Reproductive and Adrenal Endocrinology of the Giant Panda (*Ailuropoda melanoleuca*)

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LIST OF ABBREVIATIONS/SYMBOLS

ACTH: adrenocorticotropic hormone
ANOVA: analysis of variance
C: Celsius
CRH: corticotropin-releasing hormone
CV: coefficient of variation
d: day
E2: estradiol
EC: estrogen conjugate
FSH: follicle-stimulating hormone
GC: glucocorticoid
GnRH: gonadotropin-releasing hormone
h: hour
i.m.: intramuscular
LH: luteinizing hormone
m: meter
mg: milligram
ml: milliliter
mm: millimeter
mo.: month
n: number
ng: nanogram
OD; optical density
P4: progesterone
pg: picogram
r: correlation coefficient
SB: studbook
SD; standard deviation
SE; standard error of the mean
SNZP: Smithsonian's National Zoological Park
wk: week
wt.: weight
ZA: Zoo Atlanta
µl: microliter
^{3}H: tritium (hydrogen-3)
<: less than
>: greater than
~: approximate

ABSTRACT

REPRODUCTIVE AND ADRENAL EDOCRINOLOGY OF THE GIANT PANDA (*AILUROPODA MELANOLEUCA*)

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This study sought to utilize non-invasive tactics to evaluate gonadal and adrenal hormones to better understand the reproductive biology and well-being of the endangered giant panda. Specifically, the studies of this dissertation were to: (1) to develop and validate fecal hormone monitoring techniques to quantify excreted gonadal and adrenal steroid metabolites of male and female giant pandas; (2) utilize fecal hormone monitoring to analyze the hormonal milieu of periestrus in the female; (3) investigate relation between gonadal hormone excretion and reproductive physiology during the luteal phase, with particular emphasis on pregnancy/pseudopregnancy, delayed implantation and acyclicity; (4) evaluate male reproductive and adrenal biology by assessing trends in androgen and glucocorticoid excretion in the feces; (5) determine relation between excreted adrenal steroids in the feces of the female giant panda and reproductive phenomena such as, estrus, pregnancy/pseudopregnancy, lactation, seasonality and acyclicity. Strong correlations between concomitant urinary and fecal estrogen conjugate

(EC; $r = 0.69 – 0.79$; $P < 0.05$) and progestagen ($r = 0.62 – 0.79$; $P < 0.05$) metabolites across known reproductive events (periestrus and luteal phase) demonstrated biological relevance of fecal measures in tracking ovarian function. Longitudinal assessments of 17 females further revealed that, on average, fecal estrogen concentrations began to rise ($P < 0.05$) above baseline (64.5 ± 5.9 ng/g; range, 20.0 – 103.5 ng/g) 5 d before the pre-ovulatory estrogen peak (Day 0, 468.1 ± 83.9 ng/g; range, 126.9 – 1,546.8 ng/g), which was followed by a gradual 4-d descent back to baseline All females that experienced estrus exhibited a biphasic luteal fecal progestagen profile during the post-ovulatory interval that included an initial 1.6-fold "primary" increase (368.1 ± 17.7 ng/g; range, 15.9 – 1,456.1 ng/g , $P > 0.05$) above baseline concentrations (101.9 ± 4.5 ng/g; range, 4.1 – 1,245.4 ng/g) during the immediate post-ovulatory interval (88.9 ± 6.5 d; range, 63 – 122 d) which was followed by a "secondary" 8.5-fold increase (3,110.9 ± 283.5 ng/g; range, 101.4 – 16,894.5 ng/g, $P > 0.05$) in progestagen excretion lasted approximately 40 d (39.5 ± 2.8 d; range, 28 – 52 d). Additionally, there were no general differences in the duration or progestagen excretion during the secondary or primary rise of the luteal phase between parturient and nonparturient females. In the male, matched urinary and fecal androgen ($r = 0.61$) and GC ($r = 0.53$) were strongly correlated with one another in single male that was assessed over a 2-year interval. Longitudinal fecal androgen and GC excretory profiles in male giant pandas housed at North American and Chinese facilities revealed similar excretory profiles. In general, fecal androgens increased ($P > 0.05$) 2.3-fold (252.9 ± 15.9 ng/g; range, 205.5 – 294.1 ng/g; $P < 0.05$) above baseline concentrations (112.0 ± 12.6 ng/g; range, 78.8 – 156.3 ng/g) coincident with the onset of the 5-month annual breeding season. In all males ($n = 5$), androgen metabolite

concentrations declined and were basal by the end of the breeding season (June). Fecal GC measures generally tracked androgen excretion patterns ($r = 0.53 – 0.76$), with mean peak GC excretion (362.6 ± 23.1 ng/g; 302.5 – 431.5 ng/g) representing a 2.1 fold increase ($P < 0.05$) over baseline concentrations (173.8 ± 24.1 ng/g; range, 122.3 – 233.3 ng/g). Fecal androgen and GC in a single male tracked during the transition from subadult (3 years of age) to sexual maturity (6 years of age) were excreted in parallel. In this male basal fecal androgen values were positively correlated with age ($r = 0.93$; $P < 0.05$) and increased 88% ($P < 0.05$) from age 5 (70.4 ± 23 ng/g) to 6 (132.8 ± 5.0 ng/g) years. Similarly, baseline fecal GC concentrations were positively correlated with age ($r = 0.82$; $P < 0.05$) and increased 66% ($P < 0.05$) form age 5 (139.0 ± 4.7 ng/g) to 6 (231.3 ± 7.1 ng/g) years. Periestrual EC and GC measures were examined in five females, with four demonstrating a positive ($P < 0.05$) correlation ($r = 0.57 – 0.92$) between the two measures, suggesting that GC may play a facilitating role in the hormonal milieu associated with estrus. Among the reproductive states, fecal GC values for both nonparturient (495.9 ± 100.7 ng/g) and parturient (654.1 ± 106.5 ng/g) females highest ($P < 0.05$) during the periestrus interval . Further, nonparturient females excreted lower ($P < 0.05$) GC concentrations during the secondary period of the luteal phase (334.8 ± 24.8 ng/g) than nonparturient females (470.4 ± 54.0 ng/g), suggesting possible physiological differences between the two subsets of females Although fecal GC concentrations in cyclic nonparturient females were not different across all seasons ($P > 0.05$), seasonal differences were found in acyclic, nonlactational females (winter, 302.1 ± 33.4 ng/g; spring, 212.7 ± 18.1 ng/g; summer, 214.3 ± 14.8 ng/g; autumn, 155.1 ± 9.0 ng/g). Overall, fecal GC concentrations in cyclic and acyclic females were similar, which

suggests that stress may not be the primary cause of reproductive inactivity in these females. Collectively, these data are a significant contribution to the gonadal and adrenal endocrine databases of the giant panda and provide valuable insight into reproductive biology and well-being that will aid in conserving this unique endangered species.

CHAPTER 1

Background on the endangered giant panda (*Ailuropoda melanoleuca*)

1. Life History

Although the giant panda habitat is currently limited to ~ 40, small, fragmented populations in the Shaanxi, Gansu, and Sichuan provinces of China (Lumpkin and Seidensticker, 2002; Ellis et al., 2006a), their habitat once ranged throughout much of southern and eastern Asia, including as far north as Beijing, as far south as northern Vietnam, and as far west as Myanmar (Schaller et al., 1985). However, human encroachment on habitat, timber harvesting and poaching in the past century have all played a role in the decline of the giant panda (Schaller et al., 1985; Reid and Gong, 1999; Yiming et al., 2003).

The giant panda was first described by French missionary Armand David during an 1850 expedition into the Sichuan province (Perry, 1969). Due to morphological and ecological similarities of the lesser panda (*Ailurus fulgens*), the giant panda was initially thought to be large relative to the lesser panda (Lumpkin and Seidensticker, 2002). It was not until recent advances in genetic technology that the similarities between the giant and lesser (red) panda were attributed to convergent evolution (Salesa et al., 2006) and

the giant panda was found to be the only extant member of an early subfamily (Ailuropodidae) of the Ursidae lineage (O'Brien et al., 1985; O'Brien, 1987; Zhang and Ryder, 1993; Zhang and Ryder, 1994; Talbot and Shields, 1996; Salesa et al., 2006).

Although a member of the order Carnivora, the giant panda's diet consists almost exclusively of bamboo (>99%; Schaller et al., 1985; Edwards et al., 2006b). Possessing the simple stomach of a carnivore, the cellulose of bamboo is nearly indigestible and very little nutrition can be derived from this food source by the giant panda (Dierenfeld et al., 1982, Edwards et al., 2006b). However, bamboo is abundant in the habitat of the giant panda, providing a year-round food source for a bear that does not enter an annual torpor (Schaller et al., 1985). Thereby, little energy is spent in pursuit of food, yet the trade-off is that in order to meet basal metabolic requirements, the giant panda must consume large amounts (> 50% of body weight in adults) of bamboo on a daily basis (Dierenfeld et al., 1982, Edwards et al., 2006b). Despite limited gastrointestinal abilities to digest bamboo, the giant panda does posses notable adaptations for feeding on such a fibrous food source. Particularly, the thumb-like structure on each forepaw - an elongation of the radial sesamoid bone - allows for gripping and manipulation of bamboo stalks (Davis, 1969; Schaller et al., 1985; Endo et al., 1999a; Endo et al., 1999b, Endo et al., 2001). Additional adaptations, including large masseteric jaw muscles, flared zygomatic arches and a prominent sagittal crest, provide the giant panda the ability to masticate bamboo before ingestion (Davis, 1969; Schaller et al., 1985; Edwards et al., 2006b). The relative abundance of bamboo, minimal daily activity and feeding adaptations have allowed the giant panda to rely on this food source for centuries despite possessing the simple digestive tract of a carnivore.

The giant panda lives exclusively in the mountains of the Sichuan, Ganshu and Shanxi provinces of China. An original assessment of the wild giant panda population in 1988 estimated that less than 1,000 pandas remained in the wild (Ministry of Forestry, 1992). A second assessment of the *in situ* population began approximately ten years after the first. The second study used bamboo fragment size in the feces of giant pandas to extrapolate that nearly 1,600 pandas were in the wild (Ellis et al., 2006a). More recently, a study was conducted using microsatellite fecal DNA analysis collected from free-living giant pandas to suggest that the wild population may be as high as 3,000 individuals (Zhan et al., 2006). Although the last two estimates suggest that the wild population is growing, methods employed in these studies are questionable. The second estimate was never submitted for peer review or published by the primary investigators and the ability to accurately detect animal populations from the analysis of fecal DNA in the third estimate is not considered to be valid method for population estimation (Ellis et al., 2006a; Garshelis, 2006). Regardless of the current trend in wild population growth it is unlikely that the giant panda's conservation status will change in the near future.

2. Conservation status

2.1. Conservation of the giant panda in China

The factor that has been the primary force behind the decline of the giant panda is habitat loss due to deforestation and farming. Although giant panda habitat had been shrinking since the 1800s, it was not until 1963 that reserves were established in China

specifically to conserve and protect the giant panda. Currently there are approximately 40 reserves in China specifically designated for the protection of the giant panda (Ellis et al., 2006a). Further, in 1988 the giant panda was afforded the highest level of protection under the Chinese Wildlife Conservation Law (Reid and Gong, 1999). However the reserves and federal protection have not afforded the giant panda the safety-net needed to ensure species survival. Often, reserves do not cover giant panda habitat and other governmental policies that promoted farming and timber harvesting conflicted with the conservation laws designed to protect the species and its habitat (Zhang et al., 2000; Baragona, 2001; Brooks et al., 2001; Ellis et al., 2006a). Recently the government has implemented a new forest policy that will not only work with conservation laws, but also reclaim and reforest once depleted giant panda habitat (Zhang et al., 2000).

Although historical evidence indicates that poaching had played a role in the decrease of wild giant pandas, the problem is not a major factor in the current decline of the giant panda (Yiming et al., 2003). In past century captivity has posed an even greater threat to conserving pandas in the wild. The Chengdu Zoo became the first facility in the 20th century to exhibit giant pandas in 1953 (Reid and Gong, 1999). Giant pandas were a popular draw and soon afterwards pandas were being extracted from the wild to supply zoos in China with the exhibit animals. However, for much of their captive history, very little was known about giant panda biology, including diet and nutrition, reproduction, socialization, neonate care, and basic husbandry needs. As a result, giant pandas did not survive long in captivity and those that did survive did not readily reproduce (Reid and Gong, 1999). Thereby to mitigate the drain on the wild population to provide individuals for exhibit, efforts have increased in the past 20 years to improve husbandry,

management and reproduction of the captive population (Zhang et al., 2006). Currently, the *ex situ* population has sufficient genetic diversity to be sustained in captivity for a more than 100 years. However problems still remain in managing the entire captive population as a metapopulation (Ballou et al., 2006; Zhang et al., 2006). Part of the problem is logistical, concerning animal or gamete transport and ensuring that breeding and/or artificial insemination attempts are successful (Howard et al., 2006a); the other part of the problem is political.

The captive population of giant pandas inside and outside of China is primarily managed by two competing government agencies, the State Forestry Administration (SFA) and the Ministry of Construction (MoC). These two agencies manage two large breeding centers, the China Conservation and Research Center for the Giant Panda (under SFA) and the Chengdu Breeding Base for the Giant Panda (under MoC), both of which compete for government funding (Ellis et al., 2006a). In a numbers game, the center that produces the most cubs in a given year gets more funds the following year. Therefore, breeding giant pandas was not traditionally seen as an opportunity to produce a genetic self sustaining captive population, but more of a competition between the two centers to determine which had the better breeding facility (Zhang et al., 2006). However, over the past 8 years, much of that competition has faded as the centers came to the realization that genetic management was best for captive breeding and species conservation (Ballou et al., 2006).

2.2. International conservation of the giant panda

International protection was afforded the giant panda when it was listed as an Appendix III species under Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 1983. A year later CITES revised its position and listed the species in Appendix I, providing the giant panda the highest level of international protection available. CITES acts to ensure that trade in listed species and/or their parts are not conducted unless it is done within the rules enacted by the committee. Any signatory found to be in violation of CITES by dealing with animal or animal parts of listed species loses favor in the international community and runs the risk of sanctions. In 1990, the International Union for Conservation of Nature and Natural Resources (IUCN) listed the giant panda as a Red List Species. Although the IUCN has no enforcement abilities, it is recognized as the authoritative body on species status throughout the world.

2.3. United States and giant panda conservation

The United States listed the giant panda as an endangered species under the Endangered Species Act (ESA) in 1984 in the same year CITES listed the species in Appendix I. The US Fish and Wildlife Service (FWS) oversees enforcement of the ESA and is the authoritative body allowing importation of listed species and or their parts. In the case of the giant panda, where the importation of the animal was leading to exploitation of the species in China, the FWS was impelled to draft a strict policy on the

importation of giant pandas (USFWS, 1998). Currently, the FWS has only allowed 4 zoos in the United States (Zoological Society of San Diego, Zoo Atlanta, Smithsonian's National Zoological Park, Memphis Zoo) to receive pandas from China under the strict import policy.

3. General mammalian reproductive physiology and endocrinology

3.1. Mammalian reproductive endocrinology

Changes in physiology and behavior during reproductive events are primarily driven by varying sex hormone concentrations. In mammals these sex hormones can be divided into two categories: protein/peptide or steroid hormones. The protein and peptide hormones tend to serve more of a stimulatory role in the production of steroid hormones, and in turn, steroids trigger physiological and behavioral changes. Protein hormones are made from chains of amino acids, whereas steroid hormones are built upon a cholesterol backbone (Senger, 2003).

Reproductive changes are the end result of a series of events that begins at the hypothalamus. Among many functions, the hypothalamus is the primary neurohormone producer and serves to connect the nervous system with the endocrine system. Neurohormone production is contingent upon stimulation of the hypothalamus by intrinsic and extrinsic factors. The hypothalamus is sensitive to extrinsic stimuli that include, but are not limited to: (1) day length and (2) olfactory, auditory and visual stimulation (Senger, 2003). The intrinsic factors are numerous and include: presence or

absence of coitus , adrenal steroids, glucose levels, stress, and neural transmissions. Upon receiving the right stimuli the hypothalamus then produces and secretes the appropriate peptide into the hypothalamo-hypophyseal portal system (HHPS; Senger, 2003). In the case of stimuli that trigger reproductive events, the hypothalamus releases gonadotropin-releasing hormone (GnRH; Conn et al., 1998). The HHPS, a series of blood vessels, transports blood from the hypothalamus to the anterior pituitary (AP). Due to different embryonic origins, there is no direct innervation from the hypothalamus to the AP, thereby the HHPS is essential for facilitating chemical communication between the two organs (Senger, 2003).

Once in the AP, GnRH stimulates gonadotropin cells to produce follicle stimulatory hormone (FSH) and luteinizing hormone (LH). Release of GnRH by the hypothalamus can occur in either high or low frequencies. When GnRH is produced by the hypothalamus in low frequencies, the AP is stimulated to produce FSH. However when produced in high frequencies, GnRH stimulates the secretion of large amounts of LH (Levine, 1998). Structurally, LH and FSH are similar; they are both glycoproteins with an alpha and beta subunits. However, there are species differences in the beta subunits of LH and FSH, while generally the alpha subunits are well conserved (Senger, 2003).

3.2 Female reproduction

3.2.1. Follicular phase

Upon release from the AP, LH and FSH work together through different roles to regulate reproductive activity. In mammalian females, FSH stimulates the growth of follicles in the ovary. The period between initial follicular stimulatory growth and ovulation is known as the follicular stage or phase. The growing follicles produce inhibin and estrogen. Although estrogen has a positive feed-back effect upon the hypothalamus, causing more GnRH to be released and therefore more LH and FSH, inhibin works on the AP to down-regulate FSH secretion (Yoshinaga, 1998; Senger, 2003). Rather than negatively affecting follicular growth, the decreased FSH concentrations work to select the strongest follicles while other follicles regress (Senger, 2003). Upon maturation, the follicles produce a large amount of estrogen, which stimulates a surge in LH production (Yoshinaga, 1998; Senger, 2003). The surge in LH triggers the follicle to release the egg, an event better known as ovulation (Senger, 2003), and from the remaining follicular material the corpus luteum (CL) is formed.

