age and was lowest in males >15.5 yr. Frequency of behaviors observed fluctuated throughout the age groups. Social and energy expending behaviors were highest among males 4.5 yr, whereas reproductive related behaviors (scent marking) and energy expending pacing behaviors were highest in males 6.5 to 9.5 – 15 yr of age. These data indicate that while sperm production, androgen production and body size have a clear difference between age groups and appear to indicate the male panda matures at 6.5 yr of age, behavior in these pandas are still maturing. The period of behavioral change appears to be more protracted and dynamic between 6.5 – 8.5 yr in male pandas culminating at 9.5 yr with hand stand scent marking. This suggests behavior maturity is the last to change during sexual maturity. Complete sexual

maturation (body morphometric, androgens, sperm traits, and sexual behaviors) finishes between 7.5 to 9.5 yr of age in the male giant panda. These results are similar to the speculation by Kleiman [40], who indicated sexual maturity may occur between 6-7 yr in the male panda. That study followed one male giant panda [40]. This panda displayed developmental behavioral trends as he aged from 4 – 9 yr of age including the start of frequent scent marking when he was 8 yr old [40].

Our study demonstrates that a dynamic cascade of development occurs in the male giant panda allowing reproductive maturity to occur between 7.5 – 8.5 yr for reproductive success. This age of sexual maturity is somewhat later than other bear species which are reproductively mature between 3-6 yr of age depending on the species [133, 136, 137], although for these species, sexual maturity is largely described as ability to produce sperm, not reproductive success. This may also be due to the complex mechanisms of communication unique to the giant panda such as scent marking requiring protracted development for maximal reproductive fitness.

Chapter 4:

Giant panda (*Ailuropoda melanoleuca*) sperm are cryotolerant at different freezing and thawing rates

Abstract

Artificial insemination with fresh or cryopreserved sperm is used in the propagation and genetic management of the global ex situ giant panda (*Ailuropoda melanoleuca*) population. Our goal was to determine the cryotolerance of giant panda spermatozoa by comparing cryomethods with three freezing approaches and then thawing all aliquots at three differing temperatures. Ejaculates (n = 28) from five adult pandas were assessed for sperm traits, including cellular motility, forward progressive status (another indicator of motility) and acrosomal integrity. Fresh semen was diluted and washed in Ham's F10 HEPES buffered medium before resuspending the sperm pellet in a modified TEST egg yolk cryodiluent (3.3% glycerol) to yield 400 x 10^6 motile sperm/ml. Samples were cooled slowly to 4°C over 3 h, loaded into straws and cryopreserved using: 1) a manual two-step method (-35°C/min for 1 min, then -100°C/min for 1 min); 2) an automated freezer (Forma Cryomed®; -40°C/min for 1.5 min, then -100°C/min for 1 min); and 3) a dry shipper plunge approach (-196ºC/10 min). Samples frozen in each cryomethod were thawed at each of three thawing rates; 1) 22^{o}C for 30 sec; 2) 37^{o}C for 30 sec; and 3) 50^{o}C for 10 sec. Immediately after thawing at 0 h, each aliquot was washed in H-F10/HEPES medium, maintained at 37^{o}C and evaluated for sperm motility, forward progressive status and acrosomal integrity. Sperm motility traits continued to be assessed at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h and used to calculate a longevity quotient in vitro. Post-thaw sperm motility and

forward progressive status values were similar ($P > 0.05$) among the manual two-step, automated freezer and dry shipper methods. However, the sperm longevity quotient was higher ($P < 0.05$) in the dry shipper than the automated approach, whereas both were similar ($P > 0.05$) to the manual two-step. Post-thaw sperm motility was higher ($P < 0.05$) when samples were thawed at 50ºC compared to 22ºC, but both were similar ($P > 0.05$) to 37ºC. Sperm progressive status after thawing was higher ($P < 0.05$) when using 50ºC for thawing compared to 22ºC or 37ºC. Overall, optimal post-thaw sperm quality (on the basis of motility and acrosomal integrity) was achieved with the combination two-step/50ºC, two-step/37ºC or the dry shipper/50°C approach. These data demonstrate that the giant panda spermatozoon is cryotolerant, having the capacity to withstand cold stress applied by multiple means.

Introduction

Fewer than 2,000 giant pandas exist in nature in China [15]. About 10% of the global population resides in captivity (approximately 300 individuals) within breeding centers and zoos (ex situ). These individual serve both as conservation ambassadors and as a resource to generate biological knowledge and offspring for future reintroduction efforts [10]. Most of the ex situ population is managed in large breeding centers in China that emphasize both growing the population and ensuring its genetic integrity, especially maximizing genetic diversity to avoid inbreeding depression. However, controlling reproduction in the giant panda is challenging, in part, because the female displays only a single brief estrus once per year [4, 35, 80]. Additionally, arrays of other factors have been identified that limit reproductive success in this captive population [10, 13]. Among priority inhibitors are inappropriate breeding behaviors and mate aggression both of which can prevent males from copulating during the narrow window of female sexual receptivity [120]. For these reasons, the giant panda has emerged as a species that benefit from assisted reproduction and, specifically artificial insemination (AI) [8, 9, 13].

There has been significant progress in the use of AI with giant pandas since the first successful birth using fresh ejaculate in 1982 [13]. Currently, ~20 to 30 cubs are produced annually within the worldwide ex situ population by natural breeding and/or AI, mostly using fresh spermatozoa. Timing for AI is facilitated by distinctive female behaviors [40] and a hormonal profile easily discernible by monitoring urinary estrogen and progesterone metabolites [33, 80]. Giant panda semen is generally collected by electroejaculation [7], a process that has allowed substantial basic

research into understanding the physiology of giant panda sperm ultra structure [81], capacitation [82], the acrosome reaction [83] and gamete interaction in vitro [82].

