

## Urinary testosterone and cortisol metabolites in male giant pandas *Ailuropoda melanoleuca* in relation to breeding, housing, and season<sup>\*</sup>

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**Abstract** Year round baseline data was established for urinary glucocorticoid and androgen metabolites in male giant pandas. Androgen levels were highest during February, correlating with the commencement of the breeding season. There was no relationship between breeding success and androgen concentrations nor was there a relationship to different housing environments. Compared to other bear species, giant panda mating and peak androgen concentration commenced earlier in the year. This peak is expected in a polygamous species, such as the giant panda, as predicted by challenge hypothesis. Glucocorticoid levels escalated during the winter months, peaking during December. Glucocorticoids may play a preparatory role, priming the giant panda for a physically demanding breeding season [*Acta Zoologica Sinica* 52 (2): 242–249, 2006].

**Key words** Bears, Urinary hormones, Glucocorticoids, Androgens

## 雄性大熊猫尿中睾酮和皮质醇代谢物水平及其与繁殖、笼养和季节的关系<sup>\*</sup>

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**摘要** 收集了雄性大熊猫尿样肾上腺皮质激素和雄性激素代谢物年周期变化基础数据。二月份雄性激素水平最高, 与繁殖季节的开始相关。繁殖成功与雄性激素水平无相关, 与不同的笼养环境也无相关。与其他熊类物种相比, 大熊猫交配和雄性激素水平峰值在一年中的较早时候出现。在大熊猫这样一个多配制物种, 这个峰值可以看作与挑战假说相关联。在冬季, 肾上腺皮质激素水平逐月升高, 12月份出现峰值。在繁殖季节来临前, 肾上腺皮质激素可能扮演了一个预备的角色 [*动物学报* 52 (2): 242–249, 2006]。

**关键词** 熊 尿液激素 肾上腺皮质激素 雄性激素

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In recent years, significant advancements have been made in understanding the reproductive physiology of the female giant panda (Czekala et al., 2003, 2004; McGeehan et al., 2002; Lindburg et al., 2001; Zhang et al., 1998) but minimal research has been conducted on the male giant panda. Reproduction rates of captive pandas have not been self-sustaining and only recently have begun to increase (Shuling et al.\*). Previous research has focused on understanding the female physiology during estrus to improve the timing of mating or artificial insemination. An understanding of the male physiological state and readiness for mating may also aid in management practices that improve the captive population's performance.

Testosterone, the primary androgen secreted by the testis, is associated with masculine sexual activity. Testosterone stimulates the size and number of sensory receptors on the glans penis, thus influencing erections (Phoenix et al., 1976) and is also linked to such sexual behavior as mounting, neck gripping, and pelvic thrusting (Baum et al., 1987). Testosterone has indirect sexual effect on muscles, sensory receptors, respiratory metabolism, and protein production (Nelson, 2000).

Males of many species show varying androgen (i.e., testosterone) levels throughout the year with values being greatest during the breeding season (Eld's deer, Monfort et al., 1993), peak territory acquisition time (great tits, Van Duyse et al., 2003), the birthing season (red ruffed lemurs, Ostner et al., 2002) or correlated to rain patterns or food abundance (muriquis, Strier et al., 1999). In bear species androgen levels varied; androgen production increased in black bears while they were still in dens and peaked later when they were emerging from dens prior to mating and polar bears had peak concentrations during the mating season (Palmer et al., 1988). Similarly, Howell-Skalla et al. (2002) found that polar bears have the highest levels of testosterone during April (the breeding season); these values were three times those of May, July, and October. Komatsu et al. (1997) found testosterone in Japanese black bear was highest during the mating season, May-June, and lowest in Nov-Jan; active spermatogenesis was observed during May when the seminiferous tubules were the largest. Horan et al. (1993) found seasonal fluctuations in testosterone paralleled testicular size in black bear. Hesterman et al. (2005) found testosterone concentrations in sun bears fluctuated throughout the year, with no effect of season. To date, studies in the giant panda have been limited.

