Journal of Mammalogy, 91(6):1496–1507, 2010

Parallel and seasonal changes in gonadal and adrenal hormones in male giant pandas (*Ailuropoda melanoleuca*)

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The purpose of this study was to determine androgen and glucocorticoid (GC) hormonal patterns in male giant pandas (*Ailuropoda melanoleuca*) by monitoring gonadal and adrenal metabolites in feces. Initial validation experiments demonstrated comparable excretory patterns in urine versus feces for both androgen and GC measures. Matched urinary and fecal androgen and GC were correlated strongly with each other in a single male that was assessed over 2 years. A single pharmacological injection of adrenocorticotropic hormone caused a 15-fold GC increase in feces above baseline within 10 h, a peak at 12 h, and a return to baseline at 20 h, demonstrating the biological relationship between adrenal activation and GC excretion. Longitudinal androgen and GC excretory profiles in male giant pandas housed at North American ($n = 2$) and Chinese ($n = 3$) facilities were similar, with fecal androgens generally exceeding baseline coincident with the onset of the 5-month annual breeding season (January–June), after which values returned to nadir. Similarly, fecal GC excretion increased during the breeding season but was baseline thereafter. Fecal androgen and GC in a single male monitored through transition from subadult to sexual maturity also occurred in parallel. In this individual, basal fecal androgen and GC increased 88% and 66%, respectively, from 5 to 6 years of age. Collectively, these data demonstrate seasonal variations in gonadal activity in the giant panda by measuring androgen metabolites in feces, with elevations consistently occurring from January through June before a return to baseline for ~4–6 months. Findings also reveal a similar temporal rise in adrenal GC patterns associated with breeding season onset, perhaps a mechanism to enhance metabolism, maximize body energy stores, and provide a competitive advantage in achieving mating opportunities. Examination of data from a single male suggests that the ability to produce these seasonal androgen and GC elevations is age dependent and occurs coincident with puberty.
DOI: 10.1644/09-MAMM-A-404.1.

Key words: adrenocorticotropic hormone (ACTH) challenge, fecal hormones, male giant panda, puberty, reproductive seasonality

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The giant panda (*Ailuropoda melanoleuca*) exhibits reproductive seasonality, with breeding (in situ or ex situ) occurring from February through June, with a peak in April (Steinman et al. 2006). Almost all assessments of variation in sexual receptivity have focused on the female, especially its predilection for an extremely short (24- to 72-h) annual window of lordotic behavior (estrus) that almost always occurs coincident with increasing day length (Snyder et al. 2004; Steinman et al. 2006). In contrast, males are considered capable of reproducing for a more protracted interval during the breeding season (Howard et al. 2006; Snyder et al. 2004). For example, under captive management conditions, male giant pandas breed with multiple females over months of time (Xie and Gipps 2008) while producing prodigious numbers of high-quality spermatozoa (Howard et al. 2006). In nature, despite females having a short estrus, males compete for a single, estrual female, converging on her location for combat, with the winner having the opportunity to copulate (Schaller et al. 1985; Zhu et al. 2001). Males then presumably search for other sexually receptive females.

The specifics of reproductive seasonality in the male giant panda are less understood than those in the female. During the

months of sexual activity adult males produce sperm-dense ejaculates composed of mostly morphologically normal, highly motile spermatozoa (Howard et al. 2006). More recently, distinctive variations in sperm production have been associated with time of year; including some (but not all) males becoming azoospermic during periods of gonadal quiescence (C. Aitken-Palmer, University of Florida, pers. comm.). Urinary profiles of androgen and glucocorticoid (GC) metabolites have revealed that androgen excretion is seasonal, increasing coincident with the general period of anticipated female sexual receptivity (Bonney et al. 1982; MacDonald et al. 2006; Snyder et al. 2004). Temporal GC excretion in urine also yields a seasonal pattern similar to that of androgens, with GC values elevated during the breeding season and at basal levels in late spring, summer, and fall (MacDonald et al. 2006; Owen et al. 2005).

These collective efforts (conducted in a total of 13 male giant pandas) have demonstrated that both androgens and GCs appear seasonally dependent. Building on initial findings from urinary profiles, we considered an alternative idea—that such hormone metabolite fluctuations could be discernible in feces. The use of feces is advantageous because feces are much more readily available and easier to collect than urine, thereby providing the opportunity for better data interpretation through increased sample sizes. Additionally, once validated, assessments of fecal steroids can be adapted to field investigations where perturbed habitats are believed responsible for the uncertain status of this endangered species in nature (Viña et al. 2007). Giant pandas defecate frequently (~20 times a day), and it is not unusual to encounter giant panda fecal boluses in the wild (Schaller et al. 1985; Wei et al. 2000; Viña et al. 2007). Therefore, we suspected that feces could be used to estimate testicular and adrenal hormone status in free-ranging giant pandas. However, the 1st step was to validate efficacy and correspondence with behavioral and physiological changes. We were confident in this approach because fecal steroid monitoring already had been used to evaluate androgen metabolites in male brown (*Ursus arctos*—Ishikawa et al. 2002) and sun (*Helarctos malayanus*—Hesterman et al. 2005) bears, and GC excretion in sloth (*Melursus ursinus*—Young et al. 2004), brown (von der Ohe et al. 2004), and Asiatic black (*Ursus thibetanus*—Young et al. 2004) bears.

We examined longitudinal androgen and GC metabolite profiles in the feces of multiple giant pandas housed in ex situ collections in both the United States and in China. Our specific objectives were 1) to compare 2 approaches for extracting fecal hormone metabolites and then validate enzyme immunoassays for quantifying fecal androgen and GC patterns; 2) to determine the excretory route and time course of GC excretion after exogenous administration of adrenocorticotropic hormone (ACTH); and 3) to examine the correspondence between urinary and fecal androgen and GC measures and characterize gonadal and adrenal hormone seasonality across a reasonably sized cohort of giant pandas. Although our goal was to develop a comprehensive understanding of testicular and adrenal function in adult animals, we also had the unique opportunity to examine the impact of age. This effort complemented a parallel investigation that measured estrogen and progestagen metabolites in female giant pandas associated with the estrous cycle and pregnancy, and throughout the year (Kersey et al. 2010a, 2010b).

Materials and Methods

Study animals and facilities.—The Smithsonian National Zoological Park (39°N, 77°W) and Zoo Atlanta (33°N, 84°W) each managed 1 male (SB458, year of birth 1997; and SB461, year of birth 1997; respectively) throughout the course of this study (2001–2005). Water was provided ad libitum, and the giant pandas were fed a freshly cut bamboo diet (10–14 kg daily) supplemented with fruit and a diet biscuit high in fiber, vitamins, minerals, and amino acids (Edwards et al. 2006). Each animal was housed in an indoor (50- to 100-m^2)–outdoor (100- to 300-m^2) complex. Although both males were managed within auditory, olfactory, and visual proximity of a female conspecific, only the male at the Smithsonian National Zoological Park had consistent physical contact with an adult female outside of the annual estrual period.