It is well established that the damage incurred by mammalian sperm during cryopreservation does not occur during the cell's initial exposure to ultra low (-80 to-196°C) temperature, but during the intermediate temperature zone (-15 to -60°C) [91]. The rate at which the sperm pass through this interval during the freezing as well as the thawing process is critical. Injury occurring during these temperatures may be reversible, but more likely is lethal due to both disrupted membranes and cell motility both of which can decrease sperm penetration into the oocyte, largely by compromising capacitation and the acrosome reaction [92]. Furthermore, cellular reactions during freeze-thawing following classical cryo-biological principles can cause membrane damage by significant shrinking and swelling of the sperm. Any cooling of a sperm suspension below 0°C (-5 – < -10°C), triggers extracellular ice crystal formation and increased extracellular solute concentration [93]. Water within the spermatozoon then is super-cooled, but unfrozen. Because of different solute concentration gradients across the sperm plasma membrane, water flows out of the cell until the internal osmolality matches that of the surrounding, external medium that then is provoked to freeze. The loss of intracellular fluid dehydrates the spermatozoon allowing cryopreservation without cell lysis from internal ice formation. If insufficient intracellular water is removed, irreversible cell damage occurs due to ice crystal formation [93]. Therefore, research has focused on understanding sensitivity to cooling rate at the intermediate zone temperatures (-12 to -40°C) to minimize the potential for cellular destruction [93]. Cooling rates usually

are of three types, with slow change (< 10°C/min) allowing the formation of large extracellular ice crystals that causes intracellular water efflux and minimal intracellular ice. Rapid cooling (-10 to -100°C/min) inhibits water movement from the cell, which can result in some microcrystal formation within the spermatozoon inside the sperm [93]. Very rapid cooling (several hundred degrees/min) offers only brief opportunity for water to depart the cell and generally results in both internal and external microcrystal ice formation [99].

Thawing of frozen spermatozon also presents opportunities for cellular damage. The goal is for cryoprotectant to leave the spermatozoon while water enters at a rate compatible with avoiding or minimizing microcrystal formation and cellular ballooning and bursting. Although there are strong species preferences for the ideal rate, slow thawing frequently causes local, intracellular microcrystal formation that damages organelles and decreases sperm motility [94]. Thus, in some species, such as the domestic sheep, sperm that are rapidly thawed (70°C for 5 sec or 50°C for 9 sec) have higher survival and quality than counterpart cells thawed more slowly [71].

There are no comparative studies on the cryosensitivity of giant panda spermatozoa. Although offspring of this species have been produced using AI exclusively with thawed sperm (and no concomitant copulation) [13], there are no comparative data on level of cold tolerance of these cells. Thus, our first objective was to compare the motility and acrosomal integrity of giant panda sperm that were frozen at three distinctive rates following by thawing at three different speeds. This was achieved by applying three cryomethods -- a two-step manual approach (-35°C/min for 1 min, then -100°C/min for 1 min), an automated, programmable

machine (-40°C/min for 1.5 min, then –100°C/min for 1 min) and a direct plunge into a dry nitrogen vapor within a shipping container (-196ºC over 10 min). If panda sperm are especially resilient to cold temperatures, perhaps it would be possible to freeze store this germplasm simply, under field conditions using a dry shipper liquid nitrogen vapor method.

Our second objective was to examine the influence of thawing panda sperm at different velocities, including a slow (22^{o}C for 30 sec), rapid (37^{o}C for 30 sec) and very rapid (50^{o}C for 10 sec) approach. Based on the limited studies published in other ursid species about sperm cryotolerance [135, 136], and early findings that thawed giant panda sperm had the capacity to produce offspring by AI [13], our hypothesis was that the sperm of this species were cryo-tolerant and survived in vitro despite wide variance in both the applied freezing and thawing rate used.

Materials and Methods

Experimental design

The experimental plan and animal procedures were approved by the Institutional Animal Care and Use Committees at the Smithsonian's National Zoological Park, University of Maryland at College Park, Chinese Association of Zoological Gardens and the Chengdu Research Base of Giant Panda Breeding.

Animals

The five study animals were adult male giant pandas (6 – 19 yr) maintained at the Chengdu Research Base for Giant Panda Breeding or the nearby Chengdu Zoo in Sichuan Province, People's Republic of China (30°N, 104°E). All individuals were

captive born and four individuals were proven breeders as demonstrated by the production of living young within 2 yr of study onset. Each giant panda was housed individually in an enclosure with combined indoor (from 3.0 m x 3.0 m to 10 m x 20 m) and outdoor (from 12 m x 12 m to 20 m x 35 m) areas, all of which were illuminated by natural lighting. Water was available ad libitum, and freshly-cut seasonally available wild bamboo (10 – 20 kg daily) was provided throughout the day along with a biscuit supplement (~1,000 gm/day/male; range, 700 – 1,500 gm/day/male) that provided additional fiber, vitamins and minerals (proprietary recipe of the Chengdu Research Base). All males were managed within olfactory, auditory and visual proximity of adult and juvenile conspecifics of both sexes. Physical interaction between males and estrual females was permitted during the breeding season only (Feb through May) for brief (2 – 15 min) mating attempts.