Androgen values in giant pandas (Snyder, 2000; Snyder et al., 2004) were correlated with some mating behaviors in a breeding male with a peak in testosterone prior to two females entering estrus. Two isolated males that did not have access to females during the breeding season showed elevated androgen outside the breeding season—May (this male had access to a female October–December) and October (this male was adjacent to a female October–December), suggesting that androgen production might be under some environmental control. However, samples were not collected daily and no samples were collected before March. Bonney et al. (1982) found increased androgen levels to be associated with female receptivity in one year but not the following year. Again, sample collection was restricted to March–May and definitive conclusions cannot be made about baseline androgen levels in the giant panda from either of these studies. In short, testosterone levels vary in bear species, but most were associated with the onset of breeding season.

Studying the giant panda offers an opportunity to evaluate the challenge hypothesis, a theory that links testosterone levels and breeding systems (Wingfield et al., 1990). The challenge hypothesis was put forward to explain the relationship between seasonal levels of testosterone and social structure, predicting the most advantageous time for high testosterone levels. According to the challenge hypothesis, because the male giant panda is polygamous it should have maximum levels during the breeding season and additional social cues (i.e. another male or estrous female) would have minimal effect on hormone levels; additionally testosterone should not be an indicator of reproductive status or correlate with changes of aggression in polygamous species such as the giant panda. This hypothesis has been tested at length in birds (Wingfield et al., 1990) but only in a few mammal species, such as the red fronted lemurs (Ostner et al., 2002). The challenge hypothesis has not been tested in any bear species to date.

Cortisol is often associated with the “stress” response. Most cortisol studies have looked at the deleterious effects of long-term elevated concentrations of cortisol on the body and attempt to explain the physiological ramifications (Sapolsky et al., 2000). Romero (2002) found seven corticoid studies that examined hormone levels associated with season in mammals (many more in birds, reptiles, and amphibians). Six of the seven studies did show a seasonal pattern in cortisol but varied in relationship to the breeding season. He suggested seasonal fluctuating

\* Shuling Z, Zhao Q, Zhong X, Wildt DE, Seal U, 1997. Report of the Giant Panda Captive Management Planning Workshop, Giant Panda Captive Management Planning Workshop.

glucocorticoids could assist in energy mobilization, have a behavioral role, or have a preparative function for an energetically demanding time (i. e., mating, birthing, etc.).

Limited research has been conducted on cortisol levels in bear species and its relationship to the breeding season. Harlow and Beck (1990) found elevated cortisol during the winter in free ranging black bears. They concluded winter hormone increases were not associated with seasonal stress but played a role in catabolism of fat during hibernation. Palumbo et al. (1983) found corticoid levels in black bears to be similar during the active period in the fall and through hibernation, but were much less (by a magnitude of four) during the active period in the summer. Only two studies have investigated corticoid concentrations in the giant panda. Owen et al. (2004) studied the effects of noise on two captive pandas, finding chronic moderate-amplitude noise was associated with higher levels of corticoids. Additionally, Owen et al. (2005) showed the same pair of captive pandas expressed both diurnal and seasonal patterns of corticoids and these patterns were not associated with stress but suggested the patterns were endogenous, perhaps regulated by photoperiod.

This study is the first multi-institutional year round analysis of male giant panda corticoids and androgens. Hormone levels are related to breeding success in captivity to discern any differences between breeders and non-breeders. Some bear species have been studied in depth and a comparison of hormone patterns between giant pandas and other bear species is conducted. Finally, captive giant pandas offer an opportunity to investigate the challenge hypothesis and varying hormone levels during the mating season in a polygamous non-hibernating bear species.

## 1 Materials and methods

### 1.1 Animals

Nine adult males from three institutions (San Diego Zoo, AdventureWorld, Shirhama Japan, and the China Research and Conservation Center for the Giant Panda, Wolong Nature Reserve, China) were used in this study. Table 1 lists all study animals and their classifications. All males were at least 4.5 years old at the time of the study time, considered reproductively mature (Schaller et al., 1985) and housed in association with reproductively mature females. Mating season was defined as Feb-May and was based on the time frame in which females came into estrous. Males were either housed with a single female (single institutions) or in multi-male, multi-female environments (multi institutions). Samples were collected from subjects for one year. Partial data from one male (Shi Shi from the San Diego Zoo) had been

**Table 1 Giant panda demographics**

Animal	Institution	Year of birth	Successful breeder?	Housing
Pan Pan	Wolong	1985	Yes	Multi
Xin Xing	Wolong	1986	Yes	Multi
Da Di	Wolong	1992	Yes	Multi
Di Di	Wolong	1994	No	Multi
Zhuang Zhuang	Wolong	1984	No	Multi
Xi Meng	Wolong	1993	Yes	Multi
Gao Gao	San Diego	1990	Yes	Single
Shi Shi	San Diego	1982	No	Single
Yong Ming*	Adventure World	1992	No	Single

\* Androgen values only.

used in a previous study Owen et al. (2005) relating glucocorticoid concentrations to behavioral indices of stress. Males were defined as breeders if they copulated during the study year.

