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Effects of season and social interaction on fecal testosterone metabolites in wild male giant pandas: implications for energetics and mating strategies

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Abstract In the first-ever study of reproductive endocrinology in wild male giant pandas (*Ailuropoda melanoleuca*), we provide new insights into the reproductive ecology of the species. We tracked and observed pandas in Foping Nature Reserve of the Qinling Mountains for 3 years, collecting fecal samples for testosterone metabolite analysis and data on reproductive activity. Males encountered multiple potential mates and competed for reproductive access to females. Male testosterone metabolites increased in February, peaked in March and April, and fell back to baseline after the mating season. However, males did not maintain a high testosterone level throughout the mating season. Male testosterone instead peaked during encounters with potential mates and declined between encounters. These results indicate that testicular activity is typically dormant until mobilized by interactions with females and potentially by interactions with male competitors. This suggests that male pandas may be energetically constrained, elevating testosterone levels only when necessary to meet the demands of intrasexual competition and courtship and fertilization of females. Maintaining a high testosterone level is metabolically expensive and male pandas enter the mating season during a period of low food availability. If this hypothesis is correct, male panda body condition may be an important determinant of reproductive outcome, and anthropogenic activities that diminish foraging resources may have significant impacts on the mating ecology of the species, affecting its conservation.

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Introduction

Androgens, most commonly testosterone, are the major reproductive steroid hormones for males. Testosterone fluctuates in seasonally breeding species and is tightly regulated by photoperiod and temperature (Palmer et al. 1988; Clay et al. 1988) and stimuli produced by females (Rianne et al. 2003). It is also linked with aggression (Cavigelli and Pereira 2000) to the extent that testosterone levels during times of breeding are more closely associated with male aggression and competition than with other changes in reproductive physiology (Wingfield et al. 1990; Zielinski and Vandenbergh 1993).

Many testosterone-dependent behaviors may be costly in terms of immuno-competence (Folstad and Karter 1992) and energy (Marler and Ryan 1996). A positive relationship

between circulating testosterone levels and energy expenditure has been found for male lizards and sparrows (Marler et al. 1995; Buchanan et al. 2001). We know that body condition is important for testosterone production (Wingfield et al. 1987; Duckworth et al. 2001) and that individuals in good body condition should have higher levels of testosterone (Lorenzo et al. 2006). Additional factors such as food quality and availability may also play an important role in determining testosterone levels and reproductive behavior and further influence reproductive strategy (Barnes 1984; Ruiz et al. 2010).

Adult giant pandas are mostly solitary, only coming together during the breeding season for reproductive purposes (Schaller et al. 1985). Chemical and acoustic signals are used to advertise reproductive condition, attract mates, and coordinate mating activities (Swaisgood et al. 2004; Charlton et al. 2010, 2011). Usually, an estrous female is accompanied by one to several males who compete for the opportunity to copulate and the female spend the majority of the period sequestered in the branches of a tree above. The mating site is usually on a ridge and it facilitates a fight among males. Males are known to copulate with multiple females within a single mating season (Hu et al. 1985; Pan et al. 2001). While the reproductive biology of giant pandas has received some attention (reviews in Wildt et al. (2006)), nothing is known about their reproductive physiology in natural environments. The majority of reproductive research has focused on female captive giant pandas with the expressed aim of maximizing the success of ex situ breeding programs (Chaudhuri et al. 1988; McGeehan et al. 2002; Czekala et al. 2003; Lindburg et al. 2001; Hama et al. 2008; Kersey et al. 2010a). Only a handful of studies have been done on male androgen profiles, all in captivity (Bonney et al. 1982; Macdonald et al. 2006; Charlton et al. 2011; Kersey et al. 2010b).

Non-invasion fecal hormone measurements have been used in many species (Mohle et al. 2002; Mostl et al. 2002; Chang et al. 2009). Here we investigate fecal testosterone levels of wild male giant pandas. Our main goal was to obtain first-hand measures of seasonal and social influences on reproductive physiology in wild male giant pandas to contribute to the growing body of research on this endangered species (Swaisgood et al. 2010) that will aid in its conservation.

Materials and methods

Study site and animals

The study was conducted in Foping Nature Reserve (33.832–33.845° N, 107.840–107.855° E), Shanxi, China.