Semen collection and analysis

Semen was recovered from males during the months of February through May, a time interval well established as the reproductive season for the male giant panda [79, Aitken-Palmer in prep, Chapter 1]. For ejaculate collection, each male was induced into a surgical plane of anesthesia using 7 to 12 mg/kg ketamine hydrochloride (Sinceta International Trading Co., Ltd., Shanghai, China) via intramuscular dart. To increase depth and duration of anesthesia, supplemental isoflurane gas (Ningbo Samreal Import and Export Co., Ltd., Shanghai, China) was provided as necessary via a face mask and/or intubation.

Semen was collected by electroejaculation using a well-described, extensively used approach [7, 13]. In brief, this method relied on a 2.6 to 3.2 cm diameter rectal

probe with three longitudinal electrodes and a 60 Hz, sine wave stimulator (P.T. Electronic, Boring, OR) that was used to deliver standardized sets of low voltage stimulations (1– 4 V) over three series of 30 stimuli each. The electroejaculation interval generally required ~20 min to elicit an erection with semen then captured in a temperature controlled (37°C), water jacket [13].

Ejaculate volume was measured and pH determined using pH indicator strips to ensure that semen was uncontaminated with urine (Colorphast®, EM Science, Gibbstown, NJ). A 5 µl ejaculate aliquot was examined for a subjective estimate of sperm motility (0 – 100%) and forward progressive status (i.e., an indicator of the type of forward cellular trajectory and speed; scale, 0 – 5; 5 = fastest and straightest movement) using phase-contrast microscopy (200 – 400x; 37°C heated stage) [13]. After calculating sperm concentration using a standard hemocytometer method [13], total sperm per ejaculate was determined by multiplying sperm concentration/ml and ejaculate volume. This information was used to ensure that standard numbers of spermatozoa were assigned to various cryo-treatments (see below).

Sperm morphology and acrosomal membrane

Sperm morphology was assessed after storing and fixing a 10 µl sample of raw semen in 100 µl of fixative (0.3% glutaraldehyde in phosphate buffered saline) and using phase contrast microscopy (x1000) to evaluate 200 sperm per sample [13, 84, 85]. Sperm were categorized as normal or as having one of the following anomalies: 1) an abnormal head, including macrocephaly, microcephaly or bicephaly; 2) abnormal acrosome; 3) coiled flagellum; 4) bent midpiece with cytoplasmic droplet; 5) bent midpiece without cytoplasmic droplet; 6) bent flagellum with cytoplasmic droplet; 7)

bent flagellum without cytoplasmic droplet; 8) proximal cytoplasmic droplet; or 9) distal cytoplasmic droplet [13, 85]. In cases of multiple defects for a given spermatozoon, each cell was classified according to the most serious malformation [13]. For example, if a sperm was observed to contain both a bent flagellum and an abnormal acrosome, that cell would be categorized as having the latter abnormality [7].

Sperm head morphology was further delineated by detailed evaluations of acrosomal integrity using rose bengal/fast green stain [13]. Briefly, an aliquot of raw, unfixed semen was diluted 1:10 (1 part semen to 10 parts medium) in Ham's F10 medium (Irvine Scientific, Santa Anna, CA) supplemented with 20 mM HEPES buffer, 5% (vol/vol) heat-treated fetal calf serum (Irvine Scientific), pyruvate (1 mM), L-glutamine (2 mM), penicillin (10,000 IU/ml), streptomycin (10 mg/ml) and neomycin (20 mg/ml) (Sigma-Aldrich Co., St Louis, MO), collectively designated as H-F10/HEPES medium. For staining, 1 μl of this diluted semen was added to 9 μl of rose bengal (high purity biological stain, Cole-Parmer, Vernon Hills, IL) and fast green stain (certified biological stain, Sigma-Aldrich Co.), incubated for 90 sec at room temperature and smeared on a glass slide. A minimum of 100 sperm acrosomes per sample was assessed by bright-field microscopy (1,000x) for either a: 1) normal apical ridge (uniform staining of the acrosome over the anterior half of sperm head); 2) damaged apical ridge (non-uniform 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) [13] (Fig. 1).

Comparative cryo-approach

Semen was diluted 1:3 (1 part semen to 3 parts medium) and washed (200 *g*, 8 min) in H-F10/HEPES medium at 37°C and supernatant discarded. The sperm pellet was resuspended in a commercially available, modified TEST Yolk Buffer (TYB; Irvine Scientific) containing 3.3% glycerol (combination of 12% glycerol 'Freezing Medium' and 0% glycerol 'Refrigeration Medium') to yield a final concentration of 400 x 10^6 motile sperm/ml. The sample was placed in a 400 ml water jacket and allowed to cool slowly to 4ºC over 3 h [7, 13, 84, 85]. Cooled semen then was pipetted into 0.25 ml sterile, plastic straws, each of which was labeled according to donor, date of collection, method of freeze, and then heat-sealed. All straws were inventoried for later thaw. The total number of cooled straws per ejaculate (range, 9 – 30 straws) were equally divided and exposed immediately to one of three cryo-approaches. The cooling-freezing rate for each method was validated in preemptive evaluations by using the same dilution medium containing cryoprotectant (but with no spermatozoa) and fitted with a thermocouple and recording device. The first was a two-step over liquid nitrogen vapor method that has been used commonly for cryo-banking giant panda sperm in China [13]. This technique involved suspending the straws in a horizontal position on a metal rack sitting in a liquid nitrogen bath [13]. Using metal tongs, the straws first were placed 7.5 cm (for 1 min) and then 2.5 cm (for 1 min) over liquid nitrogen before plunging into liquid nitrogen, which translated into a cooling-freezing rate of -35°C/min for Step 1 and then -100°C/min for Step 2 [13]. The second approach involved placing the straws horizontally within an automated, liquid nitrogen-fed freezer unit (Forma Cryomed®, Model 8018) that was