In addition to the positive feed-back role estrogen has on the hypothalamus, it also primes the female reproductive tract for breeding by increasing uterine and vaginal secretions, and increases myometrial contractions of the uterine wall (Lindzey and Korach, 1998). Although estrogen production is controlled by the amount of circulating LH and FSH, there are a number of endocrine events that occur in the follicle that stimulate estrogen production. The first begins with LH binding to receptors on the theca interna cell layer of the developing follicle (Yoshinaga, 1998; Senger, 2003). This

binding then converts adenosine triphospahet (ATP to cyclic adenosine monophosphate (cAMP) which then provides energy for the enzyme P450scc to convert cholesterol into pregnenolone (Hinshelwood, 1998; Yoshinaga, 1998). The enzyme CYP17A1 serves two roles; first in converting pregnenolone into 17α-hydroxyprgnelone and second converting 17α-hydroxyprgnelone to dehydroepiandrosterone (DHEA; Hinshelwood, 1998). DHEA is then converted to androstenedione before being released by the theca intera cells (Hinshelwood, 1998; Yoshinaga, 1998). Although all these steps take place within cells in the female, there are analogous cells in the male called leydig cells that perform the same biosynthetic processes in converting cholesterol to androstenedione. Androstenedione then migrates from the theca interna cells across the basement lamina of the follicle to the granulosa cells where androstenedione undergoes aromatization to be converted into estradiol when FSH binds to receptors on the cell to initiate the process (Yoshinaga, 1998).

3.2.2. Luteal phase

Once ovulation has occurred and the remaining cells of the follicle have formed the CL, the female has entered the luteal phase. However, prior to becoming the CL the follicular cells form the corpus hemorrhagicum, meaning "bloody body," due to the presence of blood following the ruptured follicle oocyte expulsion (Senger, 2003). The presence of LH is necessary after ovulation to convert the remaining cells from the follicle into luteal tissue; this process is known as luteinization (Bousfield, 1998). During this process the theca interna cells and granulosa cells readily mix to form the thecal lutein and granulosal lutein cells, respectively (Bousfield, 1998). In their new state, these

cells along with connective tissue comprise the CL. The CL primarily functions in the production of progesterone, which is necessary to maintain and support pregnancy. In the absence of fertilization the CL regresses much sooner than it does in pregnant females and progesterone production drops off. Synthesis of progesterone occurs in both thecal lutein and granulosal lutein cells (Juengel et al., 1998). Both sets of cells function similarly in the production of progesterone with the initial enzymatic conversion of cholesterol into pregnenolone and then into progesterone, which is then released into the blood stream (Juengel et al., 1998).

The CL grows throughout the luteal phase by increasing granulosal lutein cell size and thecal lutein cell number (Senger, 2003). As the CL grows so does production of progesterone as well as the effects progesterone have on behavior and physiology (Senger, 2003). Progesterone works on a number of different levels; however it primarily regulates reproduction by down-regulating hypothalamic activity through a negative-feedback system. By negatively affecting the ability of the hypothalamus to produce GnRH, progesterone effectively reduces the production of LH and FSH and thereby inhibits follicular growth (Senger, 2003). Progesterone also has a dual role on the uterus: (1) to suppress or inhibit myometrial contractions and thereby provide a more suitable environment for embryonic growth; and (2) to enhance endometrial growth in order to improve the uterine environment for embryonic implantation and development (Funk and DeMayo, 1998). Additionally, vascularization of the endometrium is increased so that if implantation does occur, exchange can occur between mother and embryo (Funk and DeMayo, 1998).

The life of the CL is limited, and if implantation of an embryo does not occur the CL regresses to facilitate another wave of follicular growth. The process of regression is known as luteolysis and is regulated by the presence or absence of two hormones; oxytocin and prostaglandin F2α (PGF2α; Juengel et al., 1998). Oxytocin is produced within the granulosal lutein cells of the CL and stimulates the production of PGF2α by the endometrium of the uterus (Juengel et al., 1998; McCracken, 1998; Senger, 2003). During the initial stages of the luteal phase progesterone blocks oxytocin binding sites on the uterine endometrial cells (McCracken, 1998). However, as endometrial growth increases so do oxytocin binding sites, and progesterone is unable to block all sites. As a result PGF2α begins to be produced in small quantities by uterine cells and is secreted into the uterine vein where it then migrates into the ovarian artery. Once in the ovary, PGF2α has a positive feed-back on the granulosal lutein cells ability to produce oxytocin. However, PGF2α also negatively affects the granulosal lutein cells and thecal lutein cells ability to produce progesterone and eventually causes luteolysis (McCracken, 1998).

As luteolysis occurs, the control progesterone once had on reproduction is slowly lifted. The suppressive block it had on the hypothalamus is eased and GnRH pulses increase, restimulating LH and FSH production (Funk and DeMayo, 1998; Senger, 2003). Uterine endometrial cells regress and myometrial contractions begin again. Cytokines eventually cause cell death in the CL, a process known as apoptosis, leaving just the connective tissue to form the scar tissue of the corpus albicans (Juengel et al., 1998). Once the CL has fully regressed to the corpus albicans the female is then able to enter a new cycle and resume follicular growth.

3.2.3. Pregnancy

For pregnancy to be successful the CL cannot regress. To prevent the regression of the CL in some artiodactyls, interferons are produced by the trophoblast cell layer of the embryo and act to inhibit binding of oxytocin to binding sties on the uterine endometrium (Senger, 2003). By inhibiting the binding of oxytocin these interferons prevent the production of PGF2α and the eventual luteolysis of the CL. In other mammalian species sex steroids and proteins are produced by the developing embryo that act to either limit production of PGF2α or limit its access to the CL. However these mechanisms are not fully understood for even the few mammalian species in which they have been investigated (Senger, 2003). Once progesterone production is allowed to proceed without inhibition by CL regression, embryonic growth can continue unimpeded and implantation may take place (Norwitz et al., 2001).

The connection of embryonic tissue with maternal tissue is known as the placenta and is necessary for fetal development in eutherian mammals. The placenta has different modes of connectivity in mammalian species; however two basic components of it remain the same: (1) the embryonic portion is made from the chorion and (2) the maternal portion develops from the endometrial lining of the uterus. Finger-like projections from the chorion known as chorionic villi have four know types of attachment to the endometrium: diffuse, cotyledonary, zonary and discoid (Senger, 2003). The placenta functions to exchange materials and fluids from mother to fetus either through simple diffusion, facilitated diffusion or active transport. There are four known types of placental intimacy: epitheliochorial, endotheliolchorial, hemochorial and hemoendothelial (Senger, 2003).

The direct connection between the fetus and mother also facilitates the function of the placenta as a temporary endocrine organ. It has been shown in some artiodactyl and primate species that the placenta eventually takes over progesterone production and the CL regresses during pregnancy (Albrecht and Pepe, 1998; Senger, 2003). In the Canidae, the placenta does not secrete progesterone and therefore maintenance of pregnancy is reliant on CL function (Kiso and Yamauchi, 1984). In Equidae, the placenta secretes as hormone called equine chorionic gonadotropin (eCG), which helps maintain pregnancy by stimulating the growth of accessory CL's in addition to maintaining the primary CL (Roser, 1998). In addition to producing hormones and chemical signals that maintain pregnancy, placentas in a number of species have also been shown to produce placental lactogens, which promote mammary gland development (Senger, 2003).

3.2.4. Parturition

As the fetus develops and reaches the end of gestation it becomes stressed and produces cortisol. The fetal cortisol acts to promote the synthesis of enzymes that convert progesterone to estradiol, thereby releasing the block on myometrial contractions (Fuchs and Fields, 1998; Senger, 2003). Fetal cortisol also acts on the placenta by stimulating production of PGF2α (Fuchs and Fields, 1998). The presence of PGF2α then stimulates either the placenta or CL (depending on the species) to produce relaxin, a peptide hormone that aids in preparing the mother for parturition by relaxing the uterus and softening the cervix (Senger, 2003). Additionally, fetal cortisol increases uterine and cervical secretions to lubricate the cervical canal and vagina. Collectively the effects of fetal cortisol initiate the early stages of parturition.

As the initial myometrial contractions begin movement of the fetus out of the uterus and through the cervix, the cervical stimulation sends neurological stimuli to the paraventricular nucleus (PVN) body in the hypothalamus. The PVN then stimulates the posterior pituitary to release oxytocin. In-turn oxytocin increases myometrial contractions, which act to stimulate further fetal movement, which then provides more stimuli to the PVN in a progressive positive feed-back loop (Fuchs and Fields, 1998; Senger, 2003). During this process the fetus eventually breaks the amniotic sac providing further lubrication of the birth canal. These series of events eventually lead to the expulsion of the fetus from the womb.

The final stage of parturition involves the expulsion of the placental remnants. This requires vasoconstriction of the maternal arterioles that were once part of the placenta. The vasoconstriction releases the chorionic villi and the remaining fetal membranes are expelled. Without vasoconstriction hemorrhages at the site of attachment in the uterus can occur (Senger, 2003).

3.2.5. Delayed implantation

Delayed implantation is a phenomenon unique to mammals. The process begins soon after fertilization when a fertilized egg develops into a blastocyst and either cell division is retarded or ceases completely (Hamlett, 1935; Mead, 1989; Sandell, 1990; Renfree and Shaw, 2000). The cessation of growth is termed embryonic diapause and is known to occur in other floral and faunal species (Renfree and Shaw, 2000). However as the early development in mammals is dependent upon implantation of a blastocyst into

the uterine endometrium, the mammal differs from other life that undergoes embryonic diapause (Hamlett, 1935; Sandell, 1990; Renfree & Shaw, 2000).

Of the extant mammals, it is believed that delayed implantation has developed independently 12 to 17 times and is represented in seven orders (Conaway, 1971; Sandell, 1990). Reasons to explain how this physiological function arose numerous times within the mammals vary depending on the family and/or order in question (Canivenc and Bonnin, 1979; Renfree and Calaby, 1981; Sandell, 1990; Renfree, 1995; Benard and Cumming, 1997; Renfree and Shaw, 2000). There are two proposed reasons why a eutherian mammal would develop delayed implantation. The first proposed reason is that it evolved from the reproductive cycle and allowed the uncoupling of mating and parturition events (Sandell, 1990). This type of delayed implantation is termed seasonal, or obligate, as it occurs as part of the female's estrous cycle (Mead, 1989; Renfree and Shaw, 2000; Sandell, 1990). Obligate delayed implantation is most common in species that live in temperate climates where resources are limited during parts of the year. By developing delayed implantation in this situation, females are able to mate with males when mate choice and/or competition are high and give birth when the most resources are available (Renfree and Shaw, 2000; Sandell, 1990). The second proposed reason for the development of delayed implantation is dependent on the duration of lactation. This condition arises when a female is nursing young and is carrying fertilized eggs that will only implant and begin embryonic growth once the current young have stopped nursing (Renfree and Shaw, 2000). It is believed that this lactational, or facultative, delayed implantation evolved so that females could take advantage of available males and mate at any time.

Within the order Chiroptera, delayed implantation arose a number of times (Conaway, 1971; Bernard and Cumming, 1997). The lactational or seasonal model cannot explain the existence of delay among all the species of Chiroptera. Delay in some species can be driven by lactation, whereas other species, even within the same family, may experience delay as a result of seasonality (Sandell, 1990; Bernard and Cumming, 1997). But in addition, bats have developed two other forms of delay in reproduction: (1) delayed fertilization and (2) delayed development. During delayed fertilization sperm are stored either in the testes or in the female reproductive tract for a period of several months (Sandell, 1990; Bernard and Cumming, 1997). Once the female has ovulated, sperm are reactivated and fertilization then occurs (Sandell, 1990; Bernard and Cumming, 1997). Delayed development is a phenomenon where fertilization and implantation occur unimpeded. However, following implantation further development of the conceptus is retarded (Sandell, 1990; Bernard and Cumming, 1997). In the case of delayed development, reactivation of fetal development is driven by environmental factors (Bernard and Cumming, 1997).

Delayed implantation has also evolved a number of times within metatherian mammals. Although there are similarities between metatherian delay and the proposed seasonal and lactional delay in eutherians, aspects of delayed implantation in the metatherians conflict with both models (Conaway, 1971; Renfree, 1995). The role of delayed implantation in the red kangaroo is primarily driven by lactation, but can also be affected by season (Renfree, 1995). The role of delayed implantation in this case permits for rapid replacement of lost young but can also be modulated by resource availability (Renfree, 1995).

Within the order Carnivora there are 41 known species that experience delayed implantation, accounting for almost 2/3 of carnivores (Mead, 1989; Sandell, 1990; Lindenfors et al., 2003; Thom et al., 2004). The strong presence of delayed implantation suggests that it may be a shared derived trait, rather than one that arose multiple times (Mead, 1989; Lindenfors et al., 2003; Thom et al., 2004). The trait is completely absent from Felidae, a more primitive group of carnivores (Wyss and Flynn 1993; Lindenfors et al. 2003). Yet the trait is strongly represented within the Caniformes and is therefore believed that the common ancestor of Ursidae, Procyonidae, Mustelidae, Phocidae, Otariidae and Canidae had some form of delayed implantation (Lindenfors et al., 2003). The presence of delayed implantation within this group is driven by season and is unlikely to have evolved by lactional needs (Sandell, 1990).

During embryonic diapause CL cell division and growth are also arrested (Mead, 1993; Renfree and Shaw, 2000; Norwitz et al., 2001). Production of progesterone is limited by the CL as are other factors that would trigger either implantation or CL regression (Renfree and Shaw, 2000; Norwitz et al., 2001). During this state, embryonic growth is retarded at the blastocyst stage, still encapsulated within the zona pellucida (Renfree and Shaw, 2000; Norwitz et al., 2001; Desmarais et al., 2004; Lopes et al., 2004). Intrinsic and extrinsic factors play a role in the reactivation of the CL in both lactational and seasonal delayed implanting species, including daylength and nutrient stores (Renfree and Calaby, 1981; Mead, 1989; Sandell, 1990; Mead, 1993). These factors trigger the hypothalamus to stimulate the AP to produce prolactin (Lopes et al., 2004). Prolactin activates the CL and progesterone production is reinitiated, setting in

motion the events that normally occur following fertilization is species without delayed implantation (Mead, 1993; Lopes et al., 2004).

3.2.6. Pseudopregnancy

Pseudopregnancy is a condition where cycling non-pregnant females will experience hormonal changes normally associated with pregnant females (Erskine, 1998). As many physiological and behavioral changes during pregnancy are driven by the presence of progesterone, pseudopregnant females often display the same physical and behavioral characteristics of a pregnant female (Erskine, 1998). This condition is uniquely mammalian and is most common in carnivores. Incidents of pseudopregnancy have been recorded in a number of carnivore species including; the grey wolf (Asa and Valdespino, 1998), wolverine (Mead et al., 1993), dwarf mongoose (Creel et al., 1991), sun bear (Onuma et al., 2001), black bear (Schulz et al., 2003), ferret (Blatchley and Donovan, 1976), domestic cat (Verhage et al., 1976) and clouded leopard (Yamada and Durrant, 1989).

Despite the presence of pseudopregnancy in most extant families of Carnivora, the evolutionary benefit has not been thoroughly explained. In the Canidae, it is believed that pseudopregnant female induce a maternal response that is then expressed in the care for the offspring of those females that did give birth, even to the extent that some females lactate and nurse the young of others (Asa and Valdespino, 1998). The social structure of Canidae is such that cooperative hunting and feeding of the young is necessary for the survival of the next generation. Without the condition of pseudopregnancy in the canid

family, their social structure would most likely not have developed (Asa and Valdespino, 1998).

In species that experience pseudopregnancy, CL function can vary among pseudopregnant and pregnant females. In the cat, differences in serum progesterone concentrations can be measured in pregnant and pseudopregnant queens 21 days after fertilization (Verhage et al., 1976). Although some studies have found that progesterone profiles of luteal cycles in pregnant and non-pregnant dogs to be significantly different (Smith and McDonald, 1974; Gudermuth et al., 1998), other studies have found that progesterone and estrogen levels are indistinguishable between pregnant and pseudopregnant bitches (Smith and McDonald, 1974; Concannon et al., 1989; Onclin and Verstegen, 1997;). Studies of bear species have found that patterns in progesterone production are similar between pregnant and pseudopregnant females (Foresman and Daniel, 1983; Palmer et al., 1988; Tsubota et al., 1992; Sato et al., 2001). Further, in the Ursidae, pseudopregnancy appears to be obligate (Sato et al., 2001), so that all females that ovulate and do not become pregnant will exhibit pseudopregnancy. As many as these species are seasonal breeders, prolonging the luteal phase is at no additional cost to the female. In order to ensure survival of any possible embryos the Luteal phase is maintained similarly between pregnant and non-pregnant females (Steinman et al., 2006).

3.3. Male Reproduction

In contrast to female reproductive system, mammalian male reproduction is fairly simple. The primary function of male reproductive system is to produce the male gamete, sperm, and deliver the sperm to the female gamete.

3.3.1. Spermatogenesis

Regardless of sex, hypothalamic and pituitary physiology is essentially the same. GnRH released by the hypothalamus stimulates the AP to release LH and FSH, which then work to regulate gamete production in both the male and female. The process of producing gametes in the female is folliculogenesis, and in the male it is spermatogenesis. At the end of spermatogenesis spermatozoa are produced, which then function to fertilize the female oocyte. Spermatogenesis occurs in the seminiferous tubules of the testes. The internal lining of the tubules is composed of two cell types, germ cells and Sertoli cells. The germ cells eventually undergo proliferation, meiosis and differentiation to become spermatids (Hess, 1998). The sertoli cells provide structural support for the developing spermatids as well as necessary secretions to nurture the germ cells transformation into spermatids (Senger, 2003).