The reserve was established in 1978 primarily for the preservation of giant pandas and is located on the southern slopes of the Qinling Mountains. Across the Qinling Mountains, giant pandas live at low elevations from September to June and at high elevations at all other times (Yong et al. 1994). Foping Nature Reserve covers elevations from 980 to 2,904 m above sea level and an area of 293 km^2. It contains the highest density of wild giant pandas in the world (State Forestry Administration—China 2006) and thus presents an exceptional opportunity to study the species.

In 2007, two adult males named Xiyue (XY) and Cancan (CC) were fitted with GPS/VHF radio collars (GPS4400MU, Lotek Wireless Inc., Ontario, Canada) by the approval of State Forestry Administration, China. This made it possible for us to track them, locate their feces, conduct behavioral observations, and observe mating behavior.

Fecal collection and behavioral observations

We observed the two males from March to May 2007, February to May 2008, and October 2008 to May 2009. These periods included three consecutive mating seasons (March–May) and one non-mating season. We tracked and observed XY and CC on 147 and 159 days, respectively. We collected fresh fecal samples (0–3 h old, as determined by visual observation of defecation) every 3–5 days during the non-mating season, every 1–2 days during the mating season, and up to once daily when males accompanied a female. Fecal samples were stored in zip-lock bags, placed on ice for transport in the field within 4 h, frozen at −20°C until laboratory analysis.

We classified each fecal sample according to season and the male's association with females. Season was classified by month (October 2008 to May 2009): pre-mating ($N=58$ samples; October–February), mating ($N=76$ samples; March–April), and post-mating ($N=23$ samples; May), following earlier observatons of seasonal mating activity (Schaller et al. 1985; Pan et al. 2001). A male was considered to be *consorting* a female if (a) he remained intermittently in visual contact with the female for>24 h and (b) engaged in mutual vocal interaction with the female. It is important to note that these social interactions are in dramatic contrast to observations outside the mating season when these highly solitary animals are not found in association with any other pandas for many days at a time (Schaller et al. 1985; unpublished data from the present study). All fecal samples in the three mating seasonswere classified as follows: (1) *pre-consortship* ($N=30$; samples collected after March 1 but before the male consorted with a female), (2) *consortship* ($N=50$; samples collected on days when the male was observed to be consorting a female), (3) *interval* ($N=63$; samples collected on days

between consortships), or (4) *post-consortship* ($N=51$; samples collected at the end of the mating season after the male was no longer observed to be consorting any females but before the male migrated out of the area to the summer range at a higher elevation).

Fecal extraction and hormone analysis

We used a technique modified after those described by Wasser et al. (1994) and Li et al. (2001) wherein fecal testosterone was extracted from wet fecal samples (Barrett et al. 2002; Lynch et al. 2002; Cristobal-Azkarate et al. 2006). The samples were thawed and mixed thoroughly before 0.5 g was placed into a glass tube. To this we then added 6 ml of an 8:1 mixture of analytically pure methanol and distilled water and mixed and vibrated the tube for 3 min. To remove any possible variation in steroid–antibody binding in the radioimmunoassay caused by lipids, we also used lipid extraction. For lipid extraction, we added 4 ml of analytically pure petroleum ether and vibrated the tube for 2 min. We spun the tube in a centrifuge at 2,500g for 15 min at room temperature before transferring the 2-ml methanol layer to another tube and drying it at 70°C. We then redissolved the dried samples with 2 ml phosphate buffer solution (0.1M, pH 7.0).

We used iodinated steroid radioimmunoassay kit to determine the concentration of testosterone metabolites that cross-react with the antibody (testosterone immuno-reactivities). The assay is a commercially available RIA kit designed for serum or plasma testosterone analysis (Equate Testosterone RIA kit, Puerweiye Biological Technology Co. Ltd., Beijing, China). The assay had a sensitivity (the lowest detectable dose) of 0.02 ng/ml and the testosterone antiserum is known to cross-react at less than 0.01% with dihydrotestosterone, androstanedione, estradiol, and progesterone. There was no detectable cross-reactivity to corticoids.

We added a duplicate of 50 µl of unknown samples to sample tubes and added 50-µl aliquots of testosterone standards to other tubes, respectively. We then added 100-µl aliquots of antiserum to the standards and samples. For the steroid assay, we added a 100-µl I^{125}-testosterone solution to all tubes and incubated them for 1 h at 37°C before adding 500 µl charcoal-dextran to the tubes and spinning them at 3,500g for 15 min at 25°C; we then used an automatic radioimmunoassay r–counter to count the radio-activity of the supermatant fraction for 2 min. We estimated the loss of testosterone during the extraction procedure by the addition of ^{125}I-testosterone prior to extraction and measurement of radioactivity in the appropriate methanol fraction after separation and achieved an average recovery rate of testosterone of 87%. The intra- and inter-assay coefficients of variation for androgen were 4.9% ($N=10$) and 11.4% ($N=10$), respectively.