programmed to decrease the chamber temperature by -40°C/min for 1.5 min and then at –100°C/min for 1 min. At the end of this interval, straws were rapidly transferred and immersed in liquid nitrogen. The third approach involved placing the sealed straws vertically, a goblet on a sperm storage cane surrounded by a sleeve in a standard canister of a liquid nitrogen dry shipper (CryoPack Series, Taylor-Wharton®, Theodore, AL), commonly used for safely transporting frozen biomaterials. The internal component of the shipping unit is comprised an cryotolerant absorbent material previously infused with liquid nitrogen, thereby providing an internal core temperature of approx. -196°C.

Cryopreservation using the dry shipper method simply involved manually lowering the internal canister containing the previously cooled (4°C) straws over 1 sec into the core base of the shipping unit, previously determined to translate into a freezing rate of -196°C over 10 min. All frozen straws were transferred to appropriately labeled canes indicating donor, date and cryo-approach and then canisters stored within liquid nitrogen of a 35 L dewar [Taylor-Warton, Theodore, AL, USA] for 2-6 mo before evaluation.

Thawing rates and post-thaw sperm quality

Sperm-containing straws from each cryomethod were divided equally for evaluating three thawing rates, at ambient (22^{o}C for 30 sec), warm (37^{o}C for 30 sec) or an elevated/fast (50^{o}C for 10 sec) temperature. After thawing, both ends of the straw were cut, and the sperm sample was allowed to flow into a 5 ml sterile plastic tube and diluted immediately in 2 ml H-F10/HEPES culture medium (Irvine Scientific) at

37°C and then assessed immediately for sperm motility, forward progressive status and morphology, with a focus on acrosomal integrity (Time 0).

Immediately after thawing at 0 h, each straw aliquot was washed in H-F10/HEPES medium, resuspended to a concentration of 20 x 10^6 sperm/ml and maintained at 37^{o}C in a dry-bath incubator. The two sperm motility metrics (percentage motility, forward progressive status) were assessed at Time 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h post-thawing, or until motility was < 5%. A sperm longevity quotient was calculated by dividing the post-thaw sperm motility value at 0 h by the negative slope of sperm motility decline over 24 h.

Statistical analysis

Analysis of variance and Tukey-Kramer multiple comparisons (JMP®, Cary, NC, USA) were used to evaluate the data for male effects as well as major effects of freezing rate, thawing rate and interactions among cryomethods. The influence of cryo-approach and thawing rate on sperm motility, forward progressive status, sperm longevity of motility and sperm acrosomal integrity (including normal apical ridge, damaged apical ridge, missing apical ridge and loose apical cap) was determined as individual as well as combined effects. Data are expressed as means ± S.E.M.

Results

Every male at each anesthesia episode produced a high quality electroejaculate containing seminal volumes, sperm concentrations, sperm motility traits and morphological integrities consistent with those reported earlier by others [Howard 2006 b]. Table 8 provides summary information on mean seminal and

sperm metrics for 28 giant panda ejaculates, demonstrating the prodigious numbers of motile, mostly structurally normal spermatozoa produced by this species during the breeding season. The predominant structural defect of fresh spermatozoon was a bent midpiece either with or without a residual cytoplasmic droplet (Table 8). Also notable was that the acrosome's apical ridge was normal in >85% of spermatozoa and was damaged or missing in fewer than 15% of total cells (Table 8).

There was no impact of individual male ($P > 0.05$) on the ability of giant panda sperm to withstand freezing and then thawing using our evaluated metrics (data not shown). Additionally, in the context of sperm morphotypes, acrosomal integrity was the only cellular feature not influenced by freezing or thawing rate (see below), and other metrics were unaffected ($P > 0.05$) and, thus, not presented here. In general and when evaluating the overall influence of the three freezing approaches (without regard to thawing rate), substantial motility and forward progression was retained regardless of rate treatment (Table 9). At Time 0, there was about a 20 to 25% reduction in sperm motility and a one full score decline in progressive status rating compared to pre-cryopreservation values (Table 8 versus 9). The incidence of sperm with a normal apical ridge declined by 33% to 44 % after the freeze-thawing process across all cryomethods and thaw rates. This injury was expressed by equal proportions of sperm with either a damaged or missing apical ridge. None of the freezing methods produced a loose acrosomal cap (Table 9). When cryo-approaches were compared (without considering specific thawing treatments), there was no affect ($P > 0.05$) on sperm motility metrics. However, the slow and fast freezing rates associated with the manual two-step and dry shipper methods, respectively, improved

($P < 0.05$) both retaining normal apical ridges and increasing sperm longevity in vitro.

When results were evaluated strictly on the basis of thawing rate (and without differentiating freezing approach), results were only modestly different from the contrasting comparison. Clearly, warmer/faster thawing enhanced ($P < 0.05$) overall motility recovery traits with sperm motility for the 50ºC/10 sec treatment being only 16% less than in fresh ejaculate (Table 8, 9). The reduction in normal apical ridges again was circa 39% less than in fresh spermatozoa (Table 8, 9) and, in the absence of considering freeze rate, was unaffected ($P > 0.05$) by thaw velocity (Table 9). Again, the increased prevalence of acrosomal injury was expressed by an equally increased incidence in both damaged and missing apical ridges. Longevity in vitro (like sperm motility) increased ($P < 0.05$) with warmer (and faster) thawing temperature (Table 9).