### 1.2 Urine collection

Urine collection was attempted daily year round from all animals, with a minimum of three samples per week. To control for diurnal patterns urine samples were aspirated from enclosure floors prior to noon with plastic syringes and stored at  $-20^{\circ}\text{C}$ . After the year of collection, samples were shipped on ice to the San Diego Zoo for analysis. Samples containing fecal matter were discarded.

### 1.3 Urinary creatinine and radioimmunoassay

**1.3.1 Creatinine** Urine was analyzed for creatinine (Cr) to account for individual fluid volume differences. Samples were diluted 1:100 with distilled water, and 100  $\mu\text{l}$  were analyzed in duplicate in 96-well flat bottom microtiter plates (Costar; Cambridge, MA). A 1:1 alkaline picrate reagent (100  $\mu\text{l}$  0.04 N picric acid:0.75N NaOH) was added to all samples and standards (Sigma, St. Louis, MO), and incubated for 15 min at room temperature. Absorption was measured at 490 nm in a microplate reader (E max; Molecular Dynamics, Sunnyvale, CA). Results were expressed as mass of steroid/mg Cr, and samples with values of  $\text{Cr} < 0.05 \text{ mg/ml}$  were discarded.

**1.3.2 Testosterone** A testosterone radioimmunoassay (RIA) was used to determine testosterone concentration in urine. 10  $\mu\text{l}$  of urine was placed in  $12 \times 75 \text{ mm}$  glass tubes in duplicates. To these tubes, 500  $\mu\text{l}$  of 0.1 mol/L phosphate buffer (PBS) with 0.1% gel plus 0.9% NaCl was added and then vortexed for 10 seconds. Next, 100  $\mu\text{l}$  anti-testosterone at a 1:16 000 dilution (lot R-15P; ICN, Costa Mesa, California) and 100  $\mu\text{l}$  3H- testosterone (Perkin Elmer, Boston, MA), which yielded approximately 10 000 counts per minute, were added to the tubes. Testos-

terone standards (7.8 – 1 000 pg/tube) were run concurrently with samples. Complete mixtures of sample or standard, anti-testosterone, and  $^3\text{H}$ -testosterone were allowed to equilibrate overnight at  $4^\circ\text{C}$ . Following the overnight incubation, the unbound steroid was removed by addition of 250  $\mu\text{l}$  of Norit A charcoal/Dextran T-70 suspension. After 30 minutes at  $4^\circ\text{C}$ , the charcoal and unbound steroid were removed by centrifugation at 3 000 r/min for 10 minutes in a Sorvall RT6000B refrigerated centrifuge at  $4^\circ\text{C}$ . The aqueous phase was decanted directly into scintillation vials and 5 ml of Ultima Gold liquid scintillation fluid (Perkin Elmer, Boston, Mass.) was added. The cpm of antibody bound  $^3\text{H}$ -testosterone was then determined by a Wallac 1409 liquid scintillation counter. Raw counts were converted to ng/ml testosterone. The antibody used cross reacts 100% with testosterone, 18.75% with  $5\alpha$ -dihydrotestosterone, 3% with  $5\alpha$ -Androstane- $3\alpha$ ,  $17\alpha$ -diol, 1% with  $5\alpha$ -Androstene- $5\alpha$   $17\alpha$ -diol and less than 1% for all other steroids tested.