At the time of our study (2001, 2002, 2004) the China Conservation and Research Center for the Giant Panda at the Wolong Nature Reserve (31°N, 103°E) managed 25 adult male giant pandas (age range, 8–17 years), of which only 3 males (SB308, year of birth 1986; SB394, year of birth 1992; and SB399, year of birth 1993) had sufficient fecal collection for statistical analysis. Individuals were fed a diet composed primarily of freshly cut bamboo supplemented with a high-fiber biscuit with water available ad libitum. Indoor (30- to 60-m^2) and outdoor (100- to 300-m^2) areas were part of each enclosure. Physical interaction with estrual females was permitted during the breeding season (February–June, the interval in which females had been observed in behavioral estrus) for mating; otherwise, males were housed individually and limited to auditory, visual, and olfactory contact with conspecifics of both sexes. Care and maintenance of all animals in this study followed guidelines of the American Society of Mammalogists for use of mammals in research (Gannon et al. 2007).

Sample collection and processing.—Fresh, morning urine samples were collected 3–7 days/week and stored frozen (−20°C) in capped and labeled plastic specimen tubes (12 × 75 mm) until analysis. All urine samples were aspirated from a solid, clean substrate free from contact with feces and standing water to avoid cross-contamination and dilution. During the study period ~1,000 urine samples were collected from SB458 and ~800 from SB461. Urinary hormone concentrations were indexed with creatinine (Cr—Taussky 1954) to account for variations in water excretion and expressed as hormone mass per milligram of Cr (ng/mg Cr). In brief, urine samples were diluted (1:10 in bovine serum albumin–free phosphate buffer) and added (0.05 ml) to a microtiter plate (Dynatech Laboratories, Inc., Chantilly, Virginia) along with Cr standard (0.00625–0.1 mg/ml; Sigma-Aldrich, St. Louis,

Missouri) in duplicate. To each standard and sample distilled H_2O, 0.75 N NaOH, and 0.4 N picric acid were added (0.05 ml each), and the assay was allowed to incubate at room temperature (25°C) for 30 min prior to assessing optical density (reading filter 490 nm, reference 620 nm) on a microplate spectrophotometer (Dynex MRX; Dynex Technologies). Urine samples that were too dilute (<0.1 mg Cr/ml; ~10% of samples) were not analyzed for hormone content and were discarded.

Freshly voided feces (<1 h postexcretion), free from contact with standing water or urine, were collected each morning (1–7 days/week) and stored frozen (−20°C) in sealable plastic bags until processing. Fecal samples collected in China were shipped frozen to Smithsonian Conservation Biology Institute for processing after securing the appropriate permits for international transfer of this biomaterial. We used ~300 fecal samples from males at Wolong Nature Reserve and ~2,000 fecal samples from SB458 and SB461 combined. Prior to hormone extraction fecal samples were freeze-dried (Lyophilizer; Labconco, Kansas City, Missouri), crushed to a powder, and extracted using previously described methods (Kersey et al. 2010a). A subset of fecal samples collected during the ACTH challenge trial (see below) was used to compare hormone profiles generated from the extraction of undried (0.5 g) versus dried (0.1 g) feces. The extraction procedure followed methods described previously (Wasser et al. 1994), and fecal extractants were reconstituted in phosphate buffer (1 ml) and then stored frozen (−20°C) until analysis.

Adrenocorticotropic hormone challenge.—To artificially stimulate the adrenal cortex male SB458 was injected intramuscularly with 2 IU/kg of a synthetic ACTH (Cortrosyn; Wedgewood Pharmacy, Swedesboro, New Jersey) on a single day in October (injection at ~0800 h). Because this animal had been conditioned to enter a training cage and cooperatively allow injection and venipuncture, it was assumed that this ACTH challenge was non- (or minimally) stressful. A blood sample (~3 ml) was collected 1 h before and 1 h after ACTH injection, centrifuged within 1 h, and the serum stored at −20°C. A freshly voided fecal sample also was obtained 1 h before administering ACTH, and then samples of all urine and feces were collected over the course of the next 3 days (72 h) and stored frozen as described above. For the first 24 h this translated into 17 fecal and 2 urine samples (at 13.8 and 23.3 h).

Endocrine analyses.—Androgen metabolite concentrations in diluted urine and fecal extracts were quantified with a single-antibody testosterone enzyme immunoassay (Dloniak et al. 2004; Munro and Lasley 1988) that cross-reacted 100% with testosterone, 57.4% with dihydrotestosterone, 0.3% with androstenedione, 0.04% with androsterone and dehydroepiandrosterone, and ≤0.02% for all other tested analytes, including cortisol (Dloniak et al. 2004; C. Munro, University of California, Davis, pers. comm.). Polyclonal anti-testosterone (R156/7—C. Munro, pers. comm.) was diluted (1:7,500), added (0.05 ml) to microtiter plates (96 well, Nunc-Immuno, Maxisorp; Fisher Scientific, Pittsburgh, Pennsylvania), and allowed to equilibrate (12–18 h) at 4°C. Prior to the addition of samples in duplicate (unprocessed urine, equivalent to 0.0002–0.0025 ml; fecal extract, equivalent to 0.0005–0.005 ml) and standards in triplicate (0.05 ml; 47–12,000 pg/ml; 17β-hydroxy-4-androsten-3-one; Steraloids, Newport, Rhode Island), unadsorbed antiserum was removed with wash solution. Enzyme-conjugated testosterone (0.05 ml; C. Munro, pers. comm.) then was added to each well containing standard or sample and incubated (2 h; 25°C) before unbound components were removed. A chromagen solution was added (0.1 ml) to each well and allowed to incubate (~30 min) before optimal densities were determined (maximum binding = 1.00 optical density; reading filter 405 nm; reference filter 540 nm) on the microtiter plate reader. Interassay coefficients of variation (*CV*s) for 2 internal controls (n = 98 assays) were 12.9% (mean binding, 28.9%) and 12.6% (mean binding, 67.9%), and intraassay *CV* was <10%. Immunoreactivity of serially diluted urine and fecal extracts paralleled standard binding. A linear regression analysis of testosterone standard added to urine ($r^2 = 1.00$, y = 1.25× − 3.85; $F_{1,6} = 7,388.41$, $P \leq 0.001$) and fecal extracts ($r^2 = 0.98$, y = 0.94× − 0.01; $F_{1,6} = 371.19$, $P \leq 0.001$) to unaltered standards demonstrated significant recovery.