TABLE 8. Traits of fresh electroejaculate in the giant panda.

Ejaculate volume (ml)	1.3 ± 0.3
Ejaculate pH	8.1 ± 0.1
Sperm concentration/ml ($x10^6$)	2,189.3 ± 269.9
Sperm motility (%)	83.2 ± 1.3
Forward progressive status (0-5)*	4.0 ± 0.1
Total sperm/ejaculate ($x10^6$)	2,950.8 ± 467.9
Normal sperm morphology (%)	52.8 ± 4.8
Abnormal sperm morphology (%)	
Acrosomal defects	1.2 ± 4.2
Coiled flagellum	4.3 ± 1.5
Bent midpiece with droplet	16.3 ± 1.4
Bent midpiece without droplet	10.5 ± 1.8
Bent flagellum with droplet	8.1 ± 1.2
Bent flagellum without droplet	3.5 ± 2.1
Proximal cytoplasmic droplet	1.3 ± 0.5
Distal cytoplasmic droplet	2.0 ± 1.3
Sperm acrosomes (%)	
Normal apical ridge	86.0 ± 1.4
Damaged apical ridge	11.0 ± 3.9
Missing apical ridge	3.0 ± 1.2
Loose acrosomal cap	0.0 ± 0.0

Values are means ± SEM; n = 5 males, 28 ejaculates.
*Scale 0 to 5; 5 = best with most rapid, forward progression.

TABLE 9. Influence of cryo-approach (slow, fast, very fast) and thawing rate (22°C for 30 sec, 37°C for 30 sec, 50°C for 10 sec) on post-thaw giant panda sperm motility, forward progressive status, longevity of motility quotient and acrosomal integrity.

	Sperm motility (%)	Forward progressive status (0-5)	Sperm longevity quotient[§]	Sperm acrosomal integrity (%)			
				Normal apical ridge	Damaged apical ridge	Missing apical ridge	Loose acrosomal cap
Cryo-approach							
Slow (manual two-step)	65.7 ± 2.0	3.4 ± 0.1	21.5 ± 1.5[a,b]	53.0 ± 2.1[a]	26.5 ± 2.6	17.2 ± 2.4	0.1 ± 0.1
Fast (automated)	58.5 ± 2.4	3.0 ± 0.1	16.7 ± 1.5[b]	44.2 ± 2.5[b]	39.1 ± 4.2	23.3 ± 4.4	1.2 ± 1.2
Very fast (dry shipper)	61.1 ± 2.3	3.3 ± 0.1	25.8 ± 3.1[a]	50.6 ± 2.4[a]	29.3 ± 3.4	19.1 ± 2.8	0.0 ± 0.0
Thawing rate							
22°C for 30 sec	56.7 ± 2.2[a]	3.0 ± 0.1[a]	16.8 ± 1.8[a]	47.6 ± 2.1	28.8 ± 2.2	18.8 ± 2.6	0.1 ± 0.1
37°C for 30 sec	62.7 ± 2.2[a,b]	3.0 ± 0.1[a]	22.4 ± 2.8[b]	50.1 ± 2.3	26.5 ± 2.6	17.2 ± 2.4	0.1 ± 0.1
50°C for 10 sec	67.5 ± 2.2[b]	3.4 ± 0.1[b]	23.6 ± 1.8[b]	51.9 ± 2.5	29.3 ± 3.3	18.0 ± 3.6	0.0 ± 0.0

Values are means ± SEM measured at Time 0 (immediately post-thawing); n = 5 males, 8 ejaculates

*Scale 0 to 5; 5 = best with most rapid, forward progression.

[§]A sperm longevity quotient was calculated as post-thaw motility at 0 h divided by the negative slope of sperm motility decline over 24 h.

[a,b]Within a treatment (cryomethod or thawing rate), values within a column with different superscripts are different ($P < 0.05$).

These findings were supported when the data were combined and interactions assessed statistically. There was an overall influence of merging the freezing and thawing approaches on sperm quality ($P < 0.05$). When the warmer thaw temperatures (37ºC and 50ºC) were used, there was a tendency for increased sperm motility and progressive status with significance ($P < 0.05$) achieved in the fast freezing (i.e., automated unit) rate (Table 10). In the latter case, there was nearly a 15% advantage for sperm motility recovery when using a 50ºC versus 22ºC thawing temperature. Such enhancements were partially reflected in the sperm longevity quotients that consistently tended to be higher within freezing rate treatments for warmer thawing temperatures. Additionally, when considering thawing methodology, sperm survival duration in vitro was a clearer marker for differentiating among cryo-methods. For example, longevity values for the dry shipper and slowest thaw (22ºC) approach differed by 10% using the manual two-step and same thawing temperature (Table 10). Regardless, despite some differences among the nine treatment groups based on freezing and thawing rate interactions, it was possible to consistently recover ~78% of the original (Time 0) sperm motility and ~85% of the progressive status rating after cryo-storage. Less success was achieved in preventing acrosomal injury overall. Even optimal thaw approaches within each cryomethod allowed recovering only 54 to 64% of the original proportion of spermatozoa with this membrane intact. Nonetheless, combinations within each approach allowed at least half of all acrosomes to retain an intact apical ridge in five of nine (55.6%) treatment groups (Table 10).

TABLE 10. Combined influence of three cryo-approaches (Standard, Bench-top, Field-friendly) and three thawing rates (22°C for 30 sec, 37° for 30 sec, 50°C for 10 sec) on post-thaw giant panda sperm motility, forward progressive status, longevity of motility quotient and acrosomal integrity.