Statistical analysis

Repeated measures single-factor ANOVA was used to compare mean testosterone levels across the various sampling periods: pre-consortship, consortship, interval, post-consortship, and across the three seasons (pre-mating, mating, and post-mating), as well as monthly measurements during the non-mating season. All values used in the analysis are based on a minimum of at least three fecal sample measurements. Differences were considered as significant at $P<0.05$. The concentrations of immunoreactive testosterone are presented as mean±standard error of the mean. Due to the small sample size, we were not able to fully obtain independent measures for analysis. The results for each of the two males are combined across encounters with estrous females such that the unit of analysis is a particular male's testosterone level in each of the above-defined categories. Generalization to the larger population beyond these two males, therefore, should be done with caution.

Results

Sampling and reproductive activity

We collected 252 fresh fecal samples from the two males and observed nine giant panda consortships at 2–18 days in length. The intervals between successive consort periods ranged from 21 to 37 days. In all cases, consortships with male subjects occurred as part of larger mating aggregations involving one or more additional males (range, 1–4 males present) and only a single female. The consorting males invariably interacted with the other males present, usually involving aggression. The typical spatial configuration of a mating aggregation consisted of a female, who spent the majority of the period sequestered above in the branches of a tree, a competitive male nearby (typically at the base of the female's tree), and other males further away from the female but within 100 m. All females involved in consort-ships displayed species-typical behavioral signs of estrus (e.g., bleating, chirping, scent marking, tail up, lordosis) (sensu Kleiman 1983). Nine consortships occurred from March to April (mating season). XY consorted five estrous females across 3 years and CC consorted six estrous females during the 3-year study period. Both of them have consorted a female in two consortship events.

Seasonal patterns in testosterone levels

To reveal seasonal changes in testosterone levels, we also assigned each month to one of the following categories: pre-mating season, mating season, and post-mating season (Fig. 1). Testosterone levels in the mating season were
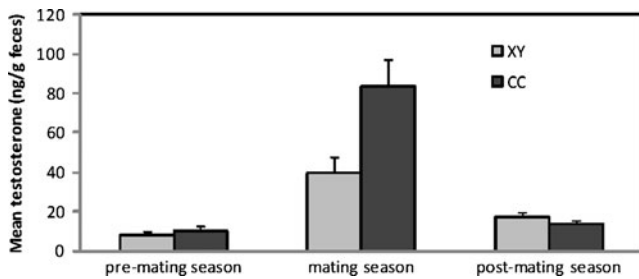
Fig. 1 Mean fecal testosterone levels for two males, *XY* (Xiyue) and *CC* (Cancan), before, during, and after the mating season

significantly higher than that observed in the non-mating season (XY: $F_{2.56}=4.17$, $P=0.02$; CC: $F_{2.81}=12.49$, $P<0.001$). Peak testosterone levels were observed mainly in 2 months (March and April) during mating seasons. In the pre-mating season, mean testosterone concentration in February was higher than that in the other months (XY: $F_{4.17}=5.98$, Oct=Nov=Dec=Jan<Feb, $P<0.05$; CC: $F_{4.18}=3.95$, Oct=Nov=Dec=Jan<Feb, $P<0.05$; Fig. 2).