A single-antibody cortisol enzyme immunoassay (Munro and Lasley 1988; Young et al. 2001) that cross-reacted 100% with cortisol, 9.9% with prednisolone, 6.3% with prednisone, 6.2% with 11-deoxycortisol, 5% with cortisone, 0.7% with corticosterone, 0.5% with 21-deoxycortisone, 0.3% with desoxycorticosterone, and 0.1% with androgens, including testosterone, dihydrotestosterone, and androstenedione (C. Munro, pers. comm.; Young et al. 2001), was used to analyze GC metabolite concentrations in all serum, urine, and fecal samples. Microtiter plates (96 well, Nunc-Immuno, Maxisorp; Fisher Scientific) were allowed to adsorb cortisol antibody (R4866, C. Munro) for 12–18 h (4°C) before adding duplicate samples (unprocessed urine and fecal extract, equivalent to 0.001–0.005 ml) and triplicate cortisol standards (0.05 ml; range 0.08–1,000 ng/ml; 17-hydroxycorticosterone; Sigma-Aldrich). Plates were incubated at room temperature (25°C; 1 h) after adding (0.05 ml) of an enzyme-linked cortisol (C. Munro, pers. comm.). Following incubation, unbound components were removed with a wash, and a chromagen solution (ABTS in citrate buffer; Sigma-Aldrich) was added (0.1 ml) to all wells. When optimal optical density (1.00) was reached, the resulting color change was quantified on the microtiter plate reader. Interassay *CV* for 2 internal controls (n = 108 assays) was 12.7% (mean binding, 37.1%) and 13.3% (mean binding, 71.9%), and intraassay *CV* was <10%. Both urine and feces demonstrated parallel displacement with the cortisol enzyme immunoassay. Significant recoveries were demonstrated when unlabeled standard was added to urine (linear regression, $r^2 =$ 0.99, y = 1.15× − 4.2; $F_{1,6} = 978.95$, $P \leq 0.001$) and fecal extracts ($r^2 = 1.00$, y = 0.90× − 3.38: $F_{1,6} = 7,320.67$, $P \leq$ 0.001).

High-pressure liquid chromatography.—Fecal androgen and GC metabolites were analyzed by reverse-phase high-

pressure liquid chromatography (Varian ProStar; Varian Analytical Instruments, Lexington, Massachusetts) with polarity gradients as previously described (Staley et al. 2007). For androgen metabolite identification fecal extracts from 6 samples containing elevated androgen concentrations were pooled, evaporated to dryness, reconstituted in 0.3 ml of phosphate-buffered saline (0.01 M $NaPO_4$, 0.14 NaCl, 0.5% bovine serum albumin, pH 5.0), and spiked with tritiated [^{3}H] testosterone (~14,000 cpm/ml) and ^{3}H androstenedione (~14,000 cpm/ml) to act as cochromatographic markers. The pooled sample (0.3 ml of phosphate-buffered saline) for GC metabolite separation was obtained from the combination of 6 extracts of a fecal sample collected ~12 h post-ACTH administration with ~14,000 cpm/ml of ^{3}H cortisol, ^{3}H corticosterone, and ^{3}H desoxycorticosterone added to the pool as radiolabeled markers. Retention times of radioactive markers were determined by combining 0.1 ml of each high-pressure liquid chromatography fraction with 3 ml of scintillation cocktail, and radioactivity was quantified on a radioactive beta counter (Beckman Coulter, Inc., Brea, California). Residual fractions were dried under air, resuspended in 0.3 ml of assay buffer, and assayed, and immunoactive peaks were compared to the retention times of radiolabeled markers.

Statistical analyses.—Baseline urinary and fecal androgen and GC metabolites concentrations were determined through an iterative process (Moreira et al. 2001). Briefly, for each data set concentrations in excess of the mean plus 2 *SD*s were removed until no values exceeded mean + 2 *SD*. The resulting mean was considered baseline and expressed as mean ± standard error of the mean (*SE*). Additionally, data sets were evaluated by calendar month and expressed as a mean value (± *SE*). All data sets were tested for normality (Kolmogorov–Smirnov test) before additional statistical tests were conducted (all tests, $\alpha = 0.05$). The correspondence between hormone standards and samples (urine and feces) spiked with known concentrations of standard was assessed using linear regression, with all residuals examined for variation to the regression line being <2 standardized residuals. Associations between data sets were evaluated using Pearson product moment correlation (r). Comparisons between fecal androgen and GC were conducted for both baseline mean and overall mean (all sample hormone concentrations in the collection period) values. Monthly trends in androgen and GC concentrations were determined via 1-way repeated-measures analysis of variance (ANOVA; with normality and equal variance assumptions accepted at $P > 0.05$), with multiple comparisons versus baseline values conducted by the Holm–Sidak method (White et al. 2005). Breeding success was determined based on a male's ability to mate with a female and produce offspring. The inclusion of SB458 in the multiyear portion of the study (starting at 3 years of age as a subadult) also allowed examining endocrine changes associated with pubertal onset. Thus, data were examined for statistically significant changes and the overall relationship of androgen and GC over time with a 1-way repeated-measures ANOVA (Holm–Sidak method). Differences between wet and dry extracts of feces, androgen, and GC values between males (SB458 and SB461) and between years of the same male were determined by the Mann–Whitney *U*-test. The Kruskal–Wallis (*H*) 1-way ANOVA test was performed to assess differences among breeding season androgen and GC values by reproductive success, with post hoc analysis conducted via Tukey's test. Baseline iterations were conducted using Microsoft Excel 2007 (Microsoft, Inc., Seattle, Washington), and all other statistical tests were conducted using SigmaStat 3.1 (Systat Software, Inc., Point Richmond, California).

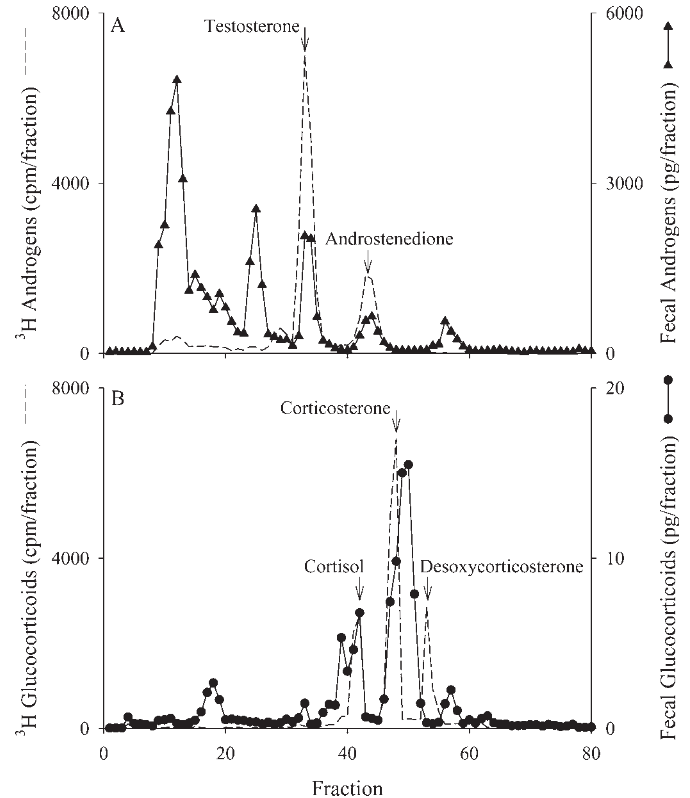

Fig. 1.—High-pressure liquid chromatography cochromatographic profiles of ^{3}H A) androgens and B) glucocorticoids with corresponding fecal androgen and glucocorticoid metabolites, respectively. Arrows mark elution of tritiated markers; A) testosterone and androstenedione, and B) cortisol, corticosterone, and desoxycorticosterone.