	Sperm motility (%)	Forward progressive status (0-5)	Sperm longevity quotient[§]	Sperm acrosomal integrity (%)			
				Normal apical ridge	Damaged apical ridge	Missing apical ridge	Loose acrosomal cap
Standard (two-step)							
22°C for 30 sec	63.8 ± 2.5[a,b]	3.2 ± 0.1[a,b]	15.0 ± 1.9[a,b]	49.8 ± 3.2	28.7 ± 2.2	18.8 ± 2.6	0.1 ± 0.1
37°C for 30 sec	68.5 ± 2.8[a]	3.4 ± 0.1[a,b]	20.6 ± 5.0[a,b]	55.9 ± 3.7	26.5 ± 2.6	17.2 ± 2.4	0.0 ± 0.0
50°C for 10 sec	70.7 ± 3.1[a]	3.5 ± 0.1[a]	24.1 ± 2.7[a,b]	55.3 ± 4.1	29.3 ± 3.3	18.0 ± 3.6	0.0 ± 0.0
Bench-top (Cryomed®)							
22°C for 30 sec	51.9 ± 3.6[b]	2.9 ± 0.1[b]	12.6 ± 1.9[a]	44.0 ± 3.5	25.8 ± 2.5	27.9 ± 2.8	0.0 ± 0.0
37°C for 30 sec	60.3 ± 3.5[a,b]	3.0 ± 0.1[b]	17.0 ± 2.6[a,b]	40.8 ± 3.5	39.1 ± 4.2	23.3 ± 4.4	0.0 ± 0.0
50°C for 10 sec	65.9 ± 3.0[a,b]	3.4 ± 0.1[a,b]	20.4 ± 3.2[a,b]	46.7 ± 5.0	29.2 ± 2.6	17.4 ± 2.6	0.1 ± 0.1
Field (dry shipper)							
22°C for 30 sec	58.6 ± 5.2[a,b]	3.2 ± 0.1[a,b]	24.7 ± 5.8[a,b]	50.3 ± 4.8	28.8 ± 2.7	23.8 ± 4.1	0.0 ± 0.0
37°C for 30 sec	63.0 ± 3.6[a,b]	3.3 ± 0.1[a,b]	27.7 ± 7.1[b]	50.9 ± 4.6	29.3 ± 3.4	19.1 ± 2.8	0.0 ± 0.0
50°C for 10 sec	69.8 ± 3.4[a]	3.6 ± 0.1[a]	27.1 ± 3.9[b]	52.1 ± 4.6	27.8 ± 2.5	22.4 ± 4.2	0.0 ± 0.0

Values are means ± SEM measured at Time 0 (immediately post-thawing); n = 5 males, 8 ejaculates

*Scale 0 to 5; 5 = best with most rapid, forward progression.

[§]A sperm longevity quotient was calculated as post-thaw motility at 0 h divided by the negative slope of sperm motility decline over 24 h.

[a,b]Within a treatment (cryomethod or thawing rate), values within a column with different superscripts are different ($P < 0.05$).

Discussion

The giant panda is a grass (i.e., bamboo) eating bear that is distantly related phylogenetically to other ursids [20]. Although the giant panda is a predominantly an herbivore it will occasionally consume various animal species, ranging from birds to small rodents [4]. This is relevant because of a remarkable variation among mammal species types in vulnerability of spermatozoa to low temperature exposure. For example, it is particularly challenging to preserve the male gamete of certain carnivores, especially those of felids (cats). A portion of this predicament has been attributed to an unusually high incidence of pleiomorphic (malformed) spermatozoa inherent to the ejaculate of certain felid species or genotypes [79]. However, it also has been asserted that felid spermatozoa are exquisitely sensitive to cold temperatures, including simply cooling the cells from core body temperature to 4ºC and before imposing a freezing stress [139]. Such hypersensitivities are not unusual as researchers have been challenged for years in developing successful gamete cryopreservation approaches for even the laboratory mouse [87]. Therefore, we found it heartening to discover empirical evidence for high cryo-tolerance in the spermatozoa of the giant panda. Regardless of freezing or thawing rate, including at ultra-rapid velocities, generally our in vitro assessments demonstrated a comparatively high incidence of motility trait recovery (circa 78 – 85%) with at least half of the sperm acrosomes escaping damage. The logistics of contemporary giant panda breeding management in China did not permit systematically testing the biological competence of sperm frozen in the different approaches of our

investigation. Nonetheless, one of our tested methods (manual two-step) routinely is used for cryo-banking and is known to result in offspring post-AI [13].

The few previous publications on sperm cryopreservation in ursids (i.e., brown bear [138] and Japanese black bear [140, 141] have largely focused on single evaluations and freezing (~20 – 100ºC/min) and thawing (~65°C for 6 min) rates. Therefore, based on our thorough multi-factorial assessment, combined with earlier in vivo AI success [13], we conclude here that the giant panda spermatozoon epitomizes a freeze-tolerant gamete. Among bears, it is difficult to know if panda sperm is more or less cryotolerant. But bear sperm has been frozen at a variety of slow and rapid rates.