Social influences on testosterone levels during the mating season

Mean testosterone concentration was higher during consortship periods than pre-consortship periods for both males (CC: $F_{1,6}=6.60$, $P<0.05$ (2007), $F_{1,7}=6.43$, $P<0.05$ (2008), $F_{1,23}=29.62$, $P<0.001$ (2009); XY: $F_{1,6}=8.98$, $P<0.05$ (2007), $F_{1,7}=6.73$, $P<0.05$ (2008), $F_{1,14}=39.72$, $P<0.001$ (2009); Fig. 3). During the interval periods, mean testosterone concentration decreased to levels similar to those during pre-consortship periods (CC: $F_{1,4}=1.17$, $P=0.34$ (2007), $F_{1,7}=0.28$, $P=0.61$ (2008); XY: $F_{1,10}=0.21$, $P=0.66$ (2008)) or even lower (CC: $F_{1,19}=6.02$, $P<0.05$ (2009); XY: $F_{1,21}=12.20$, $P<0.05$ (2009); Fig. 3). During post-consortship periods, males exhibited significantly lower mean testosterone levels than that during pre-consortship periods (CC: $F_{1,23}=6.02$, $P<0.05$ (2009), $F_{1,12}=13.73$, $P<0.05$ (2008), $F_{1,6}=6.85$, $P<0.05$ (2007); XY ($F_{1,11}=29.50$, $P<0.05$ (2008); Fig. 3).
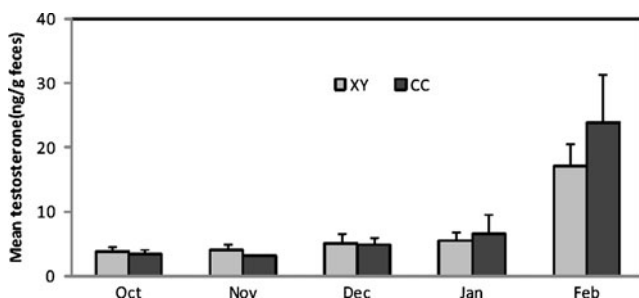

Fig. 2 Monthly mean fecal testosterone levels in pre-mating season for two males, *XY* (Xiyue) and *CC* (Cancan)
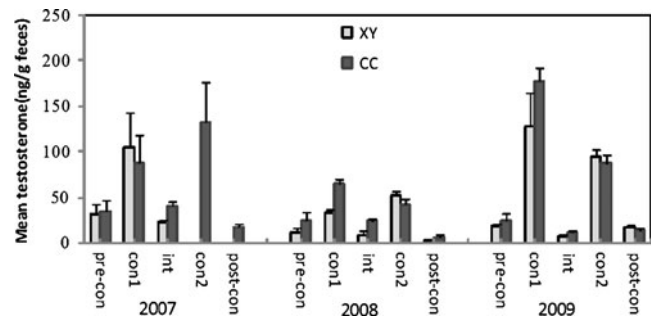

Fig. 3 Mean fecal testosterone levels as a function of reproductive context in three consecutive mating seasons for two males, *XY* (Xiyue) and *CC* (Cancan). *Pre-con* pre-consortship, *con1* consortship 1, *con2* consortship 2, *int* interval, *post-con* post-consortship

Discussion

Although our data come from only two males, they provide new insights into the reproductive ecology and biology of the giant panda and have interesting implications for our understanding of their reproductive energetics. The seasonal patterns of testosterone excretion, rising in February and falling to baseline in May, were not unexpected. Many male birds and mammals display seasonal changes in testosterone (Girard-Buttoz et al. 2009; Li et al. 2001). These seasonal rhythms are adaptive mechanisms which help synchronize breeding to ensure reproductive success (Wildt et al. 1988; Barrett et al. 2002). Likewise, the panda's ursid relatives are seasonal breeders, and testosterone levels follow seasonal rhythms similar to our findings (Palmer et al. 1988; Howell-Skalla et al. 2002; Chang et al. 2009). Our results are also consistent with the seasonal patterns of testosterone found in captive pandas (Macdonald et al. 2006; Kersey et al. 2010b). As has been demonstrated for some other bear species (e.g., Palmer et al. 1988; Tsubota et al. 1997), photoperiod changes may drive this pattern and increased testosterone levels may play an important role in spermatogenesis in giant pandas.

Our most interesting result is the apparent effects of social interaction on testosterone production. While photoperiod may prime males for testicular activity, encountering estrous females (and other males aggregating around the female) was clearly associated with elevated testosterone in all instances. As depicted in Fig. 3 and supported by statistical analysis on individual males, each and every consortship period we observed was associated with elevated testosterone levels, which always fell to baseline non-mating season levels during the intervals between consortships. Such reliable patterns across two males and multiple years increase our confidence in the predictability of this pattern in our two subjects and lead us to speculate that this may be a common occurrence in wild male giant pandas. We do not argue that all wild male pandas always show this pattern of testosterone secretion, but these

internally robust data do demonstrate that pandas maintain low testosterone levels near baseline during the mating season during periods when testosterone is not necessary to support mating behavior.