In addition to the seminiferous tubules, the testis is composed of an interstitium. The various components of the interstitium comprise the testis space between the seminiferous tubules. Included in the interstitium are the blood and lymphatic vessels, mast cells, lymphatic cells, and most important endocriniologically, the leydig cells. The Leydig cells are the primary producers of testosterone in male physiology (Akingbemi et

al., 1998). Although LH is the primary stimulator of testosterone production by the Leydig cells other factors, such as insulin, PGF2α and angiotensin II, may act to inhibit or promote the ability of the Leydig cell to produce testosterone or respond to LH stimulation (Akingbemi et al., 1998). Upon the binding of LH to receptor sites on the Leydig cell, cholesterol is synthesized from acetate by the cells (Akingbemi et al., 1998). Cholesterol is then converted to testosterone through the combined efforts of the mitochondria and smooth endoplasmic reticulum of the Leydig cell (Akingbemi et al., 1998).

Due to the proximity of the Leydig cells to the seminiferous tubules, testosterone is able to be maintained at high levels in the testis increasing the effect it has on spermatogenesis. Although testosterone is necessary for spermatogenesis, the intracellular biochemical mechanisms on which it acts in the sertoli cells and germ cells are not well understood (Zirkin, 1998). Testosterone has found to be necessary to prevent spermatocyte and spermatid degeneration, as well as promote spermatid elongation (McLachlan et al., 1996), however other factors remain unclear (McLachlan et al., 1996; Zirkin, 1998). Reductions in testis testosterone concentrations have been shown to inhibit germ cell maturation and also have negative effects on the junctions maintained between the sertoli cells and developing spermatids, yet specific cellular actions are unknown (Zirkin, 1998).

In addition to affecting spermatogenesis, testosterone exerts other physiological effects. Secondary sex characteristics, such as aggression, increase in muscle mass, and mating behaviors are caused by the presence of testosterone in the bloodstream (Johnson et al., 1998; Senger, 2003).

Circulating testosterone has a negative effect on the hypothalamic-pituitary axis. Acting both on the hypothalamus and the anterior pituitary gland, testosterone reduces the collective effort of the hypothalamic-pituitary axis to secrete LH (Akingbemi et al., 1998; Johnson et al.; 1998; Zirkin; 1998; Senger; 2003). The reduction in LH production thereby reduces the ability of the Leydig cells to produce testosterone and subsequent support of spermatogenesis. Once circulating levels of testosterone have decreased, the hypothalamus begins to produce GnRH, stimulating the AP to secrete LH, which leads to Leydig cell production of testosterone, thereby reinitiating the male reproductive cycle (Akingbemi et al., 1998; Johnson et al., 1998; Zirkin, 1998; Senger, 2003).

3.3.2. Seasonality

Seasonal changes in male behavior and physiology have been investigated in several mammalian species. In all cases, seasonal changes in male physiology are correlated with female reproductive receptivity. The varying presence of male seasonality within mammals, as well as with species spatial distribution, indicate that the trait has arisen a number of times in Mammalia. The timing of peak testosterone however does not appear to be correlated with latitude. Although, males from species of northerly latitudes did demonstrate shorter breeding seasons than those closer to the equator (Mead et al., 1991; Palmer et al., 1988; Schiml et al., 1996; Strier et al., 1999; Boonstra et al., 2001a).

Elevated testosterone levels in males indicative of seasonal trends have shown to be correlated with day length in the Eld's deer (Monfort et al., 1995). A positive correlation has been demonstrated between rainfall and increased testosterone levels in

Muriquis monkeys (Strier et al., 1999) and negatively correlated in the tufted Capuchin monkey (Lynch et al., 2002). However no conclusive studies have been conducted to identify the numerous factors possibly involved in the regulation of seasonal testosterone trends.

The tufted capuchin monkey (*Cebus apella nigritus*) and the muriquis monkey (*Brachyteles arachnoids*) are both small new world primates that share the same habitat in Minas Gerais, Brazil. Despite their presence in the same area, the breeding seasons of the two species begin at different times of the year (Strier et al., 1999; Lynch et al., 2002). Elevated testosterone levels in male Muriqui monkeys are highest between December and May, with tufted capuchin monkey males having peak testosterone levels from April to June, a period that overlaps the nonbreeding season of the muriquis monkeys (Strier et al., 1999; Lynch et al., 2002). The variation of testosterone activity in two similar species sharing similar habitat indicate that factors other than location alone play a role in timing male reproductive seasonality.

Among the studied Ursidae, the polar bear (*Ursus maritimus*) and American black bear (*Ursus americanus*) exhibit a strong seasonal trend in androgen production (Palmer et al., 1988; Garshelis and Hellgren, 1994). The polar bear mating season occurs while the bears are still on seasonal pack ice and lasts from April trough May (Palmer et al., 1988). Male serum testosterone concentrations are significantly higher during this period when compared with non-breeding season levels (Palmer et al., 1988). In the American black bear and Hokkaido brown bear (*Ursus arctos yesoensis*) peak testosterone levels were measured prior to the breeding season (Palmer et al., 1988; Tsubota and Kanagawa, 1989). The increased levels of testosterone prior to the breeding season are believed to

not only promote spermatogenesis but also play a role in metabolic function during hibernation (Nelson et al., 1978; Garshelis and Hellgren, 1994). Additionally regional differences in the onset and duration of elevated testosterone levels in a study of male American black bears have been found (Garshelis and Hellgren, 1994). From this study, it was observed that serum testosterone concentrations of male American black bears occurring in more northerly latitudes began to rise later in the year than their more southerly counterparts; additionally the northern male's testosterone levels were elevated for a shorter period of time (Garshelis and Hellgren, 1994). These data indicate that for this bear species location does affect male reproductive seasonality.

4. Adrenal physiology and endocrinology

The adrenal glands are responsible for regulating behavioral and physiological responses to stressors. A stress or is any event that perturbs an organisms physical or mental environment and affects homeostasis (Welsh Jr. et al., 1998). The inability to respond appropriately to stress places an individual in harmful situations that risk injury or mortality. Thus, an immediate, prompt response of the adrenal glands to a stressor enables an individual to cope and adapt to its environment (Charmandari et al., 2005). However production of corticoids does not always have to be caused by a stressor, other factors such as lactation, diet, bout of play and seasonal changes in light are involved in adrenal activity.

As with reproductive hormones, initiation of gland activity begins with intrinsic and extrinsic factors. These factors are numerous and not all are negative. The factors

may act either individually or collectively on the PVN of the hypothalamus (Welsh Jr. et al., 1998). Once stimulated the PVN releases corticotropin-releasing hormone (CRH), a neuropeptide hormone similar to GnRH, into the HHPS at the median eminence. The HHPS then carries CRH to the anterior pituitary where it binds to sites on corticotrope cells stimulating the conversion of proopiomelanocortin to adrenocorticotropic hormone (ACTH; Welsh Jr. et al., 1998). Once in the blood stream, ACTH acts on the cortex of the adrenal glands (Charmandari et al., 2005).

The adrenal gland is composed of two distinct cell layers, the cortex and the medulla. The medulla is located in the middle of the adrenal gland and is composed of chromaffin cells that produce the hormones epinephrine and norepinephrine. These hormones provide short-term response to a stressor, also known as fight or flight response (Charmandari et al., 2005). The production of these hormones however is not driven by ACTH, but rather central nervous system stimuli. Epinephrine and norepinephrine have a more basic hormonal structure, which allows for rapid synthesis permitting an immediate response to hazardous situations.

The cortex also acts to allow the animal to deal with stress, but more on a relatively longer-term basis in comparison with the medulla. The cortex is composed of three cell layers that produce similar but different steroid hormones; the zona glomerulosa, the zona fasciculata and the zona reticularis. The cells of the zona glomerulosa convert cholesterol to aldosterone, a mineralcorticoid, when increased levels of potassium are present (Charmandari et al., 2005). The production of aldosterone is part of the rennin-angiotensin system and aids in balancing electrolyte levels in the body.

The zona fasciculata is the cell layer that acts in response to presence of ACTH. ACTH triggers the zona fasciculata cells to convert cholesterol into glucocorticoids, primarily either cortisol or corticosterone, depending on the species. Additionally the zona fasciculata produces dehydroepiandrosterone, a weak androgen. The system that begins with the stimuli of the PVN and ends with adrenal production of cortisol is known as the hypothalamic-pituitary axis (HPA; Welsh Jr. et al., 1998).

Once in the blood stream, cortisol serves several functions, however one of the main functions is to enhance metabolism through gluconeogenesis. This process primarily occurs in the liver where released lipid and amino acid stores are rapidly converted to glucose. The sudden available glucose provides fast energy that can be used by the individual to adapt to its environment. Further, cortisol increases blood pressure and directs more blood towards the brain and heart. Cortisol also has a negative feedback on the hypothalamus, down regulating the PVN and therefore shutting down the HPA.

In instances of chronic or repeat stressors, the stressor over-rides the inhibitory effects of cortisol on the HPA, resulting in prolonged cortisol production. In this condition the individual is no longer in a healthy environment and can begin suffer the consequences of continued elevated cortisol levels. These negative effects can lead to hypoglycemia, depleted fat and muscle stores, as well as immune and reproductive suppression (Welsh Jr. et al., 1998; Pottinger, 1999; Charmandari et al., 2005).

4.1. Chronic stress and suppression of reproduction

The product of the stress response, glucocorticoids, can negatively affect the reproductive cycle of an individual. Both the stress response and the reproductive cycle are energy dependent and physiologically the stress response will suppress reproduction, as the energy response is diverted to removing the individual from a harmful environment (Predergast et al., 2002). The stress response negatively affects the reproductive cycle at the hypothalamic, pituitary and gonadal levels (Welsh Jr. et al., 1998; Sapolsky et al., 2000). At the hypothalamic and pituitary level the suppression is the same among male and females, however it is suggested that females are more sensitive to increased glucocorticoid levels due to the higher energy requirements of their reproductive cycle (Welsh Jr. et al.; 1998). In the hypothalamus, CRH and glucocorticoids inhibit GnRH production (Welsh Jr. et al.; 1998). At the pituitary level, CRH inhibits the ability of the AP to produce LH and FSH (Sapolsky et al.; 2000). In domestic cats under stress, exogenously administered GnRH produced less LH than the control group (Carlstead et al.; 1993).

At the gonadal level reproductive suppression by the stress response occurs equally between males and females, however the mode of suppression is different due to different gonadal types. In males, increased levels of glucocorticoids inhibit testis production of testosterone (Bambino and Hsueh, 1981; Cumming et al., 1983; Fenske, 1997) and induce spermatid apoptosis (Gao et al., 2002). In the female, glucocorticoid treatments have reduced the ability of the FSH induction of aromatase activity in the granulosa cells of the follicle, thereby inhibiting estradiol production. CRH has also been

found to inhibit steroidogenesis in the granulosa cell and reduce the number of recruited follicles during folliculogenesis (Welsh Jr. et al., 1998).

4.2. Seasonal adrenal activity

Seasonal trends in measured glucocorticoid concentrations are well documented in mammals (Howland et al., 1985; Schiml et al., 1996; Strier et al., 1999; Kenagy and Place, 2000; Boonstra et al., 2001a; Lynch et al., 2002; Huber et al., 2003; Schoemaker et al., 2004; Weingrill et al., 2004). The increase in seasonal glucocorticoids occurs equally during breeding efforts or after breeding (Romero, 2002). In the squirrel monkey (*Saimiri sciureus*) peak corticoids in both males and females coincided with the breeding season (Schiml et al., 1996), whereas peak corticoids were observed in male and female red deer (*Cervus elaphus*) several months after the breeding season (Huber et al., 2003).

Studies of male corticoid levels have shown to correlate with testosterone levels (Lynch et al., 2002; Strier et al., 1999). However seasonal testis function does not necessarily have to be tied with adrenal activity. The male Eld's deer exhibits a seasonal trend in testosterone concentrations but no discernable pattern in corticoid excretion (Monfort et al., 1993a; Monfort et al., 1993b). Although the complexity of gonadal/adrenal activity has not been thoroughly investigated, the interaction may work both ways, where adrenal function is influenced by sex steroid concentrations (Romero, 2002). Female seasonal corticoid trends are more complicated than male trends because female reproductive activities are generally more protracted than in males. However, glucocorticoid values in yellow-pine chipmunks are lower in cycling females when

compared to pregnant and lactating females (Kenagy and Place, 2000). Additionally similar trends in corticoid levels between the sexes of the same species indicate that other factors other than endogenous sex steroids may play a role in regulating adrenal function.

The extrinsic factors that regulate the seasonal changes in adrenal function are varied. Seasonal changes in adrenal function are driven by day length, but can also be affected by lower ambient temperature and decreased food availability (Predergast et al., 2002). Additionally negative glucocorticoid correlations have been demonstrated with rainfall in the tufted Capuhin monkey (*Cebus paella nigritis*, Lynch et al., 2002) but positively correlated with rain in the Muriquis monkey (*Brachyteles arachnoids*, Strier et al. 1999). In Alaskan brown bears (*Ursus arctos horribilis*), diet significantly altered the glucocorticoids measured in the feces (von der Ohe et al., 2004). Bears with the lowest quality diets of grasses had the highest glucocorticoid levels, whereas bears that had a mixed diet of grasses, berries and meat had the lowest glucocorticoid concentrations (von der Ohe et al., 2004). Although glucocorticoid levels may be influenced by nutrient availability, where low quality feed is more taxing on the energy demands of the animal, the influence of the feed types on gut transit rate may also play a role in the ability to measure corticoids in excreta (von der Ohe et al., 2004).

5. Giant panda reproductive biology

5.1 Reproduction in the female giant panda

The female giant panda is seasonally monoestrus (Booney et al., 1982; Murata et al., 1986; Chaunduri et al., 1988; Monfort et al., 1989; Meyer et al., 1997; Czekala et al., 1998; Lindburg et al., 2001; Zhu et al., 2001; McGeehan et al., 2002; Swaisgood et al., 2002; Czekala et al., 2003; Durrant et al., 2006; Steinman et al., 2006). Estrus generally occurs during the breeding season that lasts from February through June. However on rare occasions estrus can occur in autumn (Chorn and Hoffman 1978). The average number of ovulation sites on an ovary a female will have during estrus is not known. However evidence suggests that females are polyovular as 48% of all birthing events result in two cubs (Howard et al., 2006a). During the periestrual interval females have increased behaviors that signal impending receptivity that include; barking, bleating, tail-up, backwards walking and increased scent marking (Czekala et al., 1998). General activity also increases in the female during the periestrual interval with wild females expanding their home ranges to broadcast their receptivity and attract males from surrounding territories (Swaisgood et al., 2002). Assessments of urinary estrogens during this period demonstrate an increase in estrogen activity that can be detected 10 days prior to reaching peak levels (Monfort et al. 1989; Lindburg et al., 2001; Czekala et al., 2003). Following peak concentrations estrogens drop back down to near basal levels. The estrogen decline coincides with the time period that most matings have been observed, a time that also corresponds with captive observations of female receptivity (Monfort et al., 1989; Lindburg et al., 2001; Czekala et al., 2003; Steinman et al., 2006).

Giant pandas also undergo obligate pseudopregnancy in the absence of fertilization (Chaunduri et al., 1988; Zhu et al., 2001; McGeehan et al., 2002; Czekala et al., 2003; Steinman et al., 2006). Under this condition non-pregnant females will display

behaviors and physiological changes that are indistinguishable from those of pregnant females (Erskine, 1998; Steinman et al., 2006). However pseudopregnancy only applies to females that ovulate and do not conceive. In cases where females are anestrual pseudopregnancy will not occur. Events of anestrual females have been recorded in the literature; however the etiology of anestrus has not been thoroughly explored in healthy adult females (Masui et al., 1989; Durrant et al., 2003; Steinman et al., 2006). To date no quantifiable measure has been developed to discern pregnant females from non-pregnant females (Owen et al., 2004; Owen et al., 2005; Steinman et al., 2006). All measurable steroid and protein hormones have shown no difference between pregnant and non-pregnant females (Steinman et al., 2006). Further, as hormonal changes between pregnant and non-pregnant females are similar, all behaviors driven by hormones are the same (Steinman et al., 2006). Additionally, it is not know if failed pregnancies have been mistakenly diagnosed as pseudopregnancies (Steinman et al., 2006). The inabilities to diagnose true pregnancy from the false form have complicated reproductive studies. As a result fertilization success rates (in terms of determining effective AI methods), true twinning rates and timing of implantation have been difficult to quantify, leaving much of giant panda reproduction unknown.

There is also strong evidence to suggest that soon after an egg is fertilized it undergoes embryonic diapause (Chaunduri et al., 1988; Monfort et al., 1989; Zhu et al., 2001; Steinman et al. 2006). In this phenomenon the embryo either ceases growth or grows at a very slow rate (Mead, 1993). After a certain time period the embryo is hormonally stimulated to implant into the uterine wall and reinitiate embryonic growth (Mead, 1989; Renfree and Shaw, 2000). Other bear species, including the Asiatic black

bear, and other members of the order Carnivora experience embryonic diapause, indicating that this is likely a shared trait derived from a common ancestor (Sato et al., 2001; Chang et al., 2004). It is therefore believed that the giant panda, like other carnivore species, experiences obligate, or seasonal, delayed implantation (Sandell, 1990; Lindenfors et al., 2003; Thom et al., 2004).

Following estrus in the giant panda, urinary measures of progestins have shown an initial rise about two to three times that of basal progestin levels (Hodges et al., 1984; Monfort et al., 1989; Lindburg et al., 2001; McGeehan et al., 2002; Czekala et al., 2003; Narushima et al., 2003; Steinman et al., 2006). The duration of this initial rise is highly variable, lasting between 74 to 122 days. However progestin levels are maintained at a fairly constant rate for the duration of the initial rise (Steinman et al., 2006). Immediately after the initial rise, non-invasively measured progestin levels increase, reaching peaks between 10 and 30 times that of baseline concentrations before declining and reaching basal levels. In the females studied to date, the time period from increased progestin activity above initial rise levels back down to baseline is approximately 40 to 50 days (Hodges et al., 1984; Chaunduri et al., 1988; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Steinman et al., 2006). This pattern of an initial small rise followed by a second more substantive rise in progestin levels during pregnancy and pseudopregnancy has also been observed in the other Ursidae, including the spectacled bear (*Tremarctos ornatus*, Dehnhard et al., 2006), American black bear (*Ursus americanus*, Foresman and Daniel, 1983; Harlow et al., 1990; Tsubota et al., 1998), Asiatic black bear (*Ursus thibetanus*, Sato et al., 2001), sun bear (*Helartcos malayanus*, Schwarzenberger et al., 2004), and brown bear (*Ursus arctos*, Tsubota et al., 1992). The

end of this time frame is when parturition occurs in pregnant females. Although no difference has been measured in the magnitude nor duration of progestin levels of pregnant and non-pregnant giant pandas, only a few females have been studied leading the possibility that as more individuals and reproductive events that are studied the greater the chance that a difference in some measure will be noted.