Several investigations have examined the ability of bear sperm to simply survive freezing and thawing in a non-comparative fashion, largely by exposing sperm to a given process, ranging from simply cooling over liquid nitrogen vapor to placement in a programmable, automated freezing unit [142, 143]. Findings suggest that ursid spermatozoa abide a wide range in cooling and freezing rates. For example, Hokkaido brown bear (*Ursus artos yesoensis*) sperm were cooled to 4°C over 90 min in plastic straws and frozen at -9.8°C/min and then at -46.6°C/min using a manual two-step method over liquid nitrogen vapor [136]. Japanese black bear (*Ursus thibetanus japonicus*) sperm were frozen by suspending a vial 6 cm over liquid nitrogen vapor for 15 min before immersing directly into liquid nitrogen [25]. Sperm of the Cantabrian brown bear (*Ursus arctos arctos*) were cooled to 5°C over 80 min and frozen at a rate of -20°C/min using a programmable biofreezer [142, 143]. Although none of these studies systematically compared differing cryopreservation or

thawing [137, 140] protocols, all reported that sperm survived based on microscopic evidence of forward sperm motility. There has been minimal effort to examine other indicators of sperm viability, such as acrosomal integrity in bear species. However, the morphological normality of the sperm head of the brown bear (*Ursus arctos*) appears adversely affected by slow freezing (-20°C/min) [143].

Since acrosomal integrity was not impacted by cryo-approach and rate of thaw in the giant panda, perhaps acrosome is not the best indicator of cryodamage compared to other methods of evaluation such as motility, forward progression and longevity. Motility and forward progression is described as a good method for sperm evaluation after cryopreservation for other bear species. Although most of the sperm motility and progressive status was recoverable post-thawing in the giant panda, each of the three freezing and three thawing approaches were resulting in as little as 30-40% acrosomal membrane damage indicating relatively low amounts of temperature mitigated injury, which likely resulted from ice-crystal induced lysis.

The cold tolerance of giant panda spermatozoon has significant practical application. From an ex situ management perspective, it lends credibility to ongoing activities in China to develop sperm banking repositories for helping retain species gene diversity as well as dealing with inevitable mate incompatibilities requiring AI [13, 24]. Our findings confirmed that giant panda spermatozoa endure the stresses associated with both freezing and thawing, including preferring higher than expected warming temperatures. However, particularly fascinating was the ability to retain high levels of cellular motility and respectable acrosomal integrity by freezing a glycerol-protected aliquot in a simple dry shipping container. These devices are

inexpensive and sufficiently light weight for transport. Thus, given the ability to initially cool a diluted seminal aliquot to 4ºC (for example in a cold box powered by a battery) the 'dry shipper approach' has strong potential for field application.

A field cryo-approach could be helpful for the captive management of giant panda breeding. Beginning in the late 1990s, the Chinese government began avoiding the capture of wild giant pandas to support the ex situ breeding program. Although the captive population currently has adequate gene diversity, heterozygosity can gradually be lost over time due to genetic drift and occasional inappropriate breeding [24]. Ideally, an ex situ population could benefit significantly by the intermittent infusion of new genetic material, preferably via frozen sperm provided by wild founders. Given the high current success now enjoyed by a combination of natural breeding and AI [personal communication Zhang Zhihe] the giant panda could be a classical model of one of the first uses of frozen sperm taken from a wild male to support an ex situ breeding program. Similar success has been met in one species, the cheetah [144], but only for producing milestone pregnancies and not for routine species management. This approach may well find additional application in other ursid species.

Chapter 5:

Discussion: Research impact and significance

The giant panda is without a doubt, a flag ship species for global wildlife conservation. The panda is widely known as an endangered species and is always topical with the media. As a result, the giant panda has been the focus of study for Chinese and international scientists over the past 20 yr [10]. During those 20 yr, the first 10 yr set the stage for basic research on the physiology and health of this species and the world media watched as the panda population faced challenges with habitat loss and failure to thrive within captivity. As China, over the next 10 yr, changed logging laws and set land aside for the protection of the wild panda, large scale panda census projects have indicated the population is stable [16]. Although the wild panda population has not declined in recent years, habitat fragmentation and influence from the human population threaten long term survivorship of this species [6, 145].

The last 20 yr has been a pivotal period for the captive panda population. Research has indicated nutrition, specifically bamboo, is critical for the giant panda to thrive. It is clear that excellent nutrition allows optimal health and leads to improved reproduction for the giant panda. Improved access to high quality bamboo has elevated the health status of captive population of giant panda and even resulted in a significant increase in giant panda offspring born over the past decade. Although nutrition is critical to this boom in the captive panda population, this was not the only facet of change within the captive population. An improved understanding of giant panda reproductive physiology over the past 20 yr has resulted in a significant

increase in the number of giant panda born each year within the captive population. Extensive global research partnerships have resulted in successful sperm cryo-storage [13], artificial insemination [8], hormone evaluation [76], and environmental enrichment for the captive panda population [47]. This work has allow the ex situ panda population to reach a goal of self sustainability. There are few endangered species, with self sustainable captive populations. This again, makes the giant panda a true flag ship species and an example of what can happen when global partnerships succeed. Now that the captive panda population is self sustainable, there is ample genetic diversity within this population for the next 100 yr. As this population continues to thrive and grow, our understanding of this species and their special physiological adaptations continues to improve.

The doctoral research presented in this dissertation filled surprising gaps in our knowledge of male giant panda reproductive physiology. This work will hopefully provide information for the improved care and propagation of the captive panda population while providing information for those who study and create legislation for the wild panda.