The assay was validated for panda urine by demonstrating parallelism between dilutions of pooled urine and the standard curve ( $r = 0.98$ ). Sensitivity was 12.18 pg/tube. The intra-assay coefficient of variation was 6% ( $n = 20$ , 59% binding). Coefficient of variance for interassay controls was 7% (for 84% binding) and 11% (for 15% binding,  $n = 30$ ). Further validation was conducted using high-pressure liquid chromatography (HPLC). In brief, a reverse phase octadecylsilane column (RP-C-18 column, Beckman, Allendale, NJ) was used for analysis. 50  $\mu\text{l}$  of urine and  $^3\text{H}$ -testosterone (3 000 cpm; Perkin Elmer, Boston MA) was injected into the HPLC, eluted with a methanol: water buffer (3 : 1.4, pH 5.35) and 1ml fractions were collected for 60 min. All HPLC fractions were dried and reconstituted in PBS-gel and the metabolite immunoreactivity was quantified by RIA. Immunoreactivity was pre-

dominately free testosterone, two unidentified less polar compounds, and testosterone and androgen conjugates. Conjugates were determined by hydrolysis and HPLC. We will refer to these measurements as androgens since further purification was not made.

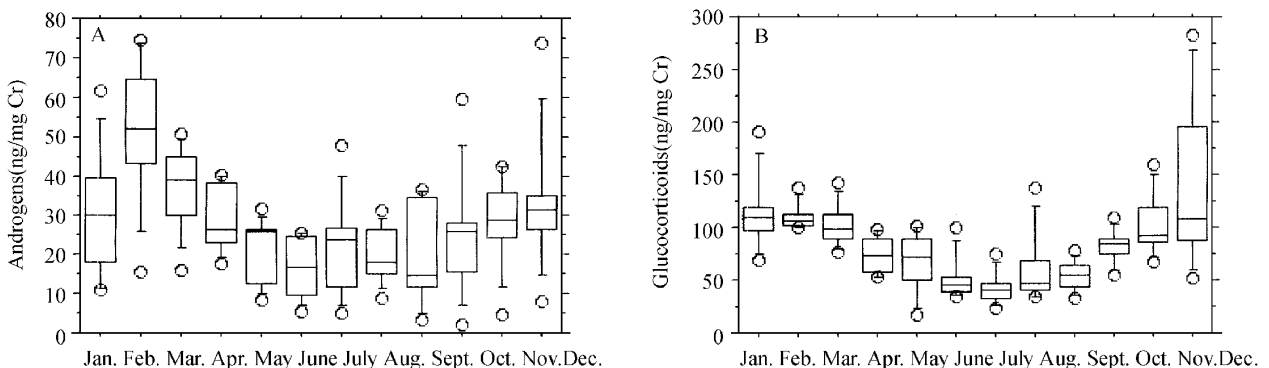
**1.3.3 Cortisol** Urinary glucocorticoids were measured by radioimmunoassay as previously described (Owen et al., 2004). In order to determine the biovalidity of measuring excreted glucocorticoid metabolites using this assay, urine samples were collected from a panda during induction of anesthesia. Urine samples were collected prior to and subsequent to the procedure (Steinman et al., 2006). During three separate procedures, glucocorticoids measured in this RIA increased eight to ten fold over baseline during the ten hours (0 to 10 hours combined) post induction (time 0 anesthesia administration).

#### 1.4 Data analysis

A monthly average for each male was generated and a repeated measures ANOVA was conducted to test for effects of month, reproductive success and housing type. Data were adjusted for homogeneity of variance by using the log value for analysis but all graphs are expressed in the actual hormone concentration. The breeding season was defined as Feb-May with all females coming into estrus during this time. Cortisol and testosterone assays were run on all animals concurrently except the AdventureWorld male in which only testosterone assays were run.