Historically, approximately 53% of females will give birth following breeding attempts and litter sizes are singleton (50%), twins (48%) or triplets (2%) (Ballou et al., 2006; Howard et al., 2006a). A recent report on the use of ultrasound in the monitoring and detection of pregnancy in a giant panda housed at the San Diego Zoo showed that two fetuses were detected up to 14 days prior to parturition, but the female only gave birth to one cub, raising the question whether this is a common occurrence (Sutherland-Smith et al., 2004). Further, mothers that birth twins will often care for only one and abandon the other (Wildt et al., 2003). Most of the time when one cub was abandoned and did not survive it was not recorded in the studbook (Ballou et al., 2006). Recent advances in husbandry have increased survival of the abandoned cub, resulting in more accurate reporting of twinning rates.

5.2 Reproductive biology of the male giant panda

In the wild, male giant pandas are solitary and rarely encounter other giant pandas outside the breeding season (Schaller et al., 1985). The male giant panda experiences seasonal changes in behavior that coincide with the breeding season. Changes in urinary glucocorticoids and androgens measures have also demonstrated circannual variations

(Kleiman, 1984; Owen et al., 2005; MacDonald et al., 2006). Free-living males have been observed fighting with other males to mate a female, and females have the choice to mate with multiple males during estrus (Schaller et al., 1985; Zhu et al., 2001). However it is not known whether males will mate with multiple females during a breeding season. Yet the ability to breed does not seem to affect the reproductive capability of the male as urinary testosterone measures from breeding and non-breeding males show no difference (MacDonald et al., 2006).

To date very few studies have been conducted on the reproductive physiology of the male giant panda. The first data on male biology were primarily from a few endocrinological measures and behavioral observations of male activity when a nearby female was in estrus (Booney et al., 1982; Kleiman, 1984). Females have been the subject of more intense study not only due to a more complicated reproductive biology, but also females have a greater impact on the population than males. A non-reproductive female can not be replaced whereas as a non-reproductive male can be substituted by a more reproductively capable male. As a result reproductively competent males have been repeatedly used for breeding and the captive population is thereby overrepresented by only a handful of males (Ballou et al., 2006).

More thorough investigations into androgen activity in the male giant panda have recently been conducted (MacDonald et al., 2006; Steinman et al., 2006). As with previous studies of male reproductive steroid measures in other bear species, MacDonald et al. (2006) and Steinman et al. (2006) found that androgens in the male peaked just prior to, or right at the onset of, the breeding season (Palmer et al. 1988; Garshelis and Hellgren, 1994). However it is not known whether testosterone needs to be elevated to

maintain spermatogenesis throughout the breeding season, if elevated testosterone is necessary to initiate the process of spermatogenesis, or if sperm are produced at the onset of the breeding season and stored in the male reproductive tract until copulation. In addition to a lack of difference in androgen levels of non-breeding and breeding males, males housed in single-male institutions had comparative androgen profiles to those males housed in multi-male institutions (MacDonald et al., 2006).

6. Giant panda adrenal physiology

Investigations into giant panda adrenal function have shown that there is a seasonal trend in glucocorticoids excreted in the urine that corresponds with the breeding season (Owen et al., 2004; Owen et al., 2005; MacDonald et al., 2006). Appreciable increases in corticoid levels however were observed outside the breeding season in a female during lactation intervals (Owen et al., 2005). Additionally glucocorticoid levels do not seem to be significantly altered by noise perturbances in either sex (Owen et al., 2004; Powell et al., 2006). In the giant panda, no difference was found in the glucocorticoid profile in terms of neither temporal pattern nor concentration between mating and non-mating males (MacDonald et al., 2006). Similar results of glucocorticoids in terms of breeding and non-breeding males were found in golden lion tamarins (Bales et al., 2006). However in the wolf (*Canis lupus*), the arctic ground squirrel (*Spermophilus parryii*), and pygmy goat (*Capra hicus*) males that mate have higher levels of glucocorticoids than those that do not (Sands and Creel, 2004). MacDonald et al. (2006) suggest that although food quality and time of year can

influence corticoid trends in other bear species, this may not be the case with the giant panda as the food source is maintained throughout the year. However, it is not known whether nutritional value of bamboo changes with the season and a comparison between the two measures may be warranted. The increase in corticoids of male giant pandas just prior to the breeding season may also be influenced by the increased energy demands of impending reproductive activities (MacDonald et al., 2006). Additionally, the promotion of gluconeogenesis to meet energy demands may also explain the high levels of corticoids measured in lactating females (Owen et al., 2005).

7. Non-invasive Endocrine Monitoring

One of the major factors in the regulation of reproductive and adrenal function is the metabolism and excretion of steroids. Steroids are produced to initiate a series of immediate changes in physiology and behavior and therefore must be metabolized to prevent recurrent effects. The liver and kidney function as the primary metabolizers of gonadal and adrenal steroids. Circulating hormones are delivered to the liver via hepatic artery, are metabolized and excreted into the bile where they mix with digested food and are excreted in the feces (Monfort, 2003). Primarily, fecal steroid metabolites are excreted unconjugated (Brown and Wildt, 1997; Monfort, 2003). The renal artery delivers steroids to the kidney where they are metabolized and excreted in the urine commonly as conjugated steroids.

Wildlife researchers have utilized steroid metabolism to monitor reproductive and adrenal hormone concentrations in a number of wild animals. As a non-invasive method,

hormone evaluations of the excreta have allowed for detailed biological studies of species without disturbing or perturbing an animal. Although hormonal studies were conducted prior to non-invasive monitoring, duration and sample frequency were often limited (Monfort, 2003). Endocrine studies relied on repeated blood sampling, which proves to be difficult in longitudinal studies on multiple animals. Further repeated blood sampling can lead to sustained, increased levels of glucocorticoids (Sakkinen et al. 2004), thus altering natural behavior and physiology. Non-invasive techniques for assessing adrenal and gonadal function have expanded the biological database for a number of species that had previously never been studied. Endocrine data derived from excreta has aided in captive propagation and has provided new insight to *in situ* populations (Monfort, 2003).

Hormones in urine and feces also represent pooled values. One blood sample represents the hormones circulating in the body at one point in time. As circulating hormones can vary diurnally, measured changes in blood samples day to day may not always reflect significant physiological changes. As circulating hormones are continually metabolized and excreted either with the feces or the urine, the hormone metabolites pool in the digestive tract or bladder. As a result one urine or fecal sample represents the circulating hormone concentrations for over several hours (Monfort, 2003)

Clearance and means of metabolism of steroids by the liver and kidney is species specific. In the African wild dog, exogenously delivered free estrogen and progestin were excreted in the urine and feces in nearly equal proportions (Monfort et al., 1997). However in domestic felids, most steroid metabolism occurs in the liver and therefore feces are the best index of circulating hormone levels (Brown et al., 1994). Conversely, steroids are predominately excreted in the urine of baboons (Wasser et al., 1991). As the

route steroids take and the form in which they are excreted vary from species to species, thorough biological, physiological and laboratory validation steps are necessary to ensure the measured hormonal changes are physiologically valid (Monfort, 2003).

The use of feces has the greatest potential to monitor endocrine function in the field. Several studies of wild populations have been conducted utilizing non-invasive measures (Wasser et al., 1991; Tsubota et al., 1992; Cavigelli, 1999; Foley et al., 2001; Lynch et al., 2002; Sands and Creel, 2004; Weingrill et al., 2004). Further, new technologies allow for individual identification through the extraction of DNA from exfoliated cells that line the fecal bolus (Garshelis, 2006). Additionally, it has become easier to locate fecal samples that are difficult to locate due to landscape or vegetation through the use of scat detection dogs (Wasser et al., 2004). However caution needs to be taken with regards to sample quality. Hormone metabolites can be degraded by bacterial activity or continued digestive enzymatic processes resulting in invalid hormone concentrations (Khan et al., 2002).

CHAPTER 2

Endocrine milieu of periestrus in the giant panda (*Ailuropoda melanoleuca*) as determined by noninvasive hormone measures

Abstract

Although urinary endocrine evaluations have determined that giant panda breeding is confined to a 4-mo period beginning in February, during which a female experiences its single annual estrus, fecal measures have not been thoroughly explored as an alternative for evaluating ovarian activity. Our results confirm that fecal excretion of estrogen and progestagen metabolites could be accurately quantified and that patterns corresponded well with urinary measures ($r = 0.79$ and $r = 0.69$ in two females, respectively) as well as with proceptive behaviors. Longitudinal assessments of 17 females further reveal that, on average, fecal estrogen concentrations began to rise ($P < 0.05$) above baseline (64.5 ± 5.9 ng/g; range, 20.0 – 103.5 ng/g) 5 d before the pre-ovulatory estrogen peak (Day 0, 468.1 ± 83.9 ng/g; range, 126.9 – 1,546.8 ng/g), which was followed by a gradual 4-d descent back to baseline. Fecal progestagen excretion increased ($P< 0.05$) approximately 2-fold above nadir (186.7 ± 27.6 ng/g; range, 75.0 – 385.0 ng/g) during the 20-d interval (410.1 ± 78.2 ng/g; range, 112.2 – 916.4 ng/g) following the pre-ovulatory estrogen surge.

Although variability within and among females precluded using a threshold of estrogen or progestagen concentrations to indicate reproductive status, hormonal evaluations conducted in feces collected 2 to 3 times a week was sufficient to identify and assess the endocrine dynamics of periestrus. These findings indicate fecal steroid metabolites are effective for monitoring reproductive-endocrine events in giant pandas living in *ex situ* collections.

1. Introduction

A substantial amount of information has been generated over the last 25 years describing the reproductive biology of the female giant panda (Bonney et al., 1982; Hodges et al., 1984; Murata et al., 1986; Chaunduri et al., 1988; Monfort et al., 1989; Mainka et al., 1990; Meyer et al., 1997; Czekala et al., 1998; Lindburg et al., 2001; Zhu et al., 2001; Durrant et al., 2002; McGeehan et al., 2002; Swaisgood et al., 2002; Czekala et al., 2003; Narushima et al., 2003; Dehnhard et al., 2006; Durrant et al., 2006; Howard et al., 2006b; Steinman et al., 2006). Males and females reach sexual maturity at ~ 5 and ~3 years, respectively (Kleiman et al., 1979; Schaller et al., 1985; Steinman et al., 2006). The species is unusual in that the adult female is reproductively active for < 1% of the year, being monoestrus during a breeding season that ranges from February through June (see review, Steinman et al., 2006). The periestrual interval appears to be hormonally-characterized by a gradual 10-day rise in urinary estrogen excretion, whereas a rapid decline in estrogen excretion signals ovulation (Monfort et al., 1989; Lindburg et al., 2001; Czekala et al., 2003). Peak estrual behaviors are observed over only a single,

annual 1 to 3 d period that generally corresponds with a peak in estrogen excretion (Monfort et al., 1989; Czekala et al., 1998; Lindburg et al., 2001; Czekala et al., 2003; Steinman et al., 2006). This abbreviated reproductive interval has historically complicated efforts to propagate giant pandas in *ex situ* collections (Zhang et al., 2006). However, as the collective knowledge describing giant panda basic biology has grown so has managed care and breeding success (Huang et al., 2001; Wildt et al., 2003; Edwards et al., 2006a; Edwards et al., 2006b; Ellis et al., 2006b; Zhang et al., 2006) with > 250 individuals now living in the world's *ex situ* collection (Xie and Gipps, 2007).

Although there are multiple publications addressing the reproductive endocrinology of the giant panda, all data have been generated collectively from studying only 23 individuals, and virtually all detailed descriptions about periestrus originate from assessing urinary steroid excretion in just three females (Bonney et al., 1982; Murata et al., 1986; Chaunduri et al., 1988; Monfort et al., 1989; Meyer et al., 1997; Lindburg et al., 2001; Durrant et al., 2002; McGeehan et al., 2002; Czekala et al., 2003; Narushima et al., 2003; Dehnhard et al., 2006; Steinman et al., 2006). There is a need to determine if these earlier findings typify norms for the species. Furthermore, although urinary hormone monitoring has been helpful for beginning to understand basic ovarian function, collecting urine can be problematic, especially when giant pandas are living on a soil substrate in a large enclosure. This disadvantage can be mitigated by assessments of steroid metabolites excreted in feces. Fecal steroid analyses have been used to study the endocrinology of other Ursidae, including the brown (*Ursus arctos*; Ishikawa et al., 2002; Ishikawa et al., 2003; Dehnhard et al., 2006; von der Ohe et al., 2004), Himalayan black (*Ursus thibetanus*; Young et al., 2004), sloth (*Melursus ursinus*; Young et al., 2004),

spectacled (*Tremarctos ornatus*; Dehnhard et al., 2006) and sun (*Helarctos malayanus*; Onuma et al., 2002; Schwarzenberger et al., 2004; Hesterman et al., 2005) bear. Fecal hormone monitoring was attempted previously in a single female giant panda after testing a diversity of extraction and assay methods (Kubokawa et al., 1992). However, this study relied on only a single sample per month from one animal, which prevented rigorous testing of the validity and feasibility of this approach for assessing reproductive status during the critical periestrual interval.

Given the strong interest in accelerating *ex situ* breeding of the giant panda, we systematically evaluated the efficacy of fecal steroid for assessing endocrine status in giant pandas during the periestrual interval. Our specific objectives were to: (1) validate immunoassays for assessing fecal metabolites of estrogen and progesterone; (2) determine the correspondence between fecal steroid excretion patterns and concomitant urinary steroid measures and sexual behaviors characteristic of periestrus; and (3) establish reproductive-endocrine norms associated with periestrus in a greatly expanded cohort of giant panda females. This study serves as a foundation for a parallel study (presented in Chapter 3) that was designed to document sex steroid parameters associated with delayed implantation, pseudopregnancy and pregnancy, and acyclicity in the giant panda.

2. Methods

2.1. Study animals and facilities

2.1.1. North America

During the study (2001 – 2006) the Smithsonian's National Zoological Park (SNZP; 39˚N, 77˚W) and Zoo Atlanta (ZA; 33˚N, 84˚W) each maintained one adult female and one adult male giant panda. The female housed at SNZP (Studbook [SB]473) was born 22 July 1998, and pubertal estrus in April 2002 (aged 3.7 y) was recorded in the collected data set. The ZA female (SB452) was born 25 August 1997 and experienced first estrus April 2001 (3.5 years old). Fresh water was available at all times, and the pandas were fed primarily a diet of bamboo (~75%), supplemented with fruit and a high-fiber biscuit that included amino acids, minerals and vitamins. All bamboo was locally harvested and kept fresh under sprinklers to prevent desiccation prior to feeding. Enclosures consisted of indoor (50 - 100 m^2) and outdoor (100 – 300 m^2) areas that allowed continuous inter-pair olfactory and auditory contact and frequent visual communication. Direct physical interaction between male and female pandas was allowed during behavioral estrus, with intermittent contact throughout the remainder of the year (but only for the SNZP pair). Dates of attempted or confirmed copulations and/or artificial inseminations were recorded and used to establish physiological relationships with hormonal profiles.

2.1.2. China

The China Conservation and Research Center for the Giant Panda at the Wolong Nature Reserve (Wolong; 31˚N, 103˚E) has consistently maintained one of the world's largest *ex situ* giant panda populations, with ~40 adult females and 25 adult males. For

the present study, fecal samples were collected from 15 reproductively active, healthy adult females (mean age, 8.8 y; age range, 3 – 16 y). Water was available *ad libitum* and, in general, the diet consisted primarily of bamboo supplemented with a high-fiber biscuit produced on site. Females were housed individually in enclosures that included indoor (30 – 60 m^2) and outdoor (100 – 300 m^2) areas. Physical contact between the sexes was limited only to periods of behavioral estrus, although all females were in auditory, visual and olfactory proximity to at least one male conspecific throughout the year.

2.2. Sample collection and processing

2.2.1. Urine

Freshly-voided urine was aspirated from the enclosure floor 3 to 7 d/wk with a clean plastic syringe and each then transferred to a 12 x 75 mm plastic tube, labeled with animal identification number and date, and stored frozen until analysis. Care was taken to avoid contamination of urine with feces or water (to avoid a dilution effect). If a fresh urine specimen could not be secured in the morning, samples were collected as excreted throughout the day. Samples excreted during the night-time were labeled as 'overnight' specimens and assigned a time of 12:00 am (midnight).

All urine samples were indexed for creatinine (Cr) to account for variations in water content (Taussky, 1954). For Cr determinations, 0.05 ml urine (diluted 1:20 in BSA-free phosphate buffer) was added in duplicate to 96-well, flat-bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA), combined with 0.05 ml each of

dH20, 0.4 N picric acid, 0.75 N NaOH, and incubated at room temperature (25ºC) for 30 min. Optical density was measured at 490 nm (reference 620 nm) using a microplate reader (Dynex MRX; Dynex Technologies, Chantilly, VA), and urine samples were compared to reference Cr standards (0.00625 - 0.1 mg/ml, Sigma; St. Louis, MO) assayed in duplicate. Samples with Cr concentrations < 0.1 mg Cr/ml were considered too dilute (probably from water contamination), a criterion that excluded ~7% of all samples. Hormone mass in a given urine sample (ng/ml) was divided by creatinine concentration (mg Cr/ml) with final hormone concentration expressed as mass of hormone/mg Cr excreted (ng/mg Cr).

2.2.2. Feces

Generally, fecal samples were collected within 1 h of excretion and from 3 to 7 d/wk. Due to the variable fiber content of giant panda feces (Edwards et al., 2006b), keepers preferentially selected feces that contained the least amount of undigested bamboo. Fecal samples were collected in re-sealable plastic bags, labeled with animal number and date and stored frozen (-20˚C) until processing.