This body of work provides insight into a remarkable gender difference in onset and duration of gonadal activity between the male and female giant panda. Because the male giant panda is actively fluctuating between hormone, gonadal and behavioral change throughout the year to accommodate the female reproductive cycle, they may be exquisitely sensitive to changes in management during the year. Our findings indicate the male pandas' reproductive patterns may be associated with photoperiod and exposure to seasonal changes in natural light. If exposed to artificial

light patterns different from natural light pattern, this could alter the male physiology [146] and result in decreased reproductive success for that male panda. It is possible that exposure to natural light patterns is equally critical to successful reproduction in the female panda. It is possible that any slight alteration in this light pattern by artificial light could completely change the length of breeding season or even the time of year that the female estrus occurs [146]. As the ex situ panda population grows and more giant panda reside in captivity, special consideration for exposure to natural light patterns is critical for the continued reproductive success of the giant panda. In male or female pandas that do not adhere to the normal panda seasonal reproductive pattern, exposure to artificial light patterns should be the first factor evaluated and remedied.

The seasonal pattern of hormone change, gonadal change and behavior change seen in the captive male pandas studied here appeared to be consistent across males and across multiple years of study. This indicates there is a strong drive to preserve these patterns for optimal reproductive success. These patterns are likely similar among wild panda, but would be an important area of study [40, 147]. Because these patterns appear robust, any ex situ male panda that displays a different pattern in reproductive hormone change, gonadal change or behavior change, probably is not as reproductive successful as others and his health and management should be evaluated.

Our study also indicates that male giant panda begin producing sperm early in the year. In this context, the systematic banking of spermatozoa can occur during Dec and Jan so as not to interfere with the female breeding season. This could be valuable information in the future, if sperm is collected from wild pandas. Allowing

for reproductive success of the wild panda is critical, if sperm is collected from wild males during the Dec/Jan this should not impact their potential to mate normally during the female breeding season Feb/Mar/Apr/May.

The second study of this dissertation systematically evaluated androgen change, gonadal change and behavioral change as the male giant panda matures. The results of this study demonstrated that a dynamic cascade of development begins prior to 4.5 yr of age in the male giant panda and continues allowing reproductive maturity to occur between 7.5 to 9.5 yr. This protracted period of reproductive development culminating with changes in reproductive behavior, may be due to the complex mechanisms of communication unique to the giant panda such as scent marking. While sexual maturity is often described as the ability to produce sperm, the development of complicated reproductive behaviors allows for optimal reproductive success and is also a critical element of reproductive fitness for the giant panda. This is especially true for the wild panda, where competition with conspecifics for mates occurs [4, 40]. As reintroduction of captive pandas into the wild panda population becomes a priority [10], male maturity becomes an important factor. Male pandas that are not mature in hormone production, gonadal function and behavior cannot be expected to thrive in the wild and compete with male conspecifics. The period of development occurring in male pandas between 4.5 to 9.5 yr is critical for reproductive fitness and success with other pandas and should not be ignored when evaluating for reintroduction.

The development of reproductive behavior in the captive male population has not been considered critical, because males to not have the same need to seek out

females, communicate with and fight with other males for the opportunity to mate. Within the ex situ population, males are house singly and are exposed to females based on relatedness and priority for breeding. Challenges associated with inappropriate male breeding behavior such as abnormal mounting and aggression toward female conspecifics continues to prevent natural mating by certain individuals within captivity. Often within ex situ populations, male panda are introduced to females for breeding younger than 9.5 yr of age. Perhaps these early introductions are not ideal because males are not fully sexually mature. It is possible that the behavioral development is more critical in some males because a few individual males have been documented to breed at a young age (i.e., 4.5 -6.5 yr). Because only few males mate naturally, especially at this young age, it is certainly possible that behavior development is critical during this period and should not be rushed. It is possible that these early introductions, during this period of behavioral development, is altering this development and predisposes the pandas to abnormal reproductive behavior. Perhaps introductions between captive male and female pandas should be reserved for males displaying androgens, gonadal function and reproductive behaviors consistent with adult sexual maturity for maximal chances of reproductive success. This is certainly an area worthy of future study since abnormal breeding behavior continues to plague the ex situ panda population.

The final study of this dissertation comparatively evaluating sperm cryopreservation has wide application for the giant panda conservation. Because few male giant pandas within captivity will naturally mate, artificial insemination has been used globally to increase the ex situ panda population. Cryopreservation of

giant panda sperm is used at nearly every zoo and breeding centers for giant pandas [13]. Many of these facilities have limited resources and training for sperm cryopreservation, indicating the necessity of a simplified cryo-approach for giant panda sperm [13]. The field-friendly dry shipper approach for giant panda sperm cryopreservation presented in this dissertation provides a simple approach to fill this need. It can be used with minimal equipment, training and resources and allows immediate transport of the cryopreserved sperm. This will become increasingly important at sperm may be exchanged between international zoos and breeding centers to preserve the genetic diversity of the giant panda as the ex situ population expands globally.

As access to the wild panda population increases, and studies on reproduction within the wild population expands, a field friendly approach for sperm cryopreservation will be critical to the collection, preservation and transport of this sperm. This may even have application for gamete exchange between the wild and captive population for artificial insemination and maximal genetic diversity among populations. This may provide an option for small in situ populations challenged with habitat fragmentation and genetic isolation from other populations.

Beyond the use of the cryo-approaches presented in our studies, we demonstrate giant panda sperm are cryotolerant during the freezing and thawing process. This may provide insight during further research on the tolerance of sperm within the family Ursidae and the osmotic changes associated with freezing and thawing. Further study on what mechanism allows the giant panda sperm to be

particularly cryotolerant may provide clues to what limits sperm cryotolerance in other mammalian species.

The application of the present research certainly has implications for the giant panda, both the ex situ and in situ populations. Broader application of this body of research on other species continues to demonstrate why the giant panda serves as flag ship species for endangered species conservation.

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