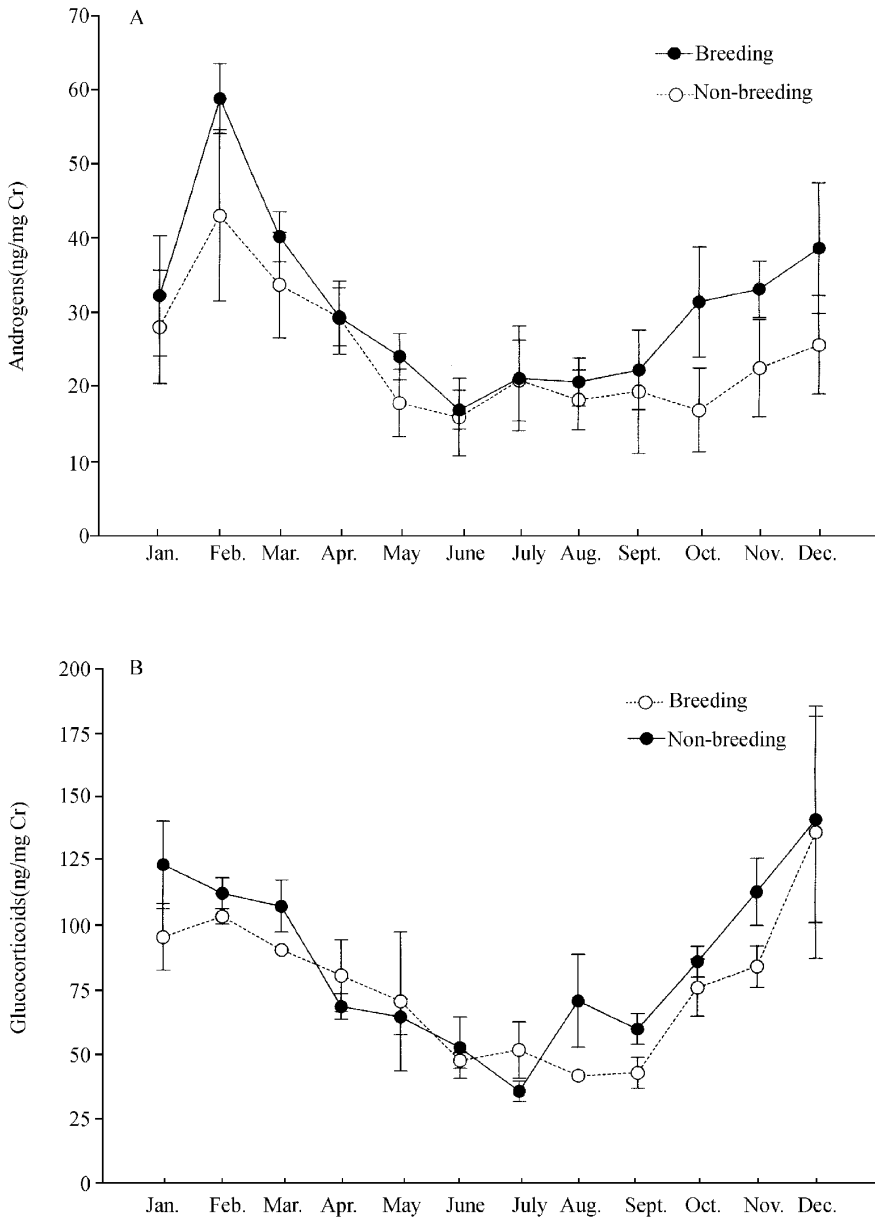
## 2 Results

All males showed similar seasonal patterns in both monthly androgen (Fig. 1A) and glucocorticoid (Fig. 1B) concentrations. Androgens were greatest in Feb, the beginning of the breeding season, and gradually decreased to the lowest levels in the summer months. Glucocorticoid concentrations began to increase in the winter with highest levels in December then decreased rapidly, reaching the lowest levels in



**Fig. 1** Monthly patterns of A) androgens and B) glucocorticoids

Quartile box plots are shown with the vertical line representing the median and the notches are the 95% confidence interval. Outliers are depicted as circles.



**Fig. 2** Monthly mean value of breeding (solid circles) or non-breeding males (open circles) of A) androgens and B) glucocorticoids ( $\pm$  SE)

the summer months.

There was no significant difference between the males that mated and those that did not mate in androgen levels ( $F_{1,11} = 0.909$ ,  $P > 0.05$ ) or glucocorticoid concentrations ( $F_{1,11} = 0.433$ ,  $P > 0.05$ ) across the twelve months (Fig. 2A, 2b).