Results

High-pressure liquid chromatography.—Immunoreactive fecal androgen peaks quantified after high-pressure liquid chromatography separation co-eluted with ^{3}H-testosterone and ^{3}H-androstenedione at fractions 33 and 43, constituting 11.0% and 2.9% of total immunoreactivity, respectively (Fig. 1A). An additional 5 unidentified immunoreactive peaks were detected by androgen enzyme immunoassay. The largest unidentified peak (fractions 9–13) represented 38.0% of total immunoreactivity, with the additional peaks occurring at fractions 15 (10.8%), 19 (7.4%), 25 (15.6%), and 56 (2.2%).

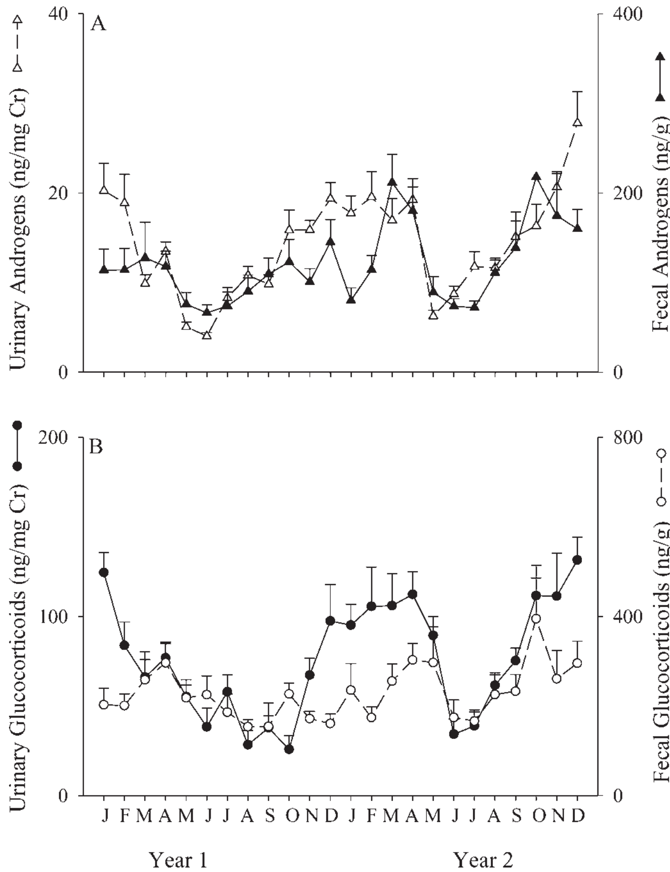

Fig. 2.—Matched A) monthly (± *SE*) urinary (open triangles, dashed line) and fecal (closed triangles, solid line) androgen metabolites, and B) monthly (± *SE*) urinary (closed circles, solid line) and fecal (open circles, dashed line) glucocorticoid metabolites for 24 months in a single adult male giant panda (SB458). Data are aligned to month of the year.

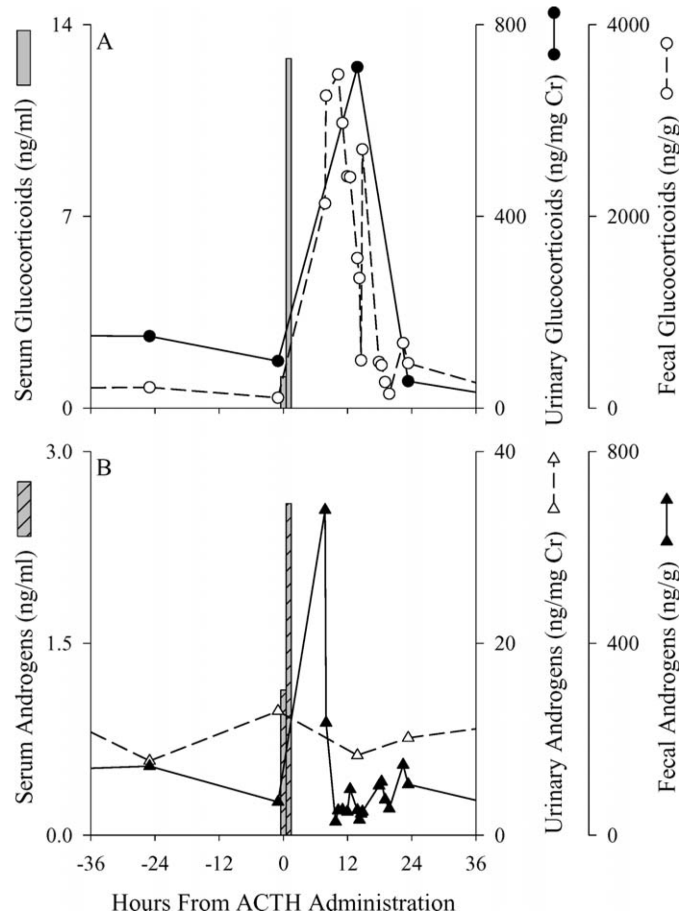

Fig. 3.—Serum, urinary, and fecal A) glucocorticoid and B) androgen concentrations before and after exogenous adrenocorticotropic hormone (ACTH) administration to a single male giant panda (SB458). Data are aligned to the hour time of ACTH administration.

Five distinct immunoreactive peaks were identified by cortisol enzyme immunoassay after high-pressure liquid chromatography, with the largest peak (fractions 46–52; 52.2% total immunoreactivity) co-eluted with ^{3}H corticosterone (fraction 48; Fig. 1B). The 2nd most prominent immunoreactive metabolite (fraction 42; 12.8%) co-eluted with ^{3}H cortisol, whereas 3 additional unidentified peaks occurred at fractions 18, 39, and 57, representing 6.5%, 5.8%, and 4.1% of total immunoreactivity, respectively.

Matched urinary and fecal endocrine measures.—Androgen patterns in urine versus feces of giant panda SB458 were similar temporally (Pearson product moment correlation, $r = 0.61$, $n = 24$, $P = 0.001$; Fig. 2A), as were urinary and fecal GC profiles ($r = 0.53$, $n = 24$, $P = 0.008$; Fig. 2B). We also found statistical correspondence between androgen and GC excretion in urine ($r = 0.74$, $n = 24$, $P = 0.00003$) and feces ($r = 0.62$, $n = 24$, $P = 0.001$).