Fecal samples were freeze-dried in a lyophilizer (Labconco, Kansas City, MO) and crushed in plastic bags to separate feces (powdered material) from undigested dry matter. Fine, powdered feces was stored frozen (-20˚C) in labeled and capped 12 x 75 mm plastic tubes and later thawed to allow 0.1 g of each sample to be extracted for hormonal metabolites using the procedures of Brown et al. (1994), Wasser et al. (1994) and Monfort et al. (1997). Extracts then were dried under air and resuspended in 1 ml of

BSA-free phosphate buffer before being stored frozen. The efficiency of hormone recovery rate for extraction from fecal powder was 84.9 ± 1.3% (based on a preliminary trial involving the addition of tritiated [^{3}H] estradiol [E2]).

2.3. Endocrine analyses

2.3.1. Estrogen conjugate (EC) EIA

Estrogens were assessed in unprocessed urine and fecal extracts using a single antibody estrogen conjugate (EC) enzyme immunoassay (EIA; Stabenfeldt et al., 1991). Microtiter plates (96-well, Nunc-Immuno, Maxisorp; Fisher Scientific, PA) were coated with an estrogen conjugate antibody (R583; C. Munro, University of California, Davis, CA) 12 to 18 h (4°C) before samples (unprocessed urine, equivalent to 0.2 – 2.5 µl; fecal extract, equivalent to 0.5 – 5.0 µl) and standards (39 – 10,000 pg/ml; 1,3,5(10)-estratrien-17-one 3-sulfate [E1S]; Sigma-Aldrich, MO) were added (0.05 ml) in duplicate and triplicate, respectively. An enzyme conjugated estrogen (C. Munro, University of California) then was added (0.05 ml) and, after a 2 h incubation (25°C) and removal of unbound components, 0.1 ml of substrate solution (ABTS in citrate buffer; Sigma Chemical Co., St. Louis, MO) was added to each well. Plates were read on a microplate reader (Dynex MRX; Dynex Tehnologies, VA) when optimal optical density (reading filter, 405 nm; reference filter, 540 nm) of the maximum binding wells was reached (1.00 OD). Inter-assay coefficients of variation (CV) for two internal controls (n = 87 assays) were 13.8% (mean binding, 41.8%) and 11.4% (mean binding, 74.5%), and intra-assay

CV was < 10%. Both urine and feces demonstrated parallel displacement with the EC EIA. Recovery of added standard to urine ($y = 0.76x + 4.38$, $r = 0.99$) and fecal extracts ($y = 1.24x + 19.54$, $r = 0.99$) both demonstrated significant ($P < 0.05$) recovery.

2.3.2. Progesterone EIA

Fecal and urinary progestagen concentrations were determined using a single antibody progesterone (P4) EIA (Graham et al., 2001). The sensitivity of the assay was 15.6 pg/ml with a standard range of 15.6 to 4,000 pg/ml. Microtiter plates were coated (12 – 18 h at 4°C) with P4 antibody (CL425, C. Munro, University of California) before the addition of 0.05 ml of sample (duplicate; equivalent to 0.04 – 2.5 μl of unprocessed urine and fecal extract) and P4 standard (triplicate; 4-pregnen-3,20-dione; Sigma-Aldrich, MO). Conjugated P4 (C. Munro, University of California) was added (0.05 ml) to all wells containing sample or standard and allowed to incubate at room temperature for 2 h before removal of all unbound components. Substrate solution then was added (0.1 ml) to all wells and absorbance read on a microtiter plate reader when optimal OD achieved. Inter-assay CV for two internal controls ($n = 54$ assays) was 12.2% (mean binding, 37.0%) and 13.2% (mean binding, 67.8%), and intraassay CV was < 10%. Parallel displacement was demonstrated in both serially diluted, unprocessed urine and fecal extracts. Recovered progesterone added to urine and fecal extracts was $y = 0.88x – 1.13$, $r = 0.99$ ($P < 0.05$) and $y = 0.67x – 2.03$, $r = 0.99$ ($P < 0.05$), respectively.

2.3.3. High pressure liquid chromatography (HPLC)

HPLC analyses (Varian ProStar; Varian Analytical Instruments, MA) were conducted to identify fecal estrogen and progestagen metabolites as previously described (Monfort et al. 1991; Monfort et al. 1997). Pooled fecal extracts with high estrogen and progestagen immunoreactivity were concentrated 20-fold and spiked with respective hormone tracers (estrogen run, ~14,000 cpm/ml each ^{3}H-E1S and ^{3}H-E2; progesterone run, ~14,000 cpm/ml ^{3}H-P4) to act as co-chromatographic markers. Fractions collected during HPLC analysis were measured for radioactivity to identify the retention time for radioactive markers. Eluates were evaporated to dryness, resuspended in 0.13 ml buffer and hormone mass quantified in fractions by EIA (estrogen conjugate and progesterone). Fractional immunoactivity was compared to retention times for known radioactive markers to establish the presumptive identity of excreted steroidal metabolites.

2.5. Data and statistical analysis

Baseline concentrations of urinary and fecal estrogen concentrations and fecal progestagens were determined through an iterative process as previous described (Moreira et al., 2001) with minor modifications. In brief, baseline values were assessed yearly for each female for each hormone. Values in excess of two standard deviations (SD) of baseline were removed from a data set until no values exceeded 2 SD of the baseline mean. Mean baseline hormone concentrations were expressed as mean ± standard error of the mean (SE). The duration of hormonal periestrus was considered the day from initial increase in estrogen values above baseline range (mean + 2 SD) to the

day estrogen values returned to within baseline range. Estrual behaviors were defined as pacing, scent marking, vulval swelling, bleating, tail up, backwards walking and lordosis (McGeehan et al., 2002)

To standardize profiles for the periestrual interval, urinary and fecal hormone data were aligned to day of peak estrogen value (Day 0). However to demonstrate the correspondence between urinary and fecal measures data presented in Figure 1 were aligned to urinary estrogen measures. However, day of year (based on Julian calendar) was used to assess differences in time of onset of periestrus within and among females. Correlations between standard and samples with added standard during validation were assessed using linear regression; however relation between urinary and fecal hormone data were determined with Pearson correlation coefficient. Data sets were tested for normality (Kolmogorov-Smirnov test) prior to assessment for statistical difference. Data sets that passed normality were tested for statistical significance using a Student's *t*-test or one-way analysis of variance (ANOVA). Statistical significance for data sets that failed normality were assessed using a Mann-Whitney test or Kruskal-Wallis one-way ANOVA. All analyses were conducted with SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA).

3. Results

3.1. HPLC

Fecal EC immunoreactivity quantified after HPLC separation co-eluted with ^{3}H-E1S (fraction 17) and ^{3}H-E2 (fraction 64), which constituted 11.9 and 21.7 % of total immunreactivity, respectively. HPLC analysis of fecal progestagen metabolites revealed seven prominent immunoactive peaks. The largest peak (fraction 10) constituted 8.7% of total immunoreactivity and exhibited the same retention time as pregnanediol-3-glucuronide (PdG). A small proportion of the total immunreactivity (2.6 %) was associated with 4-pregnen-3,20-dione (P4, fraction 66). The presumptive identities of the six other major immunoreactive peaks (12.4 % of total immunoactivity) detected by the progesterone EIA was not established.

3.2. Urinary and fecal estrogen comparison

Concurrent urinary and fecal EC profiles from a single periestrual event from two female giant pandas housed at SNZP (SB473) and ZA (SB452) are presented in Figure 1 with hormone excretion aligned to day of the urinary estrogen peak (Day 0). The correlation between urinary and fecal EC excretion was significant for SB473 ($r = 0.79$, $P < 0.05$) and SB452 ($r = 0.69$, $P < 0.05$).

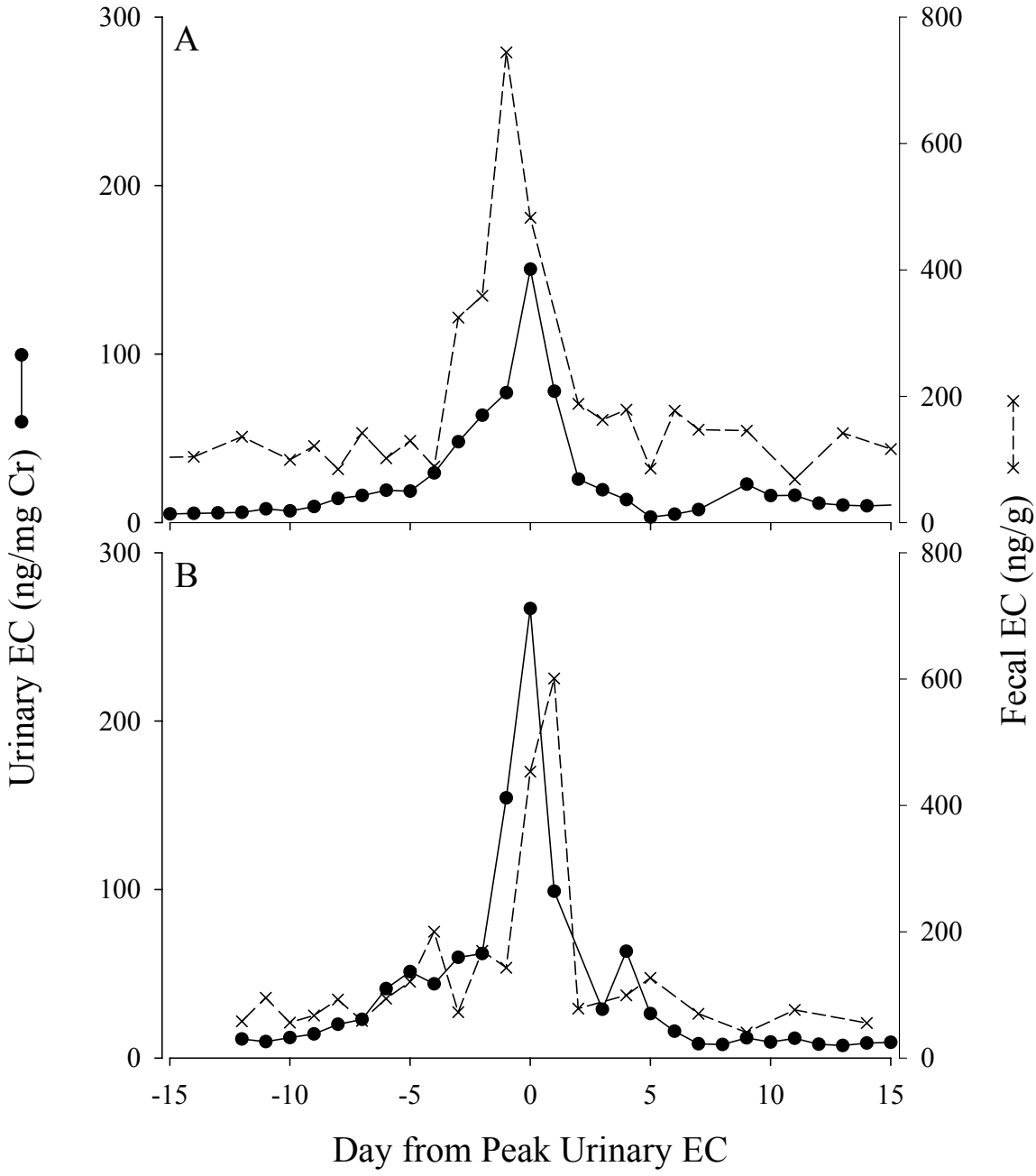

Figure 1. Urinary (closed circles, solid line) and fecal (cross, dashed line) EC concentrations during periestrual intervals from females (A) SB473 and (B) SB452. Data were aligned to day from peak urinary EC excretion.

For SB473, the initial increase (> baseline + 2SD) in urinary EC above baseline (10.1 ± 0.5 ng/mg Cr) occurred on Day –6 (19.1 ng/mg Cr), whereas fecal EC exceeded (> baseline + 2SD) basal concentrations (98.6 ± 4.8 ng/mg Cr) on Day -3 (324.1 ng/g). Fecal EC peaked (744.4 ng/g) one day before urinary EC (150.4 ng/mg Cr). Urinary EC excretion subsequently declined to baseline by Day +4, whereas fecal measures were basal one day later (Day +5).

Urinary EC in female SB452 increased (14.3 ng/mg Cr, > baseline + 2SD) above baseline (9.7 ± 0.5 ng/mg Cr) on Day –9, whereas fecal EC exceeded (> baseline + 2SD) baseline concentrations (72.5 ± 4.8 ng/g) on Day -5 (120.9 ng/g). Female SB452 excreted peak urinary (266.8 ng/mg Cr) and fecal (600.9 ng/g) EC concentrations on Day 0 and Day +1, respectively. Urinary EC excretion declined to baseline by Day +7, whereas fecal measures were basal five days earlier (Day +2).

3.3. Mean periestrual profiles

Mean (± SE) periestrual concentrations of fecal EC and progestagen profiles for female SB473 (years, 2002, 2003, 2004, 2005) and female SB452 (years, 2002, 2003, 2004) are depicted in Figure 2. For SB473 (Fig. 2A), fecal EC increased ($P < 0.05$) above baseline (153.0 ± .2.4 ng/g) by Day -6 (167.2 ng/g), peaked at 687.9 ± 56.0 ng/g and then decreased precipitously to baseline (110.7 ng/g) by Day +1. Fecal progestagen excretion during the 20 d before (297.1 ± 24 ng/g) and after (730.7 ± 45.0 ng/g) the EC peak were different ($P < 0.05$). For panda SB452, periestrus onset coincided with a rise

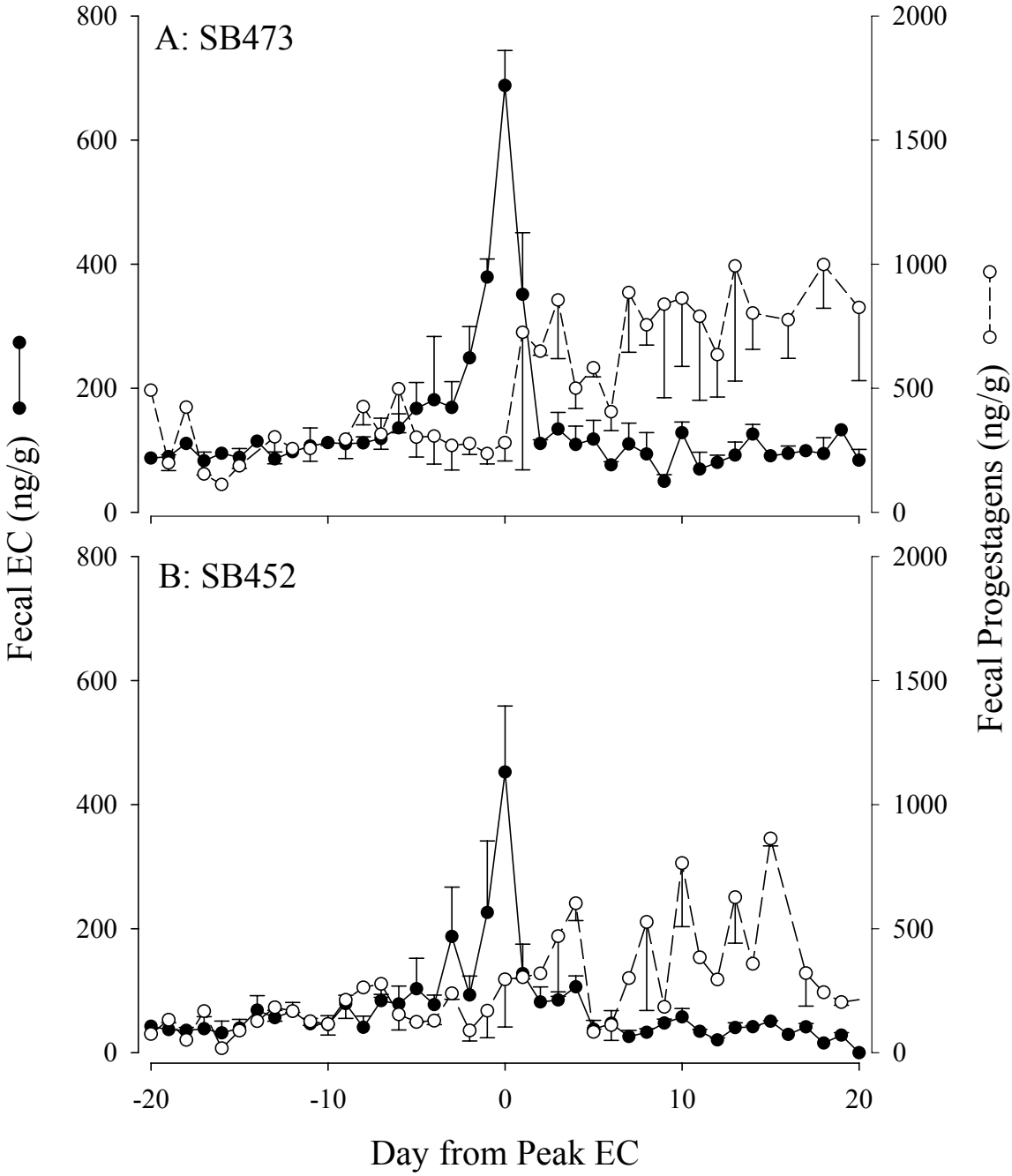

Figure 2. Mean (± SE) fecal EC (closed circles, solid lines) and progestagen (open circles, dashed lines) immunoreactivity during periestrual intervals for females (A) SB473 (years 2002, 2003, 2004, 2005) and (B) SB452 (2002, 2003, 2004).

($P < 0.05$) in mean EC excretion above baseline (36.7 ± 1.3 ng/g) to 79.0 ng/g on Day -9, peaked (Day 0, 452.5 ± 106 ng/g) and then declined to baseline (37.9 ng/g) by Day +5. Progestagen excretion 20 d before (146.7 ± 14 ng/g) and after (381.7 ± 48 ng/g) the EC peak were different ($P < 0.05$).