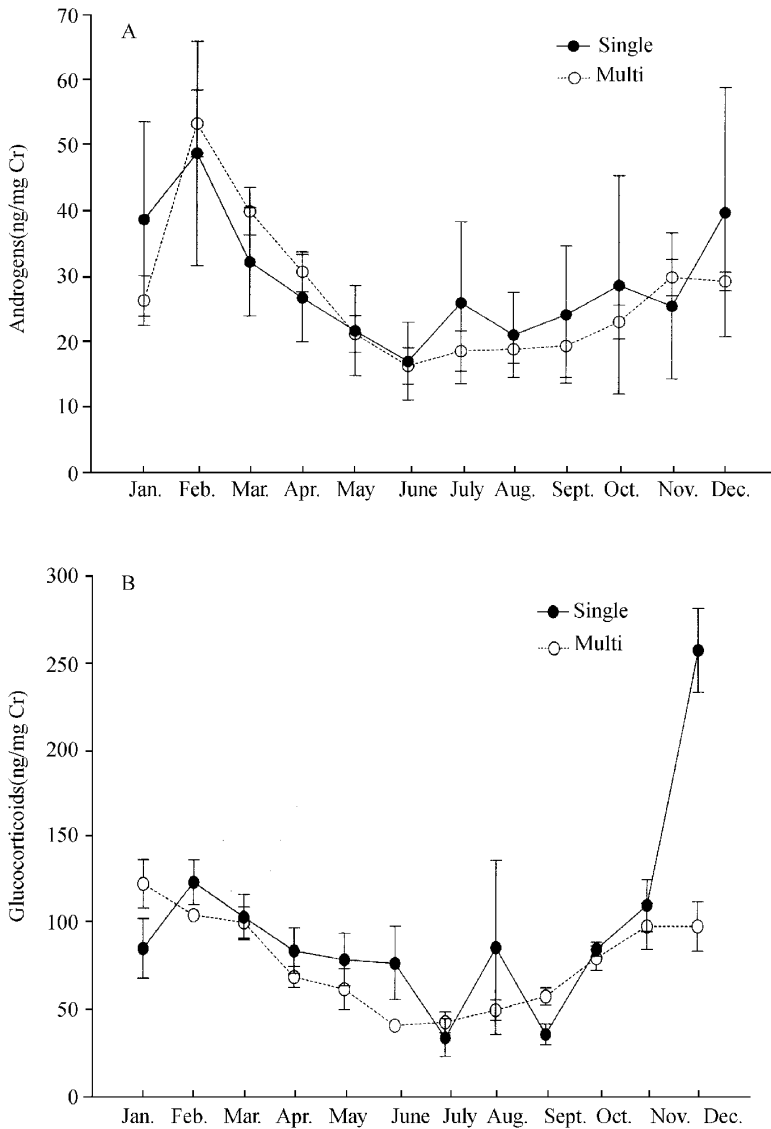
Similarly, there was no difference throughout the year between males that were housed in multi-male institutions and single male institutions in androgens ( $F_{1,11} = 1.064$ ,  $P > 0.05$ ) or glucocorticoids ( $F_{1,11} = 1.61$ ,  $P > 0.05$ ) (Fig. 3A, 3B).

There was no relationship between the age of the male and glucocorticoid concentration during the breeding season ( $r^2 = 0.071$ ,  $P > 0.05$ ) nor was

there a correlation between age and androgen during the breeding season ( $r^2 = 0.195$ ,  $P > 0.05$ ).

### 3 Discussion

Males showed a seasonal increase in androgen concentrations that corresponded to the beginning of the mating season. These findings are in accordance with the challenge hypothesis. Being polygamous, male pandas should be at near maximum androgen levels during the breeding season compared to the rest of the year (birthing season, etc.). In this study androgen concentrations increased two-fold during the first month of breeding season and gradually decreased to low levels during summer, fall and winter.



**Fig.3** Monthly mean value of males housed in single-male institutions (solid circles) or in multi-male institutions (open circles) of A) androgens and B) glucocorticoids ( $\pm SE$ )

Additionally, the challenge hypothesis predicts overall there should be little difference between males with differential reproductive success (i. e., breeder and non-breeders) as “T (testosterone) is largely genetically determined and there has been selection for males that have maximum levels of T regardless of social situations” (Wingfield et al., 1990). This study found there was not a significant difference between males that mated and those that did not mate. Of note, the male with the overall lowest androgen levels still had pronounced seasonal patterns and sired a cub after the study, suggesting a tenuous association between mating success and androgen concentrations. These results suggest that the increase in androgen production at the commencement of the breeding season is established by photoperiods or endogenous rhythms.