Adrenocorticotropic hormone challenge.—Serum, urine, and fecal GC and androgen concentrations in SB458 before and after ACTH administration are depicted in Fig. 3. Serum GC concentrations increased 11-fold (from a baseline of 1.1 to 12.8 ng/ml) within 1 h post-ACTH administration, whereas circulating androgen increased 2.3-fold (from a nadir of 1.1 to 2.6 ng/ml). Of the 2 urine samples collected within 24 h of ACTH administration, the 1st at 13.8 h represented a GC value (711.1 ng/mg Cr) that was 5.6-fold above the pre-ACTH nadir of 128.7 ± 11.9 ng/mg Cr (range, 91.4–176.3 ng/mg Cr). The androgen content (8.3 ng/mg Cr) in this same sample was within the pre-ACTH nadir range ($\bar{X}$, 11.0 ± 0.56 ng/mg Cr; range, 4.6–17.0 ng/mg Cr). The 2nd urine sample (at 23 h post-ACTH) had GC (56.1 ng/mg Cr) and androgen (10.1 ng/mg Cr) concentrations below and within the range of pre-ACTH values, respectively. The 1st post-ACTH fecal sample (at ~8 h) yielded a 14.9-fold increase in GC (from a baseline of 143.6 ± 15.7 to 2,133.6 ng/g) and an 8.2-fold increase in androgen (from a baseline of 82.8 ± 8.1 to 677.9 ng/g). Peak fecal GC (3,479.6 ng/g) was detected ~12 h post-ACTH and remained elevated for ~7 h more before declining to preinjection concentrations at 20 h. In contrast, fecal androgen concentrations were reduced by 8 h post-ACTH (234.1 ng/g) and were basal (27.7 ng/g) by 9.8 h. Although fecal GC had declined by 20 h post-ACTH, several fecal samples after this period contained GC concentrations above baseline, with the sample at 36 h being at nadir.

Temporal fecal GC excretion patterns in dried versus undried giant panda feces were highly correlated (Pearson product moment correlation, $r = 0.92$, $n = 20$, $P < 0.001$). However, mean metabolite concentration for the sample set ($n = 20$) was 7.8-fold greater (Mann–Whitney $U = 58.0$, $n = 20, 21$, $P \leq 0.001$) in dried (64.6 ± 13.4 ng/g) versus undried feces (8.2 ± 1.2 ng/g).

Seasonality in male giant pandas maintained in North America.—We observed no within-animal differences in average endocrine values for either of the 2 giant pandas monitored over 2 years (2003 and 2004) in North America (SB458—androgens: $U = 12{,}711.0$, $n = 149, 185$, $P = 0.222$; GC: $U = 12{,}082.0$, $n = 149, 166$, $P = 0.724$; SB461—androgens: $U = 13{,}008.0$, $n = 170, 173$, $P = 0.065$; GC: $U = 14{,}424.0$, $n = 170, 173$, $P = 0.76$). Therefore, to allow a more detailed examination of the existence of gonadal seasonality, hormonal data for both years were combined and averaged by month for each individual (± *SE*; Fig. 4). For SB458 temporal trends in fecal androgen and GC excretion were similar (Pearson product moment correlation, $r = 0.74$, $n = 12$, $P = 0.006$; Fig. 4A). Fecal androgens were elevated above baseline (156.3 ± 4.1 ng/g; $F_{12,322} = 5.89$, $P < 0.001$) during February (243.5 ± 29.6 ng/g; Holm–Sidak method, $P = 0.006$), March (260.2 ± 25.9 ng/g, $P < 0.001$), October (256.5 ± 21.9 ng/g, $P < 0.001$), November (255.6 ± 26.4 ng/g, $P = 0.001$), and December (298.9 ± 35.6 ng/g, $P < 0.001$). The seasonal trend in fecal GC excretion was less pronounced with concentrations of this metabolite only exceeding baseline concentrations (233.3 ± 5.1 ng/g; $F_{12,303} = 2.89$, $P < 0.001$) in October (403.8 ± 31.2 ng/g, $P < 0.001$) and December (459.1 ± 96.8 ng/g, $P = 0.001$). Likewise, temporal trends in fecal androgen and GC excretion were similar ($r = 0.61$, $n = 12$, $P = 0.04$) in SB461 (Fig. 4B). Fecal androgen concentrations were elevated above baseline (111.8 ± 2.4 ng/g; $F_{12,330} = 14.31$, $P < 0.001$) in January (150.9 ± 9.2 ng/g, $P < 0.001$), February (184.9 ± 15.2 ng/g, $P < 0.001$), and March (226.1 ± 14.0 ng/g, $P < 0.001$). Fecal GC concentrations only exceeded baseline (159.4 ± 2.8 ng/g; $F_{12,328} = 6.35$, $P < 0.001$) in March (326.5 ± 40.8 ng/g, $P < 0.001$) and May (233.2 ± 20.0 ng/g, $P = 0.002$).

When overall fecal hormone concentrations were compared between males, SB458 produced more androgen (209.1 ± 7.3 ng/g; $U = 36{,}759$, $n = 334, 343$, $P \leq 0.001$) and GC (314.4 ± 12.6 ng/g; $U = 27{,}539$, $n = 315, 343$, $P \leq 0.001$) than SB461 (133.6 ± 3.5 ng/g and 194.4 ± 5.8 ng/g, respectively), a ~1.6-fold difference. This variation in overall concentration over time also extended to a higher basal excretion level for SB458 for androgens (156.3 ± 4.1 ng/g; $U = 24{,}033$, $n = 268, 287$, $P \leq 0.001$) and GC (233.3 ± 5.1 ng/g; $U = 15{,}168$, $n = 242, 284$, $P \leq 0.001$) compared to SB461 (androgens, 111.8 ± 2.4 ng/g; GC, 159.4 ± 2.8 ng/g).

Seasonality in male giant pandas maintained in China.—Fecal androgen and GC metabolite profiles of 3 representative males from Wolong Nature Reserve are presented in Fig. 5. Androgen and GC measures for male SB308 (Fig. 5A) were

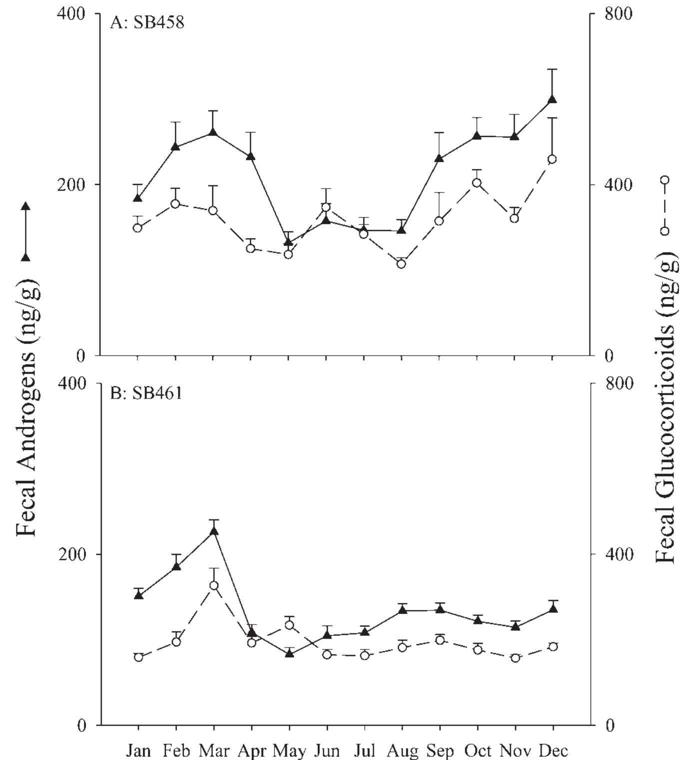

FIG. 4.—Seasonal trends in fecal androgen (closed triangles, solid line) and glucocorticoid (open circles, dashed line) excretion averaged by month (± *SE*) over a 2-year interval in male giant pandas A) SB458 and B) SB461. Data are aligned by month of the year.