Fecal data collected throughout the year were examined for variation within and between animals with respect to the onset of periestrus and the duration of estrus. For SB473, increased reproductive behaviors commenced 24 April 2002, 2 April 2003, 1 May 2004 and 11 March 2005 for a range in periestrus onset of 53 d. This female exhibited overt estrual behaviors (e.g. tail-up, backwards walking, bleating) that lasted 10 d (2002), 8 d (2003), 9 d (2004) and 8 d (2005) demonstrating within animal variation in estrus duration of 2 d over the 4 year study period. For SB452, reproductive behaviors commenced 2 April 2002, 25 March 2003, 14 March 2004 for a range in periestrus onset of 18 d). SB452 exhibited overt estrus behaviors for 9 d (2002), 8 d (2003) and 13 d (2004) for a within animal variation in estrus duration of 5 d over the 3 year study period. Mean peak fecal EC concentrations for SB473 (506.7 ± 116 ng/g) and SB452 (452.5 ± 106 ng/g) were not different ($P > 0.05$).

3.4. Individual periestrual profiles

Representative fecal EC and progestagen profiles in four periestrual females housed at the China Conservation and Research Center for the Giant Panda (Wolong Nature Reserve) are presented in Figure 3. The temporal excretory dynamics of hormone excretion in this cohort were similar to what was observed in the more intensively

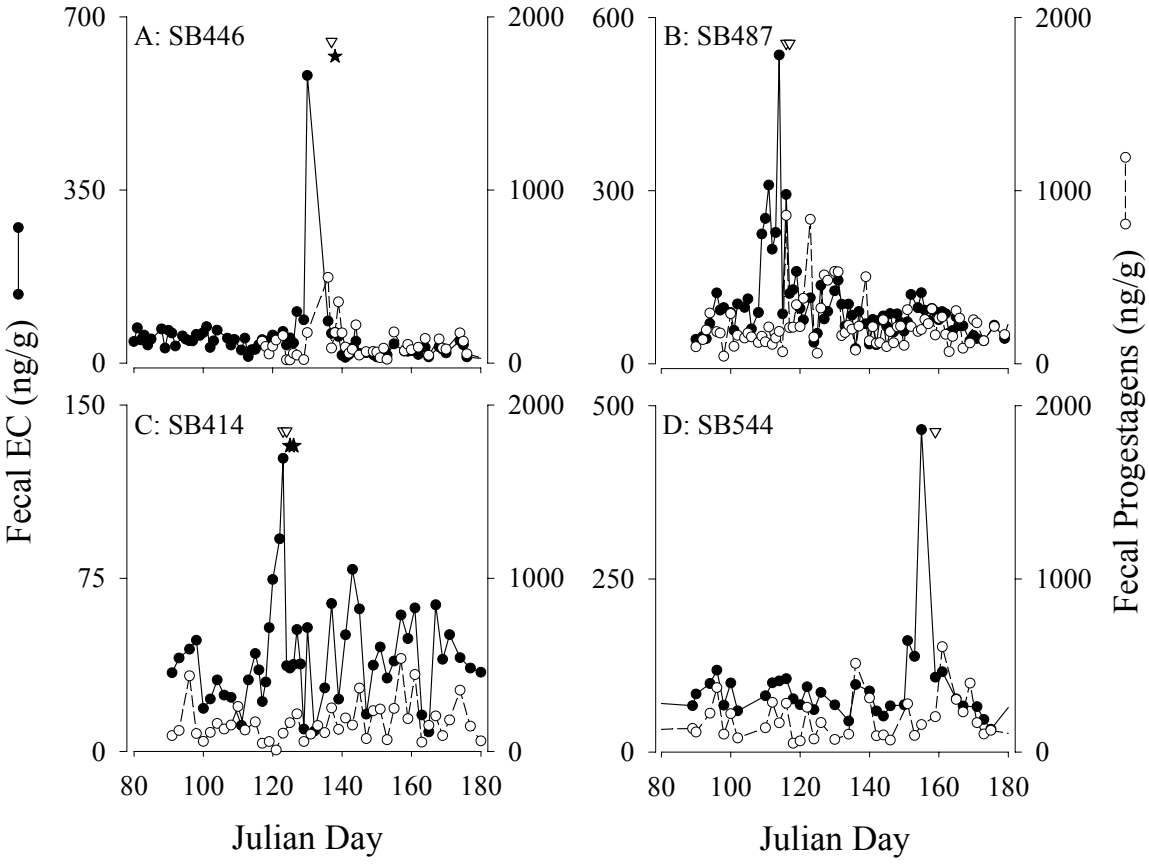

Figure 3. Representative fecal EC (closed circles, solid lines) and progestagen (open circles, dashed lines) excretion profiles for females (A) SB446, (B) SB487, (C) SB414 and (D) SB544. Open triangles denote day(s) of natural breeding and closed stars represent the timing of artificial insemination(s). Data are aligned to the Julian day. Note differences in the fecal EC scales among individuals.

sampled giant pandas (Figs. 1-2). The between-animal range (n = 4) in the onset of periestrus behaviors of 42 d (from Day +109 for SB587 to Day 151 for SB414). Due to limited sampling for SB466 during the periestrual interval, only a single EC increase (4-fold above baseline) was detected (Fig. 3A; 581.2 ng/g, Day 130), which occurred 7 d before onset of behavioral estrus and copulation. Progestagen excretion in SB466 20 d before (114.8 ± 35.0 ng/g) and after (112.2 ± 21.8 ng/g) mating were not different (P > 0.05). Female SB487 (Fig. 3B) mated on Day 116, 5 d after estrogen metabolites increased above baseline and 2 d after the fecal EC peaked (534.8 ng/g) 7-fold above baseline.. Mean progestagen excretion for this panda 20 d before (151.2 ± 13.6 ng/g) and after (355.5 ± 53.8 ng/g) mating were different (P < 0.05). For female SB414, the fecal EC peak coincided with behavioral estrus and copulation (Day 123; Fig. 3C), but peak EC concentrations (126.9 ng/g) were reduced compared to other females and exceeded baseline by only 4-fold. Progestagen concentrations for SB414 20 d before (121.3 ± 22.8 ng/g) and 20 day after (146.9 ± 18.9 ng/g) were not different (P > 0.05) between. Peak EC in SB544 (Fig. 3D, 465.4 ng/g) increased 6-fold increase above baseline on Day +155, with copulation on Day +159 (no fecal samples were collected from Days 156 through 159). Progestagen concentrations for this female for the 20 days before (202.1 ± 54.6 ng/g) and after (267.7 ± 59.9 ng/g) mating were not different (P > 0.05).

4. Discussion

This study validated a new, noninvasive approach for monitoring the single, annual periestrual interval of the endangered giant panda. This is important for

understanding the hormonal factors that trigger sexual receptivity, ovarian follicle development, behavioral estrus and ovulation in this rare species. Findings demonstrated that it was possible to extract and then measure key steroidal metabolites in the fibrous feces of the giant panda, including identifying the estrogen surge associated with presumed maximal follicle activity. Quantifying changes in progestagens were more challenging, less so because of technical limitations to the assay, but more related to the nature of the subtle, yet significant post-ovulatory rise in this metabolite. Urinary hormonal assessments previously have documented that ovulation in the giant panda is associated with a small but discernable rise in progestagen with a more significant elevation occurring at the presumptive time of implantation (Steinman et al., 2006). Fecal metabolite tracking in a larger cohort of subjects revealed a clearly identifiable, significant rise in fecal progestagens during the immediate post-ovulatory interval. Fecal assessments conducted at 2 to 3 d intervals were highly effective for tracking the endocrine milieu from the onset of ovarian follicle development to peak estrogen production that drives estrus behavior.

From a management perspective, the increased processing time associated with fecal hormone methods – as compared to urinary methods – may limit the usefulness of this approach for "real time" scheduling of natural mating or AI. However, fecal collection is simplified compared to harvesting urine from enclosure substrates, which makes compliance with longitudinal sampling regimens more feasible (i.e., 2 - 3 samples/wk). Fecal hormone monitoring can be applied under field conditions and has tremendous potential for evaluating reproduction and stress physiology in wild pandas

(Monfort, 2003; Schwartz and Monfort, 2008), where animals are rarely seen, but feces are often encountered (Steinman et al., 2006).

Despite the extremely fibrous nature of giant panda feces, we were able to adapt established fecal extraction methods (Brown et al., 1994; Wasser et al., 1994; Monfort et al., 1997) to achieve excellent metabolite extraction efficiency (circa 85%) comparable to what has been reported in the Malayan sun bear (*Ursus malayanus*, 81.1%; Hesterman et al., 2005) and other diverse mammalian species, including the maned wolf (*Chrysocyon brachyurus*, 89.7-104.4%; Velloso et al., 1998), black-footed ferret (*Mustela nigripes*, >90 %; Brown, 1997), cheetah (*Acinonyx jubatus*, >90%; Brown et al., 1996), moose (*Alces alces*, 108%; Monfort et al., 1993c) and African elephant (*Loxodonta Africana*, 75 – 86%; Wasser et al., 1996). Achieving uniformly high extraction efficiency is important for generating data sets that can be compared within and among animals over prolonged time intervals. This is especially important because giant panda feces can vary substantially, even within the same individual, in quality and consistency, ranging from hard, compact bamboo boluses to loose mucoid stools (see photos, Edwards et al., 2006b). We also demonstrated that dried feces can be readily pulverized into a powder that facilitates consistency in weighing, processing and extracting feces. Overall, these data demonstrated that fecal hormones can be reliably quantified in giant pandas to provide new scholarly information about the reproductive endocrinology of the giant panda. Additionally, these endocrine methods were readily adapted for use in China at the Chengdu Research Base of Giant Panda Breeding, to augment reproductive efficiency via timed-mating and AI.

Due to their hydrophobic nature, steroids are often conjugated to water-soluble moieties (i.e., sulfate or glucuronic acid) in the liver to facilitate excretion in the water-soluble environment of the gut (Whitten et al., 1998). Bacterial enzymes hydrolyze steroid conjugates in the large intestine, which results in the excretion of 'free', unconjugated steroids (Monfort, 2003; Schwartz and Monfort, 2008). However, HPLC analysis demonstrated that a majority of estrogen and progestagen metabolites in giant panda feces were excreted as steroidal conjugates. This observation may be explained by the fact that giant pandas exhibit rapid (6 - 7 h) gut transit time (Dierenfeld et al., 1982; Edwards et al., 2006b), which may result in reduced enzymatic hydrolysis and an increased proportion of excreted steroid conjugates (Edwards et al., 2006b). This hypothesis also is consistent with the fact that fecal estrogen and progestagen can be accurately assessed in the giant panda using immunoassays with antisera directed at steroid conjugates.

For more than 25 years urinary estrogens have been measured in the giant panda to track reproductive changes associated with the short, annual periestrual interval (Bonney et al., 1982; Hodges et al., 1984; Chaudhuri et al., 1988; Monfort et al., 1989; Mainka et al., 1990; Lindburg et al., 2001; McGeehan et al., 2002; Czekala et al., 2003; Steinman et al., 2006). Peak estrual behaviors and sexual receptivity, including tail-up and backwards walking, are most often correlated with peak and declining urinary estrogen concentrations. Hormonally, periestrus is characterized by estrogen values that gradually rise (1 – 2 wk) from baseline to peak excretion, then decline precipitously at the presumptive time of ovulation (see review, Steinman et al., 2006). The urinary EC profiles during periestrus from females SB473 and SB452 (Fig. 1) conformed to the

previous descriptions, including peak estrual behaviors at and after the peak in estrogens. However, of importance to this study was the same basic trend in EC excretion was measured concomitantly in feces, yet the specific dynamics between urinary and fecal measures were different. For both females (SB473, SB452) the duration of elevated periestrual EC was longer in the urine than in the feces (range, 2 – 9 d). Additionally, the estrogen onset of periestrus was detected in the feces 3 to 4 d after it was measured in the urine. Both measures showed that estrogen is quickly metabolized as all EC values dropped sharply after the peri-ovulatory peak and returned to basal range within a week. The immediate decline of hormonal measures in the feces following ovulation has practical application for infrequently collected fecal samples. As there is no protracted excretion of the hormone, any peaks in estrogen are likely due to significant ovarian activity. For Wolong female SB446 (Fig. 3A) a 15-fold increase in fecal EC above baseline was measured in the only fecal sample collected during the week before peak breeding behaviors were observed. Although differences were observed in the dynamics of urinary and fecal EC excretion, both measures were effective for tracking the endocrine dynamics during periestrual physiology. Fecal EC measures, however, provided a useful approach for investigating the incidence of periestrus in a large *ex situ* population and have tremendous potential for studying the reproductive endocrinology of wild populations of giant pandas.

Fecal estrogen metabolite excretion appeared similar in amplitude and duration within and among females; however variation was enough to prevent establishing quantitative thresholds. For every periestrual profile assessed, the peak in estrogen excretion represented a minimum 3.6 fold increase over nadir, however the peak estrogen

concentrations varied just as much within the two North American females that were monitored over successive years (SB473; CV, 57%; range, 214.3 – 744.4 ng/g; SB452; CV, 41%; range, 245.9 – 600.85 ng/g) as it did among the cohort of females at Wolong (all females; CV, 48%; range, 126.9 – 581.2 ng/g). Therefore, each female experienced a significant increase in fecal EC values during periestrus, but that change was unique to each female each year. The average duration of elevated periestrual fecal EC during the periestrual interval (8.3 ± 0.8 d; range, 4 – 13 d) was consistent among all assessed females. Taken together, these data suggest that longitudinal fecal EC assessments can be used to track the temporal endocrine changes predictive of ovulation in giant pandas.

In the giant panda, urinary progestagens increase coincident with the rapid decline in estrogen excretion and confirms that ovulation has occurred and a corpus luteum (CL) has formed (Hodges et al., 1984; Monfort et al., 1989; Mainka, et al., 1990; McGeehan et al., 2002; Czekala et al., 2003; Steinman et al., 2006). Fecal progestagens in 3/7 females (SB473, SB452, SB544) increased >2.4 fold during the 20-d interval after peak EC excretion. In the American black bear (Wimsatt, 1963), morphological studies on the ovary following estrus have indicated the presence of corpora lutea, however CL size in females with implanted conceptuses were significantly greater than females with diapause embryos. As the giant panda similarly experiences delayed implantation, it is likely that immediately following ovulation CL formation is slow and not uniform in all females. Yet it is more likely that following the presumptive time of implantation that CL size would increase and progestagen production would be significantly greater than 20 d post estrual concentrations. From previous studies we know that urinary progestagen excretion in the giant panda accelerates substantially 74 to 122 d post

ovulation, presumably the time implantation and fetal growth takes place (see review, Steinman et al., 2006). Thereby, methods reported in this study have been employed in a parallel study to examine fecal steroid metabolites during pregnancy and pseudopregnancy in this species. For both nonpregnant and pregnant females, significant elevations in progestagens (more than 30 fold over baseline) have been observed by 63 to 122 d after predicted ovulation. To study the early post-ovulatory/early luteal phase mechanisms urinary measures will likely provide the most dynamic data. However longitudinal studies of complete luteal phase function can be provided through evaluation of progestagens in the feces.

All females in the present study experienced a single estrus during the interval from February to June, which was similar to what has been reported previously for this species (Kleiman et al., 1979; Bonney et al., 1982; Chaunduri et al., 1988; Czekala et al., 1998; Lindburg et al., 2001; McGeehan et al., 2002; Durrant et al., 2003; Steinman et al., 2006), and for other Ursidae (Ferguson and McLoughlin, 2000; Spady et al., 2007). For the two most intensively monitored females, there was a relatively narrow window of time during the year (Julian Days 70 – 123 for SB473 and Julian Days 92 – 105 for SB452) when sexual activity was observed. Although autumnal estrus has occasionally been observed in the giant panda (Chorn and Hoffman, 1978; pres. comm., A. Kuba), the incidence of this phenomenon has not been hormonally documented and occurrence in the wild is unknown; fecal hormone methods may now permit such rare events to be studied in wild pandas.

In summary, we demonstrated that ovarian steroid metabolites could be quantified in feces as a viable alternative to urine for accurately monitoring ovarian activity during

periestrus in the giant panda. Furthermore, periestrus could be tracked by assessing fecal steroids in as few as 2 to 3 samples per week. Prolonged processing precludes using fecal measures as a tool for timing natural mating or artificial insemination (Czekala et al., 2003), but fecal assessments could be extremely useful for retrospective examinations of reproductive activity in *ex situ*, especially in situations whereby frequent urine collections are impractical. Also intriguing is the potential of using this approach for understanding physiological fitness in free-living populations. Monfort (2003) and Schwartz and Monfort (2008) have summarized the value of assessing steroidal metabolites in the excreta of a host of wildlife species in nature. For example, fecal hormones have been used to understand the influence of dominance on reproductive success in African wild dogs (*Lycaon pictus*, Creel et al., 1997), the relationship of moose (*Alces alces*) ecological carrying capacity and carnivore colonization (Berger et al., 1999), and the causes of reproductive suppression in meerkats (*Suricata suricatta*, Young et al., 2008). Although giant pandas are observationally elusive in the wild, their fecal samples are not. Frequent defecation (i.e., up to 20 times/d) by the giant panda increases the likelihood of finding samples without observing individuals. The use of scat detection dogs (Wasser et al., 2004) and radio-telemetry further increases the feasibility of collecting fecal samples under field conditions, and when combined with genetics, one could identify the individual animal and attribute hormonal measures to specific individuals. Therefore, although it would likely be impractical to collect serial samples at a sufficient frequency to plot a periestrual profile, it may be possible to determine that free-ranging females are 'reproductive active' on the basis of even a single estrogen or progesterone value. Additionally, there is a growing database demonstrating the potential

of measuring adrenal corticoid (stress-related) hormones in mammals (von der Ohe and Servheen, 2002; Monfort, 2003; Millspaugh and Washburn, 2004; Young et al., 2004). The present study demonstrated the usefulness of fecal hormone measures for accurately quantifying gonadal hormone patterns in the giant panda. We predict that it will be possible to extend these methods to permit accurate assessments of adrenal status by quantifying corticosteroid metabolites excreted in feces, which would be a powerful means for assessing the impact of various perturbations, including human disturbance, on well-being of wild giant pandas.