Snyder et al. (2004) found two males not

housed with females during the breeding season had androgen peaks in May and October. Females were placed next to these males during October – December and the authors suggested the female’s presence might account for the Oct peak found for one of the males. However, androgen concentration was not examined prior to March, and thus it is possible the males experienced their highest androgen concentrations in February, as was found in our study. Similarly, Bonney et al. (1982) examined urinary androgen concentration in a male giant panda and found a peak in May which coincided with the female’s peak estrus, but again sample collection was limited to March – May. Our study did not evaluate solitary males (i. e., with out a female) but the effect of solitary housing on androgen levels in males and the effect of introducing females outside of the breeding season should be investigated in future studies for

husbandry purposes.

Compared to other bear species (Asiatic black bear and north American black bear), the breeding season of the giant panda commences earlier in the year (Feb compared to April, May, and June respectively). Additionally, all the above species except the giant panda show androgen increases during the same month (April), regardless of different behavioral patterns during that time frame, i.e., emerging from den, mating, etc. The giant panda does not hibernate and has a year round food source (Schaller et al., 1985). Thus the early onset of the breeding season and earlier rise in androgens in the giant panda compared to other bear species may be linked to the same ecological factors that also stimulate year round activity in the giant panda. This generates a new question about androgen production: are seasonal fluctuations of androgens related to ecological factors associated with year round activity? It is necessary to study the hormone patterns of other non-hibernating bear species (i. e., spectacle and sun bear), to expand on the differences between seasonal hormone levels in hibernating and non-hibernating species and the cues (whether environmental, behavioral, or others) that may stimulate an increase in production in androgens.

The lack of difference in androgens between males housed in single male facilities versus multi male facilities suggests this type of housing does not influence hormone levels. Schaller et al. (1985) noted that many captive males failed to mate and showed either indifference or extreme aggressiveness towards estrous females. In some species, placing males in captivity in suboptimal environments can affect breeding success. Swanson et al. (2003) found that among small felids, breeders had higher testosterone levels than non-breeders. But in many species there is no relationship between mating males and non-mating males or subordinate and dominant males (lemurs: Ostner et al., 2002; muriquis: Strier et al., 1999; capuchin, Lynch et al., 2002). In giant pandas, the lack of motivation and breeding does not appear to be linked to androgen deficiencies and thus reproductive hormonal suppression may be ruled out.

Glucocorticoid concentrations were highest during the fall and winter with maximum levels during January, similar to the results found by Owen et al. (2005). It has been suggested that in some species glucocorticoid levels are elevated during the winter to aid in survival when food abundance is decreased. Harlow et al. (1990) found black bears had elevated corticoid levels that were associated with metabolic need during winter and showed little difference in seasonal stress exposure or influence by the mating season. In the red deer, Huber et al. (2003) found corticoid levels inversely correlated to nutritional in-

take during the winter and suggested corticoids play a role in catabolizing stored body tissue to ensure an adequate energy supply. However unlike many animals, giant pandas do not suffer a shortage of food during the winter months as bamboo (their main staple) is available year round. Although pandas shift their preference for parts and species of bamboos throughout the year, food sources are not greatly reduced in the winter months (Schaller et al., 1985). Thus glucocorticoids may not function to regulate energy requirements in the giant panda to the same degree as it does in species that are affected by drastic reductions in winter food supply. However, this interpretation may be skewed by the fact this was a captive study and the food supply may not have mimicked food supply in the wild. To confirm this, analysis of hormones from wild pandas is necessary.

Instead, although not mutually exclusive, an increase in glucocorticoids prior to the breeding season may have a preparatory function: "annual changes in GC (cortisol) concentrations serves to modulate the priming of the stress pathways during periods with different potential exposures to adverse conditions. For many species, breeding can be a period when stress is more common" (Romero, 2002). During the mating season of the giant panda males may travel long distances and fight with other males over access to the estrous female (Schaller et al., 1985). Zhu et al. (2001) found males to associate with females for up to a month, terminating right after copulation. Additionally, three to five males competed for the opportunity to mate. Sapolsky et al. (2000) states that glucocorticoids help stimulate glycolgenesis and gluconeogenesis, increasing blood glucose levels, and presumably energy. Thus the elevated levels in glucocorticoids in December, just prior to the beginning of the mating season (February), may prime the generally sedentary male for the increased physical demands that come with the breeding season.

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