correlated significantly (Pearson product moment correlation, $r = 0.76$, $n = 10$, $P = 0.01$) throughout the collection period. Androgen concentrations were elevated above baseline (78.8 ± 2.9 ng/g; repeated-measures ANOVA, $F_{10,61} = 3.10$, $P < 0.001$) only during February (294.1 ± 87.9 ng/g; Holm–Sidak method, $P < 0.001$); however, GC values exceeded baseline (122.3 ± 4.3 ng/g; $F_{10,97} = 6.20$, $P < 0.001$) throughout most of the breeding season (February, 440.5 ± 86.0 ng/g, $P < 0.001$; March, 341.6 ± 72.1 ng/g, $P < 0.001$; April, 295.2 ± 68.9 ng/g, $P = 0.002$). The correlation between androgen and GC excretory patterns for male SB394 (Fig. 5B) was not significant ($r = 0.53$, $n = 10$, $P = 0.10$). Fecal androgen metabolites were greater than basal values (112.8 ± 7.5 ng/g; $F_{10,56} = 6.72$, $P < 0.001$) for the first 2 months of the breeding season (February, 209.4 ± 17.9 ng/g, $P < 0.001$; March, 256.7 ± 36.8 ng/g, $P < 0.001$) and for 3 months during autumn (September, 238.6 ± 41.2 ng/g, $P < 0.001$; October, 221.7 ± 73.5 ng/g, $P < 0.001$; November, 207.1 ± 41.3 ng/g, $P < 0.001$). Fecal GCs were elevated above baseline (228.0 ± 8.3 ng/g; $F_{10,92} = 6.58$, $P < 0.001$) for only the month of March (638.7 ± 121.8 ng/g, $P < 0.001$). Although appearing similar, trends in androgen and GC excretion for male SB399 (Fig. 5C) were not correlated ($r = 0.66$, $n = 10$, $P = 0.09$). Androgen values were not greater ($F_{10,61} = 1.98$, $P = 0.052$) than baseline (100.5 ± 5.0 ng/g) throughout the collection period; however, monthly GC

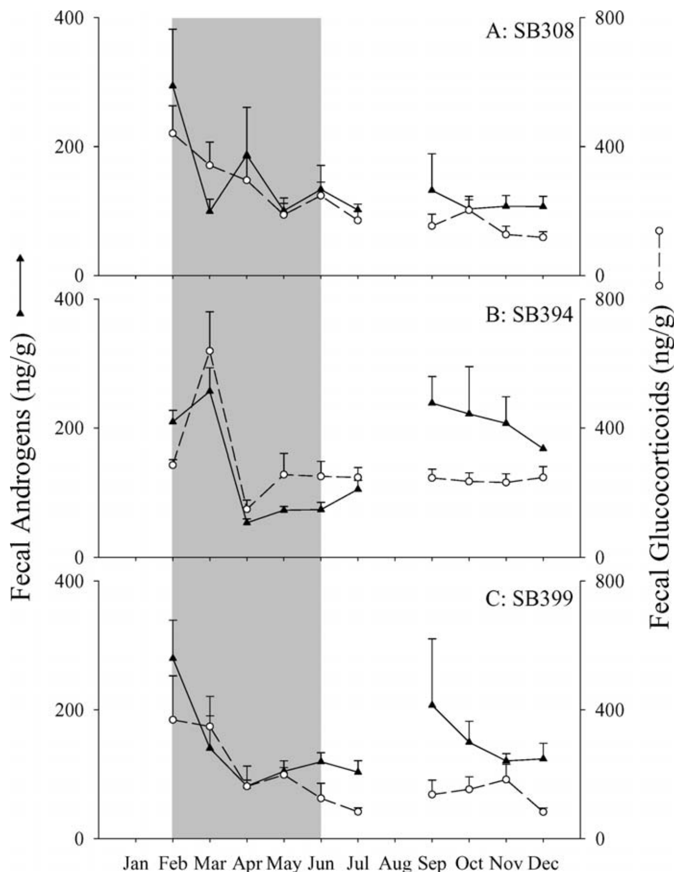

FIG. 5.—Representative fecal androgen (closed triangles, solid line) and glucocorticoid (open circles, dashed line) excretion profiles averaged by month (± *SE*) in male giant pandas A) SB308, B) SB394, and C) SB399 living ex situ in the Wolong Nature Reserve. Shaded area represents the known interval of the species' breeding season. Data are aligned to month of the year.

concentrations exceeded baseline (125.8 ± 6.2 ng/g; $F_{10,97} = 3.86$, $P < 0.001$) during the breeding season months of February (368.8 ± 136.6 ng/g, $P < 0.001$) and March (348.2 ± 93.7 ng/g, $P < 0.001$).

Relationship of androgen and GC concentrations to breeding success.—Androgen concentrations during the breeding season (February–June) did not differ according to overall reproductive success in the years of evaluation or during the reproductive life span of a given giant panda ($H_5 = 11.04$, $P = 0.051$). Conversely, GC concentrations differed ($H_5 = 18.65$, $P = 0.002$) by reproductive success, with SB458 (a male that never mated) excreting lower (161.5 ± 22.1 ng/g) during the breeding season (Tukey's test, $P < 0.05$) than SB308 (618.7 ± 66.1 ng/g) and SB394 (599.8 ± 148.3 ng/g, two males that had successfully mated).

Male pubertal onset.—On average, overall and baseline androgen concentrations of a single male (SB458) that was evaluated intensively for 5 years and through puberty onset differed (overall: $F_{4,564} = 207.83$, $P < 0.001$; baseline: $F_{4,420} = 741.53$, $P < 0.001$) with age (Table 1). Most notable increases (Holm–Sidak method, $P < 0.05$) in androgen concentrations occurred between ages 3 and 4 (1.7- and 1.3-fold increase in overall and baseline androgens, respectively), and 5 and 6 (1.7- and 1.9-fold increase in overall and baseline androgens, respectively).

Mean overall and baseline GC concentrations (Table 1) varied with age (overall: $F_{4,548} = 81.67$, $P < 0.001$; baseline: $F_{4,419} = 715.56$, $P < 0.001$). Although overall GC concentrations increased ($P < 0.05$) 1.3-fold from 3 to 4 years of age, no differences ($P > 0.05$) were detected in overall GC excretion or basal values from 3 to 5 years of age. As observed for androgen measures, overall (1.4-fold) and baseline (1.6-fold) fecal GC excretion increased ($P < 0.05$) from 5 to 6 years of age.