CHAPTER 3

Fecal progestagens for assessing luteal activity in the giant panda (*Ailuropoda melanoleuca*)

Abstract

The luteal phase is the most complicated and least understood aspect of the giant panda's reproductive biology. To better understand the hormonal dynamics of the luteal phase, fecal progestagen metabolites were quantified in feces (3 - 7 d/wk) from 14 females sampled over a 3-yr interval. Progestagen metabolite concentrations from matched urine and fecal samples across known luteal phases from two females demonstrated strong correlations ($r = 0.79$; $r = 0.62$). All females that experienced estrus exhibited a biphasic luteal fecal progestagen profile during the post-ovulatory interval. An initial 1.6-fold "primary" increase (368.1 ± 17.7 ng/g; range, 15.9 – 1,456.1 ng/g , $P > 0.05$) above baseline concentrations (101.9 ± 4.5 ng/g; range, 4.1 – 1,245.4 ng/g) during the immediate post-ovulatory interval lasted for approximately 90 d (88.9 ± 6.5 d; range, 63 – 122 d). A "secondary" 8.5-fold increase (3,110.9 ± 283.5 ng/g; range, 101.4 – 16,894.5 ng/g, $P > 0.05$) in progestagen excretion lasted approximately 40 d (39.5 ± 2.8 d; range, 28 – 52 d). Although the primary rise in fecal progestagen excretion was higher ($P <$

0.05) for nonparturient (460.9 ± 28.0 ng/g; range, 60.8 – 1456.1 ng/g) compared to parturient (291.0 ± 20.9 ng/g; range, 15.9 – 1,279.2 ng/g) females, there were no differences ($P < 0.05$) in fecal progestagen concentrations between nonparturient (3125.5 ± 339.4 ng/g; range, 149.7 – 12326.5 ng/g) and parturient (3,096.5 ± 455.3 ng/g; range, 101.4 – 16,894.5 ng/g) females during the secondary rise interval. Additionally, the durations of the primary (nonparturient, 95.5 ± 10.0 d; parturient, 82.3 ± 8.4 d) and secondary rise (nonparturient, 42.3 ± 5.4 d; parturient, 36.8 ± 2.2 d) intervals were not different ($P > 0.05$) between the two subsets. Fecal progestagens monitoring permitted longitudinal assessments of corpus luteum activity, but there were few substantive differences in hormonal excretory patterns between nonparturient and parturient giant panda females. Overall, these data confirmed that fecal progestagens can be used to study the endocrine dynamics associated with delayed implantation, pseudopregnancy, and anestrus in the giant panda.

1. Introduction

The reproductive biology of the female giant panda has received considerable attention over the past 30 years (Kleiman et al., 1979; Bonney et al., 1982; Hodges et al., 1984; Kleiman, 1984; Murata et al., 1986; Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; Meyer et al., 1997; Czekala et al., 1998; Lindburg et al., 2001; Zhu et al., 2001; Durrant et al., 2002; McGeehan et al., 2002; Swaisgood et al., 2002; Czekala et al., 2003; Narushima et al., 2003; Dehnhard et al., 2006; Durrant et al., 2006; Howard et al., 2006a; Steinman et al., 2006; Howard et al.,

2008). Particularly, research emphasis has focused on defining and explaining the physiology and behavior of estrus, in large part, because giant pandas are seasonally monoestrus and conservation efforts sought to maximize reproductive success during a narrow window of fertility. However, the luteal phase of the giant panda reproductive cycle has remained relatively unstudied with the current endocrinological knowledge of this reproductive phase based on 16 individuals, of which repeated, complete luteal cycles have been examined in only 3 females (Bonney et al., 1982; Hodges et al., 1984;Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Narushima et al., 2003; Dehnhard et al., 2006; Steinman et al., 2006). These studies were important for providing the first descriptions of delayed implantation and pseudopregnancy in this species, yet much remains to be learned about the hormonal dynamics associated with these phenomena.

Pseudopregnancy occurs when a female experiences the same physiological and behavioral changes as a pregnant female even though fertilization does not occur (Erskine, 1998). Within the Family Ursidae, pseudopregnancy is fairly common and hormonal dynamics of this prolonged luteal phase in the American black bear (*Ursus americanus*; Schulz et al., 2003), Asiatic black bear (*Ursus thibetanus*; Sato et al., 2001), brown bear (*Ursus arctos*; Tsubota et al., 1992), and sun bear (*Helarctos malayanus;* Schwarzenberger et al., 2004) are indistinguishable from those of pregnant females. Although there is evidence that some bears are induced ovulators (Wimsatt, 1963; Herrero and Hamer, 1977; Palmer et al., 1988; Boone et al., 2003; Boone et al., 2004), which implies that pseudopregnancy can only be induced through coitus. Giant pandas, however, have been shown to ovulate spontaneously, including in the absence of a male

(Mainka et al., 1990), and it appears that a prolonged luteal phase is an obligate feature of the their reproductive cycle (Monfort et al., 1989; Steinman et al., 2006). Progestagen excretion following estrus in nonpregnant females are consistent with those of pregnant females suggesting that pseudopregnancy is common in the giant panda (Monfort et al., 1989; Mainka et al., 1990; Narushima et al., 2003; Steinman et al., 2006). Thereby, excreted hormone measures have, thus far, been ineffective as a means for diagnosing pregnancy in the giant panda (Steinetz et al., 2005; Steinman et al., 2006).

Luteal activity in the giant panda is characterized by a two-phase increase in progestagen activity following ovulation (Hodges et al., 1984; Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Narushima et al., 2003; Steinman et al., 2006). A similar biphasic progestagen pattern following ovulation has been reported for a number of other bear species, including the American black bear (Foresman and Daniel, 1983; Palmer et al., 1988; Hellgren et al., 1991; Tsubota et al., 1998; Schulz et al., 2003), brown bear (Tsubota et al., 1992), spectacled bear (*Tremarctos ornatus*; Dehnhard et al., 2006) and Asiatic black bear (Sato et al., 2001). Immediately following ovulation in the giant panda, a "primary rise" in urinary progestagen concentrations occurs lasting 74 - 122 d (Hodges et al., 1984; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Czekala et al., 2003; Steinman et al., 2006). A subsequent "secondary rise" in progestagens excretion occurs during the 40 - 50 d interval preceding parturition or the end of a pseudopregnancy (Hodges et al., 1984; Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Steinman et al., 2006). Although this biphasic luteal progestagen activity seems to characterize the Ursidae, it is

also found in other species that experience delayed implantation, including the mink (*Mustela vison*; Allais and Martinet, 1978; Papke et al., 1980), European badger (*Meles meles*; Canivenc and Bonnin, 1981), western spotted skunk (*Spilogale gracilis;* Mead, 1981), and northern fur seal (*Callorhinus ursinus*; Daniel Jr., 1981).

Delayed implantation is strictly a mammalian strategy and prominent in the Order Carnivora. Following gamete fertilization, embryonic development is ceased or severely retarded at the blastocyst stage for an extended period of time, and then when conditions permit, implantation occurs and normal development is resumed (Hamlett, 1935; Conaway, 1971; Weir and Rowlands, 1973; Renfree and Calaby, 1981; Mead, 1989; Sandell, 1990; Lindenfors et al., 2003). Detailed studies of the mink (Allais and Martinet, 1978; Papke et al., 1980), European badger (Canivenc and Bonnin, 1981; Yamaguchi et al., 2006) and spotted skunk (Mead, 1981) have shown that the secondary rise in progesterone is coincident with the onset of embryo nidation. As the luteal progestagen pattern in giant pandas closely resembles that of species known to experience delayed implantation, it is believed that the giant panda experiences embryonic diapause and that the secondary rise in post-ovulatory progestagen concentrations approximates the time of implantation (Hodges et al., 1984; Monfort et al., 1989).

Although some endocrinological studies of the giant panda have been thorough, none have sought to evaluate the endocrine dynamics of the luteal phase by examining temporal patterns in fecal progestagen metabolite excretion. As a viable alternative to blood and urinary evaluations, fecal hormone monitoring has been used successfully to study luteal activity in other Carnivora, including the clouded leopard (*Neofelis nebulosa*;

Brown et al., 1995), cheetah (*Acinonyx jubatus*; Brown et al., 1996), sun bear (Schwarzenberger et al., 2004), maned wolf (*Chrysocyon brachyurus*; Velloso et al., 1998; Songsasen et al., 2006), red wolf (*Canis rufus*; Walker et al., 2002), and black footed ferret (*Mustela nigripes*; Brown, 1997; Young et al., 2001). We previously (Kersey et al., unpublished) demonstrated the validity of fecal steroids for assessing the endocrine milieu associated with periestrus in the giant panda, and we now seek to use these same methods to elucidate the endocrine dynamics of the giant panda luteal phase. Our specific objectives were to: (1) demonstrate that fecal progestagens could be useful for assessing the endocrine dynamics of the luteal phase; (2) establish and compare luteal progestagen parameters for parturient and nonparturient females; (3) determine if the pattern in fecal progestagen excretion is similar to that reported for other species that experience delayed implantation, and (4) examine gonadal hormone activity in females that were behaviorally classified as experiencing weak estrus.

2. Methods

2.1. Study animals and facilities

2.1.1. North America

Female SB473 (DOB 7/22/98) was housed at the Smithsonian's National Zoological Park (SNZP; 39˚N, 77˚W), and female SB452 (DOB 8/25/97) was housed at Zoo Atlanta (ZA; 33˚N, 84˚W) throughout the course of this study (2001 – 2005). The

pandas were provided *ad-libitum* water and were fed a diet that consisted primarily of freshly cut bamboo (>75%) that was supplemented with fruit and a diet biscuit that was high in fiber, vitamins, minerals and amino acids. Enclosures consisted of indoor (50 - 100 m^2) and outdoor (100 – 300 m^2) areas. A single adult male at SNZP was allowed intermittent physical contact with the female throughout the year including during behavioral estrus, except in 2002 to prevent mating during the first estrus of SB473. The male and female pair at ZA were kept separate, but still within olfactory, visual and auditory contact, throughout the year with physical contact only allowed during behavioral estrus. Records were kept of dates of attempted or confirmed copulations and/or artificial inseminations, and parturition to establish physiological relation with hormone concentrations.

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2.1.2. China

One of the world's largest captive populations of giant pandas is at The China Conservation and Research Center for the Giant Panda at the Wolong Nature Reserve (Wolong; 31˚N, 103˚E). The facility houses approximately 40 adult females and 25 adult males, of which 12 reproductively active, healthy adult females (age range, 4 – 16 y) were used for our study. All animals at this facility had fresh water available at all times and were provided a diet that consisted primarily of bamboo supplemented with a high-fiber biscuit. Enclosures for the individually housed females consisted of indoor (30 – 60 m^2) and outdoor (100 – 300 m^2) areas. For breeding purposes, females and males were allowed physical contact during behavioral estrus, however outside of these periods, males and females were limited to auditory, visual and olfactory contact.

2.2. Sample collection and processing

2.2.1. Urine

Urine samples were collected fresh (3 – 7 d/wk) after being aspirated from a clean substrate and stored frozen in 12 x 75mm plastic tubes until analysis. Urine was primarily collected in the morning, however if a sample was not available then one would be collected later in the day. Urine samples excreted overnight were collected and marked as "overnight" specimens and assigned an excretion time of 12:00 am (midnight).

To account for variations in water content all urine samples were indexed for creatinine (Cr; Taussky, 1954). Urine (0.05 ml urine; diluted 1:20 in BSA-free phosphate buffer) and Cr standards (0.00625 to 0.1 mg/ml, Sigma; St. Louis, MO) were added in duplicate to 96-well flat bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). Each sample and standard received 0.05 ml each dH20, 0.4 N picric acid, 0.75 N NaOH, and incubated at room temperature (25ºC) for 30 min and then read at Optical density 490 nm (reference 620 nm) on a microplate reader (Dynex MRX; Dynex Technologies, Chantilly, VA). Urine samples with CR mass was less than 0.1 mg Cr/ml (~7% of all samples) were considered too dilute and were excluded from hormone analyses. Following hormone concentrations (ng/ml) were divided by creatinine concentration (mg/ml), and final hormone concentrations were expressed as mass of hormone per mg of Cr excreted (ng/mg Cr).

2.2.2. Feces

Freshly voided feces that contained the least amount of undigested bamboo were collected (within 1 h of defecation) 3-7 d/wk. Fecal samples were placed in re-sealable plastic bags, labeled with animal identification and date, and stored frozen until processing.

To obtain the 0.1g of fecal powder needed for extraction all fecal samples were freeze-dried, gently crushed in plastic bags to separate undigested dry matter from the feces, and powdered feces were transferred to 12 x 75 mm plastic tubes, capped and stored frozen until extraction. Feces were extracted using previously described methods (Brown et al., 1994; Wasser et al., 1994; Monfort et al., 1997) and extractants were dried under air, resuspended in 1 ml of BSA-free phosphate buffer and stored frozen until immunoassayed.

2.3. Endocrine analyses

2.3.1. Estrogen conjugates (EC) EIA

Fecal estrogens metabolites were quantified using a single antibody estrone conjugate (EC) EIA (Stabenfeldt et al., 1991). An estrogen conjugate antibody (R583; C. Munro, University of California, Davis, CA) was allowed to adsorb (12 to 18 h; 4°C) to microtiter plates (96 well, Nunc-Immuno, Maxisorp; Fisher Scientific, PA) prior to the addition of duplicate samples (unprocessed urine, 0.2 – 2.5 µl; fecal extract, 0.5 – 5.0 µl) and triplicate standards (39 – 10,000 pg/ml; 1,3,5(10)-estratrien-17-one 3-sulfate [E1S];

Sigma-Aldrich, MO) were added (0.05 ml). An enzyme conjugated estrogen (C. Munro, University of California, Davis, CA) was immediately added (0.05ml) and allowed to incubate (2 h; 25°C) before unbound components were removed. To each well, 0.1ml of substrate solution (ABTS in citrate buffer; Sigma Chemical Co., St. Louis, MO) was then added and when optimal optical density (reading filter 405 nm; reference filter 540 nm) of the maximum binding wells was reached (1.00 OD), plates were read on a microplate reader (Dynex MRX; Dynex Tehnologies, VA). Interassay coefficients of variation (CV) for two internal controls (n = 83 assays) were 14.5% (mean binding 40.0%) and 14.9% (mean binding 74.2%), and intraassay CV was < 10%.

2.3.2. Progesterone EIA

Progestagen concentrations for urine and feces were determined with a single antibody progesterone (P4) EIA (Graham et al., 2001). Progesterone antibody (CL425, C. Munro, University of California, Davis, CA) was allowed to adsorb to microtiter plates 12 to 18 h (4°C) before the addition of duplicate samples (unprocessed urine and fecal extract, 0.04 – 2.5 µl) and triplicate P4 standards (0.05 ml; range, 16 – 4,000 pg/ml; 4-pregnen-3,20-dione; Sigma-Aldrich, MO). To the standards and samples a conjugated P4 (C. Munro, University of California, Davis, CA) was added (0.05 ml) and allowed to incubate at room temperature for 2 h before removal of all unbound components. Chromagen reaction was initiated with the addition of the substrate solution to all wells(0.1 ml), with resultant color change read on a microtiter plate reader when optimal optical density of the maximum binding wells was reached (1.00 OD). Interassay CV for

two internal controls (n = 104 assays) were 14.3% (mean binding 39.0%) and 14.6% (mean binding 71.2%), and intraassay CV was < 10%.

2.3.3. High pressure liquid chromatography (HPLC)

Reverse phase HPLC (Varian ProStar; Varian Analytical Instruments, MA) was used to identify progestagen metabolites present in the fecal extracts (Monfort et al. 1997). Pooled extracts of samples that contained high concentrations of progestagens from parturient (n = 2; 1 ml from 3 samples from each female) and nonparturient females (n = 2; 1 ml from 3 samples from each female) were concentrated (20-fold) and spiked with ^{3}H 4-Pregnen-3, 20-dione (P4: ~14,000 cpm/ml) to act as co-chromatographic marker. Following separation using a reverse phase C18 HPLC column (Varian Analytical Instruments, MA), collected fractions were quantified for radioactivity to identify radioactive marker elution times. The fractions were then dried, resuspended in 0.3mls of assay buffer and assayed on the Progestagen EIA to identify fraction immunoactivity. Fraction immunoactivity were analyzed alongside radioactive marker elution times to identify predominate steroid metabolites.

2.4. Statistical Analysis

Baseline fecal progestagen excretion during the anestrus phase of the reproductive cycle was determined using an iterative process as previously described (Moreira et al., 2001). The first day of the luteal phase was considered the day after the pre-ovulatory EC peak. The end of the luteal phase was designated as the first day that progestagen

concentrations returned to baseline range (baseline mean +2SD) for two or more consecutive days. The subset of samples that constituted the luteal phase was evaluated using an iterative process to eliminate fecal hormone concentration outliers that that exceeded 2SD of the mean. The duration of the primary rise in progestagen concentrations was defined as the time from the onset of the luteal phase to when fecal progestagens concentrations were elevated 2SD above mean hormone concentrations for at least two consecutive days. The duration of the secondary rise was defined as the interval from the end of the primary rise to the end of the luteal phase. All hormonal concentrations and interval durations were expressed as mean ± standard error of the mean (SE).

For statistical comparisons, urinary and fecal progestagen data were aligned to the urinary EC peak. For all other fecal hormone analyses, data were aligned to the Julian calendar. Correlations between concomitant urinary and fecal progestagen concentrations and in the HPLC analysis of parturient and nonparturient progestagen metabolites were determined with Pearson correlation coefficients. Relation between date of onset of the luteal phase and the duration of the luteal phase was determined with a linear regression. All data were tested for normality (Kolmogorov-Smirnov test) before subsequent testing. Normal data comparisons were tested for statistical significance using a Student's *t*-test and one-way ANOVA, whereas non-normally distributed data were evaluated using a Mann-Whitney test or Kruskal-Wallis one-way ANOVA. All statistical tests were conducted at alpha = 0.05. Iterations were conducted in Microsoft Excel 2007 (Microsoft, Inc., Seattle, WA) and all other statistical analyses were conducted in SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA).