Clearly, male panda tersticular activity is not governed solely by photoperiod. Testosterone peaks coincident with mating interactions have been reported in a single captive male that was not exposed to other males (Bonney et al. 1982). One plausible hypothesis is that female signals of estrus activate testosterone production. Indeed male pandas are known to prefer vocal (Charlton et al. 2010) and odor (Swaisgood et al. 2002) signals from sexually receptive females and, moreover, female odors activate male sexual motivation (Swaisgood et al. 2000). Female-induced sexual arousal and hormonal responses in males have been reported in many species (Sachs 1997; Nelson 2000; Briand et al. 2004). In addition, male–male competition can result in increased testosterone levels (Wingfield et al. 1990) and the male pandas in our study may have experienced increased testosterone to mobilize energy and potentiate aggression with male competitors vying for access to females (i.e., the "challenge hypothesis"). While it is possible that winning a contest or copulation itself affected the androgen levels, this does not appear to explain our results, as males that lost contests and did not copulate also showed increased testosterone levels.

It is possible that male pandas in good body condition are able to maintain testosterone levels for longer periods. In this light, it is interesting to note that studies on captive pandas, which are not food-limited, do not report this pattern of testosterone reduction during the mating season (Madonald et al. 2006; Kersey et al. 2010b). Likewise, male pandas in good body condition in nature may also maintain more sustained elevated testosterone levels.

High testosterone is known to increase metabolism and energy expenditure (Wingfield et al. 1990; Marler et al. 1995; Tobler et al. 2007). With supplemental food, sagebrush lizards (Sceloporus graciosus) enhanced testosterone production, demonstrating the importance of nutritional status for sustaining a testosterone-mediated reproductive activity (Ruiz et al. 2010). In male African elephants, musth is an energetically expensive state characterized by elevated testosterone, aggression, and sexual motivation as well as increased travel. Males in poor body condition either do not commence musth or maintain musth for shorter periods (Poole 1989).

Might giant pandas experience similar trade-offs between energetic demands for survival and reproduction? As bamboo specialists, pandas are completely dependent on the quality and quantity of bamboo forage. Having retained a carnivore-like digestive tract from their ursid ancestors, pandas have poor digestive efficiency (<20% digestibility) and so they must consume 6% of their body mass in bamboo dry weight to meet daily requirements (Dierenfeld et al. 1982). To do this, they spend about 14 h each day foraging (Schaller et al. 1985). Bamboo availability declines throughout the winter months and early spring (Schaller et al. 1985; Wei et al. 1999). Schaller et al. (1985) conclude that early spring may be the nutritionally limiting time of the year. Thus, it suggests that bamboo might provide the least energy and nutrition just as the mating season commences. These factors may explain why males do not sustain high testosterone levels when energy is not immediately needed for encounters with reproductive females. Male pandas roam over large distances, compete with other males, court females, and appear to invest a great deal of energy in mate acquisition (Schaller et al. 1985). Reliant on poor-quality forage, many aspects of panda natural history appear to be governed by energetic constraints (Schaller et al. 1985). Our testosterone data suggest that the male mating strategy may be also energy-limited. Male pandas that cannot sustain elevated testosterone levels may not only be at a disadvantage in intra-male competition but may also be less preferred by females. Recent evidence indicates that male bleat vocalizations are affected by androgen levels (Charlton et al. 2011) and could therefore play a role in female mate choice.

Our study has implications for conservation, contributing to the significant body of work recently available to assist with conservation management of this endangered species (Swaisgood et al. 2011). If our hypothesis is substantiated, male giant panda reproductive effort may be constrained by energetics. Already facing a limited food supply and contending with poor body condition, male panda reproduction may be compromised by anthropogenic effects—such as habitat loss, degradation, and fragmentations—that diminish the supply of bamboo or make it more difficult to access. Management activities in protected areas should attempt to maintain a sufficient level of bamboo forage, particularly the bamboo stands utilized by pandas in late winter and early spring. Our data also have implications for ex situ conservation, showing the potential importance of social interaction on male reproductive ability. Male panda testosterone secretion and sexual motivation may be stimulated by the presence, cues, or signals of females or by male–male interactions. Indeed many of the recent successes in breeding giant pandas can be attributed to the provision of appropriate opportunities for males and females to exchange chemical signals (Swaisgood et al. 2004, 2006). The current study suggests that keeping males in good nutritional condition may also facilitate androgen production and, perhaps, reproductive success.

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