DISCUSSION

This work demonstrated, for the 1st time, the feasibility of monitoring seasonal fluctuations in testicular and adrenal hormonal metabolites in the highly fibrous feces of the giant panda. Excreted androgens were elevated during what has typically been identified as the 5-month-long breeding season for the male of this species (Snyder et al. 2004; Steinman et al. 2006), which contrasted substantially to the remarkably short (24- to 72-h) interval for the conspecific female (Czekala et al. 1998, 2003; Lindburg et al. 2001; Monfort et al. 1989; Steinman et al. 2006). This prolonged interval of maximal testosterone production no doubt supports protracted prodigious sperm production (Howard et al. 2006) and likely provides incentive for multiple breeding opportunities with various females (Pan et al. 2004; Schaller et al. 1985; Zhu et al. 2001). The synchronized elevations in GC could be related to greater metabolism that assists wild giant pandas sustain energy, ambulate longer distances, and express aggressive behaviors important for winning breeding competitions. Our results also suggested that physiological puberty appeared to be occurring at ~4 years of age. Finally, it was evident that male giant pandas generally expressed the same endocrine profiles (temporally and in amplitude) regardless of whether they lived in North American zoos or in a Chinese breeding center, thereby confirming that ex situ conditions were influencing endocrine patterns minimally, if at all. Male pandas maintained in urban Washington, D.C., and Atlanta, Georgia, and exposed to millions of visitors annually, demonstrated endocrine profiles no different than counterparts living in a remote, high-occupancy breeding center within a Chinese national reserve.

The circannual fecal androgen patterns observed in our study were similar to fluctuations measured in urine from 9 giant pandas (MacDonald et al. 2006) and other ursids, including black (*Ursus americanus*—Garshelis and Hellgren 1994; McMillin et al. 1976; Palmer et al. 1988), polar (*Ursus maritimus*—Palmer et al. 1988), and brown (Tsubota and Kanagawa 1989) bears. Regardless of species or use of the urinary or fecal approach, androgen concentrations were highest by mating season onset and basal by end of the breeding period. From the intensive monitoring of the pandas in North American zoos, the seasonal androgen peak in males (February) was preceded by a gradual rise that began as early

TABLE 1.—Comparison of overall and baseline fecal androgen and glucocorticoid concentrations (mean ± *SE*, ng/g) over successive years in giant panda male SB458.

	Androgen		Glucocorticoid	
Age	Overall	Baseline	Overall	Baseline
3	61.5 ± 3.6a (*n* = 120)	46.8 ± 2.1a (*n* = 99)	170.1 ± 7.7a (*n* = 120)	145.2 ± 4.1a (*n* = 105)
4	104.9 ± 6.2b (*n* = 145)	61.7 ± 2.6a (*n* = 97)	228.9 ± 13.3b (*n* = 150)	137.2 ± 4.3a (*n* = 96)
5	113.2 ± 5.8c (*n* = 203)	70.4 ± 2.3b (*n* = 143)	213.4 ± 11.9ab (*n* = 176)	139.0 ± 4.7a (*n* = 125)
6	192.5 ± 9.8d (*n* = 160)	132.8 ± 5.0c (*n* = 120)	296.0 ± 14.1c (*n* = 139)	231.3 ± 7.1b (*n* = 110)
7	182.5 ± 9.9d (*n* = 143)	130.0 ± 4.3c (*n* = 108)	297.7 ± 19.4c (*n* = 143)	219.0 ± 7.6b (*n* = 112)
F-test[a]	207.83	741.53	81.67	715.56
d.f.	4,564	4,420	4,548	4,419
r^2[b]	0.88*	0.86*	0.85*	0.67

[a] One-way repeated-measures ANOVA of within-column means ($P < 0.001$ for all tests); different lowercase letters within a column indicate significant differences (Holm–Sidak method, $P < 0.05$).
[b] Linear regression between age (explanatory variable) and mean hormone concentration (response variable).
* $P < 0.05$.

as September. Although this pattern has been inconsistent in other species, prebreeding increases in urinary androgens have been measured in brown (Tsubota and Kanagawa 1989) and American black (Palmer et al. 1988) bears. This persistent, steady rise is believed important for promoting spermatogenesis while playing a role in renal and gastrointestinal physiology during hibernation (Garshelis and Hellgren 1994; Nelson et al. 1978). However, variations among species clearly exist, because the giant panda does not experience hibernation, perhaps because the primary food source (bamboo) is available year-round (Garshelis 2004). Although increased androgen production is a prerequisite for testicular activity and spermatogenesis in the giant panda, other factors that also likely assist in evoking a full repertoire of sexual behaviors in this species must be examined, such as olfactory and auditory cues or simply female presence.

Based on our experiences, assessing hormonal metabolites in giant panda urine required less time and expense than fecal analysis. However, urine collection can be complicated and laborious, especially in situations requiring simultaneous monitoring of multiple animals living on soil substrates or in facilities where urine becomes contaminated with water (e.g., from cleaning). The fecal strategy is better suited to centers managing many pandas where feces can be recovered at the convenience of the care-givers rather than watching and waiting for individual animals to urinate. Furthermore, giant pandas experience rapid gut transit of only 6–7 h (Dierenfeld et al. 1982; Edwards et al. 2006), which results in frequent defecations that occur more often than voiding urine. Repeated defecation is advantageous, because samples are readily available, easy to collect, and represent discrete periods of physiological activity (Monfort 2003; Schwartz and Monfort 2008). The disadvantage is that fecal processing and analysis are cost- and time-intensive in the laboratory, usually requiring 15–20 h/100 samples to generate data. Nonetheless, for large-scale characterization and comparative investigations (as in the present study), the fecal approach is most practical. This assertion also was supported by our discovery that results were unaffected by the need for preemptive drying of feces. This process normally requires another 120–240 h for lyophilization per batch of fecal samples. The ability to eliminate this step reduces turnaround time and obviates the need to purchase expensive lyophilizer devices that generally have not been available in Chinese giant panda breeding centers. Finally, results revealed that fecal sampling 2–3 days per week was sufficient to identify trends in androgen and GC excretion over time.

Although high-pressure liquid chromatography indicated the presence of several GC metabolites, the group-specific antibody in our cortisol enzyme immunoassay had the capacity to quantify basal and peak GC activity. The validity of this assay was confirmed by ACTH challenge testing. Within hours of ACTH administration, clear evidence of adrenal activation and a subsequent increase in GC excretion were seen, with the lag time being <24 h for both urine and feces. The interval for a detectable response in feces (~10 h) was shorter than for urine (~13 h), which no doubt reflected the rapid movement of the bamboo diet through the gut (Dierenfeld et al. 1982; Edwards et al. 2006). Our observations also suggested that the ability to identify an acute perturbation or stressful event on the basis of fluctuating GC in a giant panda required at least once- or twice-daily fecal sampling. However, adrenal longitudinal tendencies or patterns can be determined by collecting only a few samples per week over a sustained period of time.

To maximize information learned we evaluated androgen excretion in serum, urine, and feces pre- and post-ACTH. Although urinary androgens did not change, ACTH appeared to stimulate androgen metabolite excretion into panda feces within 2 h. A short-term androgen burst in response to ACTH also has been observed in the cheetah (*Acinonyx jubatus*—Wildt et al. 1984), pudu (*Pudu puda*—Bubenik and Reyes-Toledo 1994), fallow deer (*Dama dama*—Bartoš et al. 2004), white-tailed deer (*Odocoileus virginianus*—Bubenik et al. 1990), spotted hyena (*Crocuta crocuta*—Lindeque et al 1986), and red squirrel (*Tamiasciurus hudsonicus*—Boonstra et al. 2008). For the giant panda the difference between the urine and fecal reaction likely was due to preferential metabolism and excretion of androgen metabolites by the gastrointestinal tract rather than via renal clearance. Regardless, the acute rise

and fall in fecal androgen content post-ACTH helped substantiate minimal androgen cross-reactivity in the cortisol enzyme immunoassay. Although some analyte cross-reactivity could have occurred between the 2 assays, fecal GC values were distinguished by a longer elevation post-ACTH than fecal androgens (20 versus 8.9 h, respectively). If cross-reactivity had been a factor, the GC profile would have declined coincidentally with the androgen pattern. Also, our findings were consistent with a wealth of information in the human literature demonstrating differential androgen and GC production by the adrenals after ACTH administration (McKenna et al. 1997).

As many as 42% of adult, male giant pandas have failed to breed in ex situ collections (Zhang et al. 2006). Our androgen excretion data were uninformative in predicting males that were reproductively competent versus those that were not. Individuals that were proven breeders had androgen levels and temporal profiles no different (and in some case substantially less) than males failing to copulate. Such comparisons can be challenging due to natural variations and dynamism within or among males that are partially due to individual animal differences in metabolic, excretory, or hormonal clearance, or a combination of these. Yet, others have found that older American black bears (>8 years of age) secrete less androgen than younger counterparts, and that perhaps this condition reduces the ability to compete for females (Garshelis and Hellgren 1994). Although we found no indication of a relationship between androgen concentrations and overall breeding success, determining the linkage (if any) between this hormone and explicit reproductive behaviors is a rich area for future investigations. Although this topic has received considerable attention in female giant pandas (Bonney et al. 1982; Kleiman 1984; Kleiman et al. 1979; Lindburg et al. 2001; Murata et al. 1986; Schaller et al. 1985; Snyder et al. 2004; Swaisgood et al. 2002, 2003), including being a major factor limiting breeding programs in China (Ellis et al. 2006), the relationship among male behaviors and reproductive success largely is unknown. Aggression toward estrual females is common among nonbreeding males (Ellis et al. 2006), which might reflect abnormal androgen patterns. Greater understanding could result from longitudinal monitoring of androgen metabolite profiles and concentrations in pandas that display usual versus aberrant or unusual behaviors, especially during the period surrounding estrus.

Two earlier investigations reported a seasonal and coincidental rise in urinary androgen and GC in the giant panda (MacDonald et al. 2006; Owen et al. 2005). We observed a similar annual rhythm via fecal monitoring, with GC peaking during the breeding season (February–June) and returning to baseline by summer. Seasonal GC fluctuation is believed to be related to changes in reproductive behavior, energy mobilization, and exposure to seasonal stressors (Romero 2002). However, the mechanisms regulating the interplay of these events, including seasonal adrenal activation, is not well studied or understood. Increased GC during or just before the reproductive season is a common feature in the gray wolf (*Canis lupus*—Sands and Creel 2004), southern muriqui (*Brachyteles arachnoides*—Strier et al. 1999), tufted capuchin monkey (*Cebus apella nigritus*—Lynch et al. 2002), squirrel monkey (*Saimiri boliviensis boliviensis*—Schiml et al. 1996), and arctic ground squirrel (*Spermophilus parryii*—Boonstra et al. 2001a, 2001b). One of the primary physiological effects of GC is to convert stored protein and lipids into carbohydrates (Ferin 2006), and this metabolic property may be necessary to facilitate competition and breeding behaviors observed in reproductively seasonal males. Ex situ studies have demonstrated that male giant pandas generally increase activity patterns and reduce appetite as the breeding season approaches, especially when nearby females enter estrus (Kleiman et al. 1979; Snyder et al. 2004). Furthermore, wild male giant pandas engage in battles with conspecifics in pursuit of mating opportunities (Pan et al. 2004; Schaller et al. 1985; Zhu et al. 2001). Therefore, androgens and GC might act synergistically—with anabolic effects of androgens augmented by metabolic effects of GC—to enhance virility.

Although only 1 animal was available for exploring pubertal transition, we measured the ontogeny of an important hormonal change for the 1st time. It was apparent that androgen concentrations increased steadily in male giant pandas from 3 to 7 years of age, with adult concentrations achieved by 6 years. The latter age also coincided with the 1st annual increase in GC production. These endocrine data fit well with studbook records indicating that the youngest male ever to have mated successfully and sired a cub was 5.5 years of age (Xie and Gipps 2008). Onset of male sexual behaviors (e.g., depressed appetite, increased vocalizations, scent marking, and locomotion) occurs at 5 years of age (Kleiman et al. 1979; Snyder et al. 2004), and sperm have been collected by electroejaculation from pandas 5.5 years of age, although it is of lesser quality than that from older counterparts (Howard et al. 2006). The male grizzly bear (*Ursus arctos horribilis*) is considered adult at 5.5 years when spermatozoa accumulate in the seminiferous and epididymal tubules (White et al. 1998). By contrast, serum testosterone concentrations, mate pairings, and body weight are greatest in American black bear males that are 4 years of age or older (Garshelis and Hellgren 1994). A detailed and longitudinal investigation of giant panda puberty that integrates endocrine data with behavior and sperm production is underway in our laboratory.

In conclusion, we have demonstrated the usefulness of fecal monitoring for characterizing seasonal excretion patterns of androgen and GC metabolites in the giant panda. This has increased our fundamental understanding of male reproductive biology, but it is only the 1st step in exploiting this noninvasive monitoring approach for this endangered species. Historically, important findings have been made from conducting endocrine monitoring in free-ranging wildlife (Monfort 2003; Schwartz and Monfort 2008). Opportunities exist for studying wild giant pandas to determine the variability (or similarity) among males in reproductive function and competency, or if seasonal reproductive patterns in nature mimic those observed in captive individuals.

Particularly exciting would be determining adrenal function in the context of reproductive fitness and if endocrine patterns are altered by disturbances to the local environment. Such studies now are feasible given molecular advances in identifying individuals and paternity (David et al. 2006) and improved abilities to locate quality fecal samples in nature, including with dogs (Lasley and Kirkpatrick 1991; Millspaugh and Washburn 2004; Monfort 2003; Schwartz and Monfort 2008; von der Ohe and Servheen 2002, Wasser et al. 2004).

Acknowledgments

We thank N. Parker, C. Aitken-Palmer, A. Crosier, N. Presley, K. Steinman, R. Stewart, and S. Walker for logistical support. Special thanks are extended to C. Bazlett, J. Beckman, S. Enloe, V. Parkman, N. Savageau, and B. von Holdt for laboratory assistance. We also acknowledge the enormous contributions made by the keeper and curatorial staffs at the Smithsonian National Zoological Park, Zoo Atlanta, and the China Conservation and Research Center for the Giant Panda in frequent and accurate sample collection. This study was supported by The Friends of the National Zoo and the Smithsonian National Zoological Park.

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Submitted 6 November 2009. Accepted 8 July 2010.

Associate Editor was Jane M. Waterman.