3. Results

3.1. Urinary and fecal progestagens during the luteal phase

Matched urine and fecal progestagen luteal profiles for females SB473 and SB452 are presented in Figure 4. Correlations between the two measures were strong for SB473 ($r = 0.80$; $P < 0.05$), but lower, yet still significant, for SB452 ($r = 0.62$; $P < 0.05$). For SB473, total luteal phase duration estimated using urinary measures was 13 d longer (149 d) than when estimated using fecal progestagens (136 d). Further, the length of the primary rise in progestagens was 14 d shorter when assessed using urinary measures (88 d) compared to fecal progestagens (102 d). In contrast, the duration of the secondary rise in urinary progestagens was nearly twice (61 d) the duration derived from assessments of fecal progestagens (34 d). For SB452, total luteal phase duration estimated using urinary measures was 7 d shorter in (141 d) than when estimated using fecal progestagens (148 d). The length of the primary rise in progestagens was similar when assessed using urinary (88 d) and fecal (89 d) measures. Likewise, the duration of the secondary rise in urinary progestagens was 6 d shorter (53 d) than the duration derived from assessments of fecal progestagens (59 d).

Although progestagen concentrations for both females were always lower during the primary rise compared to the secondary rise (all comparisons, $P < 0.05$), the magnitude of increase during the secondary rise concentrations was not consistent among hormonal measures (i.e., fecal vs. urinary progestagens) or between females. Mean

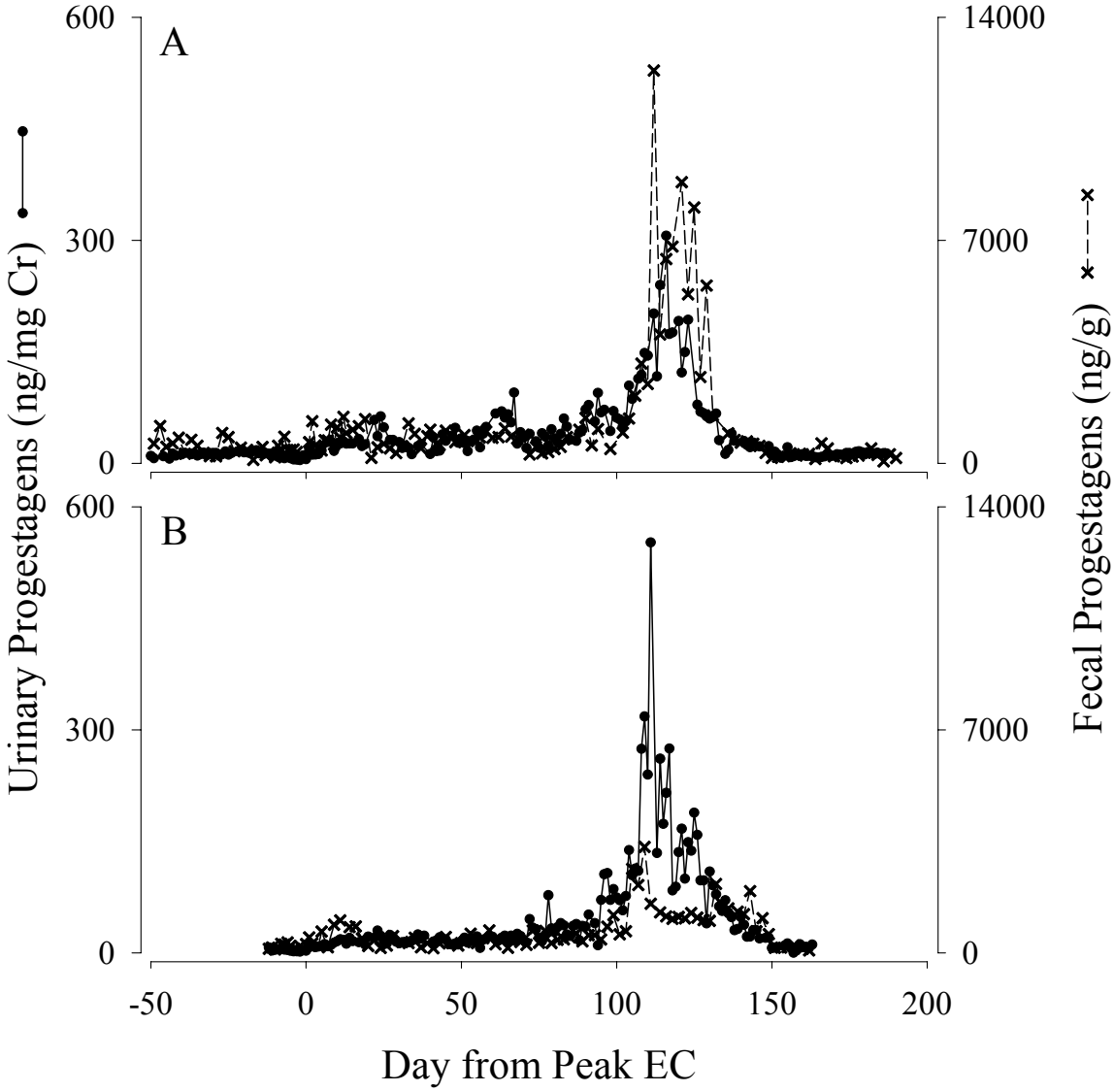

Figure 4. Matched urinary (closed circles, solid lines) and fecal (crosses, dashed lines) progestagen metabolite concentrations during luteal phases from females (A) SB473 and (B) SB452. Data are aligned to day peak urinary EC excretion.

urinary progestagen concentrations during the secondary rise (88.6 ± 9.4 ng/mg Cr; range, 12.8 – 306.1 ng/g) for female SB473 were 3-fold higher than concentrations during the primary rise interval (27.4 ± 1.1 ng/mg Cr; range, 11.5 – 43.9 ng/g). Similarly, mean fecal progestagen concentrations during the secondary rise (5,048.3 ± 853.3 ng/g; range, 1,405.7 – 12,326.5 ng/g) were 7-fold higher than concentrations during the primary rise interval (733.8 ± 37.0 ng/g; range, 169.6 – 1,252.0 ng/g). For SB452, mean urinary progestagen concentrations during the secondary rise (116.9 ± 13.2 ng/mg Cr; range, 10.0 – 551.9 ng/g) were 7-fold higher than concentrations during the primary rise interval (16.7 ± 0.6 ng/mg Cr; range, 6.6 – 25.6 ng/g). Mean fecal progestagen concentrations during the secondary rise (1,342.7 ± 125.6 ng/g; range, 580.5 – 3,327.2 ng/g) were 3-fold higher than concentrations during the primary rise interval (428.5 ± 29.7 ng/g; range, 145.6 – 1,019.1 ng/g).

3.2. Year-to-year evaluations of fecal progestagens excreted during the luteal phase

Year-to-year parameters for luteal phase duration and fecal progestagen excretion for SB473 and SB452 are presented in Table 1. Comparisons of the durations of the primary rise, secondary rise and total luteal phase duration did not differ between the two females (all comparisons $P > 0.05$). Between-female differences ($P < 0.05$) in fecal progestagen concentrations were evident during the primary but not during the secondary rise interval ($P > 0.05$). Progestagen concentrations during the primary and secondary rise intervals for SB473 did not differ during 2003 and 2004 ($P > 0.05$), but both years were elevated ($P < 0.05$) compared to 2002. For SB452, fecal progestagens excreted

Table 1. Year-to-year evaluations of mean fecal progestagens concentrations (± SE ng/g) and durations (± SE d) during the luteal phases of two adult female giant pandas.

			Primary Rise		Secondary Rise		
Female	Year	Day of year of estrogen peak	Duration	Pg Conc.	Duration	Pg Conc.	Total luteal duration
SB473	2002	117	109	424.6 ± 16.7[a]	52	1338.2 ± 154.8[c]	161
	2003	94	103	648.7 ± 37.7[b]	40	2996.8 ± 545.5[d]	143
	2004	123	98	777.9 ± 42.0[b]	39	4536.3 ± 821.2[d]	137
	mean	**111.3 ± 8.8**	**103.3 ± 3.1**	**572.6 ± 19.5†**	**43.7 ± 4.2**	**2302.8 ± 253.1***	**147.0 ± 7.2**
SB452	2002	92	105	238.7 ± 23.8[e]	42	2583.3 ± 662.7	147
	2003	84	61	369.4 ± 38.8[f]	64	2548.2 ± 382.9	125
	2004	74	95	427.3 ± 30.4[f]	53	1342.7 ± 125.6	148
	mean	**83.8 ± 5.2**	**87.0 ± 13.3**	**542.6 ± 55.3**	**53.0 ± 6.4**	**2008.5 ± 197.8***	**140.0 ± 7.5**

Progestagen concentrations within reproductive state within females with different subscripts differ ($P < 0.05$)

* indiacte difference ($P < 0.05$) in hormonal concentrations within females between primary and secondary rise values

† indicates difference ($P < 0.05$) in mean progestagen concentrations within columns between females

during the 2002 primary rise were lower ($P < 0.05$) compared to 2003 and 2004 progestagen values (which were similar; $P > 0.05$), but no between-year differences ($P > 0.05$) in fecal progestagens were detected in secondary rise progestagen concentrations. There was no correlation between the date of onset of the luteal phase (i.e., day of the EC peak) and the duration of the luteal phase either SB473 ($r = 0.08$; $y = 96.3 + 0.10x$; $P > 0.05$) or SB452 ($r = 0.10$; $y = 93.3 – 0.07x$; $P > 0.05$). Fecal progestagen concentrations for all females and years (SB473 2002, 2003 2004; SB452 2002, 2003) were higher ($P < 0.05$) during the 50-d interval after compared to the 50-d before the urinary EC peak.

3.3. Fecal progestagens during the nonparturient luteal phase

Fecal progestagens during the luteal phase of four nonparturient females are presented in Figure 5. Female SB414 (Fig. 5A) excreted peak fecal EC on the Day 156 and natural mating occurred three days later. Fecal progestagens indicated that the luteal phase ended on Day 273, providing an estimated luteal phase duration of 117 d. Although infrequent sample collection during the luteal phase prevented an accurate assessment of the primary and secondary rise durations, a significant increase ($P > 0.05$) in progestagen excretion occurred from Day 245-273. Progestagen concentrations ($n = 10$; 3,620.3 ± 905.6 ng/g; range, 299.9 – 9,255.2 ng/g) measured during this 28-d interval were increased ($P < 0.05$) compared to luteal progestagen excretion during the preceding interval ($n = 35$; 299.5 ± 33.1 ng/g; range, 103.6 – 607.5 ng/g). Peak fecal EC for female SB544 (Fig. 5B) were measured on the Day 115 and natural mating occurred one and two days later. Based on the fecal progestagen data, the estimated duration of the luteal phase

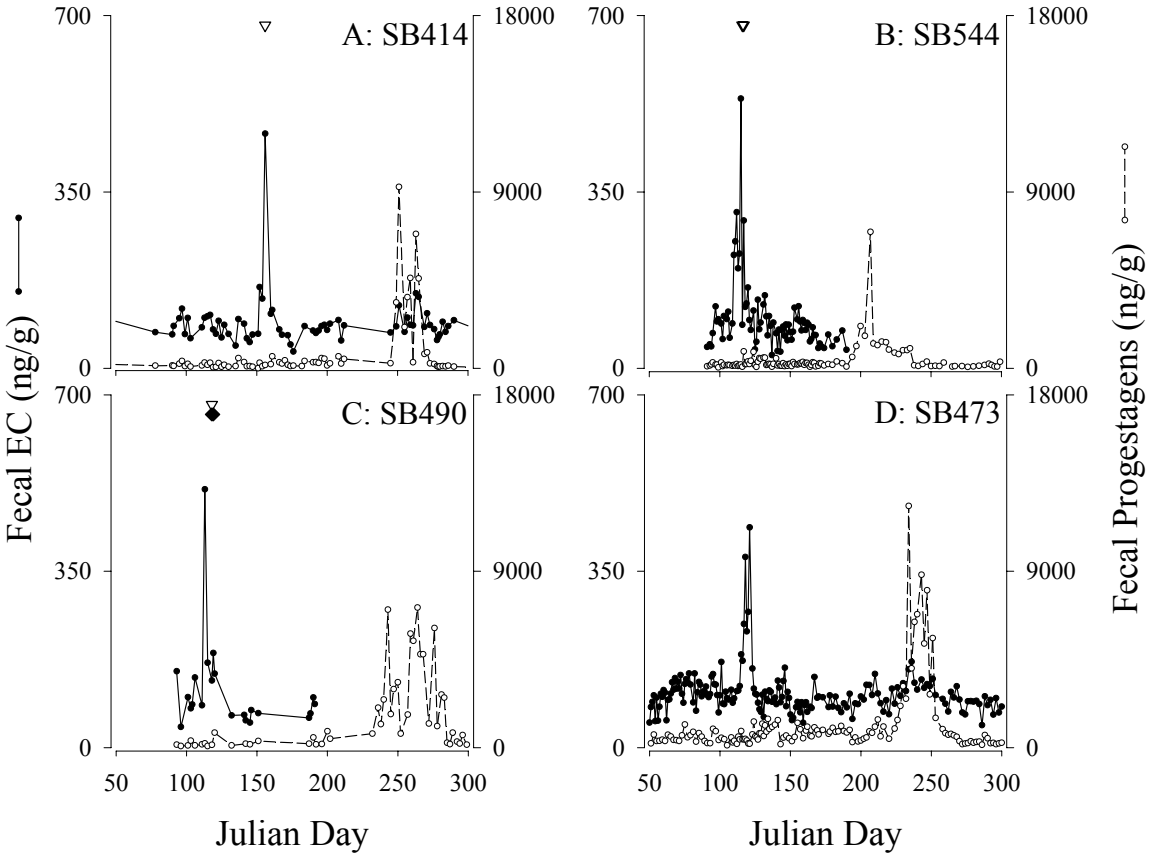

Figure 5. Representative profiles of fecal estrogen (closed circles, solid lines) and progestagen (open circles, dashed lines) metabolites of nonparturient females (A) SB414, (B) SB544. (C) SB490 and (D) SB473. Open triangles denote natural breeding and closed diamonds represent artificial insemination. All data are aligned to the Julian Day.

approximated 123 d. Progestagen concentrations (1,437.6 ± 384.5 ng/g; range, 149.7 – 6,952.5 ng/g) measured during a 49-d secondary rise were greater ($P < 0.05$) than during the 74-d primary rise (246.7 ± 21.3 ng/g; range, 60.8 – 856.7). Female SB490 (Fig. 5C) excreted peak EC concentrations on Day 113, followed by natural mating on Day 118 and artificial insemination (AI) on Days 118 and 119. The luteal phase concluded on Day 287, with a primary rise (342.2 ± 69.8 ng/g; range, 78.5 – 828.9 ng/mg) duration of 122 d and a secondary rise (3101.1 ± 485.4 ng/g; range, 173.0 – 7145.6 ng/g; $P < 0.05$) of 52 d. The pre-ovulatory EC peak for female SB473 (Fig. 5D) occurred on Day 123 and the total luteal phase duration was 137 d. The primary rise in fecal progestagens (777.9 ± 42.0 ng/g; range, 169.6 – 1456.1 ng/g) lasted 97 d, whereas the secondary rise (4,536.3 ± 821.2 ng/g; range, 940.8 – 12,326.5 ng/g; $P < 0.05$) lasted 40d. Comparisons of the primary rise in fecal progestagen concentrations from the three complete luteal profiles indicated no differences between females SB544 and SB 490 ($P > 0.05$), however fecal progestagens in SB473 were higher the other two females (both comparisons $P < 0.05$). No differences were measured in the secondary rise in progestagen concentrations between females SB490 and SB473 ($P > 0.05$), but SB544 excreted less progestagens compared to the other two females.

3.4. Fecal progestagens during the parturient luteal phase

Luteal progestagen profiles that ended in parturition for four females are shown in Figure 6. Female SB473 (Fig. 6A) was artificially inseminated once (Day 69) and gave birth after a 121-d pregnancy. Duration of the primary rise in luteal progestagens (657.8

± 38.4 ng/g; range, 276.1 – 1,279.2 ng/g) was 80 d, after which a marked increase ($P <$ 0.05) in fecal progestagens (3,988.7 ± 618.3 ng/g; range, 392.3 – 16,451.4 ng/g) began, ending with parturition 41 d later. Female SB487 (Fig. 6B) naturally mated on two consecutive days (Days 123, 124), was artificially inseminated on two additional consecutive days (Days 125, 126), and gave birth after a 121-d gestation. Mean fecal progestagens excretion during the 39-d secondary (1,567.4 ± 333.9 ng/g; range, 299.2 – 3,685.3 ng/g) was 8-fold ($P < 0.05$) higher than during the 82-d primary rise (194.6 ± 19.8 ng/g; range, 52.4 – 536.2 ng/g). For female SB414 (Fig. 6C), natural mating on Days 139 and 140 was followed a day later by artificial insemination, and parturition occurred after a 99-d gestation. Mean fecal progestagens excretion during the 36-d secondary (2,997.4 ± 1,634.3 ng/g; range, 173.0 – 16,894.5 ng/g) was 20-fold ($P < 0.05$) higher than during the 63-d primary rise (144.6 ± 16.4 ng/g; range, 40.8 – 556.0 ng/g). Female SB446 (Fig. 6D) was artificially inseminated a day after being naturally bred (Day 138) and gave birth after a 135-d gestation (Day 266). Mean fecal progestagens excretion during the 31-d secondary (1,744.2 ± 1,377.6 ng/g; range, 101.4 – 10,000.0 ng/g) was 9-fold ($P < 0.05$) higher than during the 104-d primary rise (148.2 ± 19.0 ng/g; range, 15.9 – 614.8 ng/g). The mean of the average secondary rise fecal progestagen concentrations for all females in the represented profiles (2,574.4 ± 568.8 ng/g; range, 1,567.4 – 3,988.7 ng/g) were approximately 9-foldgreater ($P < 0.05$) than the mean progestagen averages of the primary rise (286.3 ± 124.3 ng/g; range, 144.6 – 657.8 ng/g). Two females gave birth to twins (SB487, SB414), however fecal progestagen values (622.8 ± 182.3 ng/g; range, 40.8 – 16894.5 ng/g) were lower ($P < 0.05$) for those two

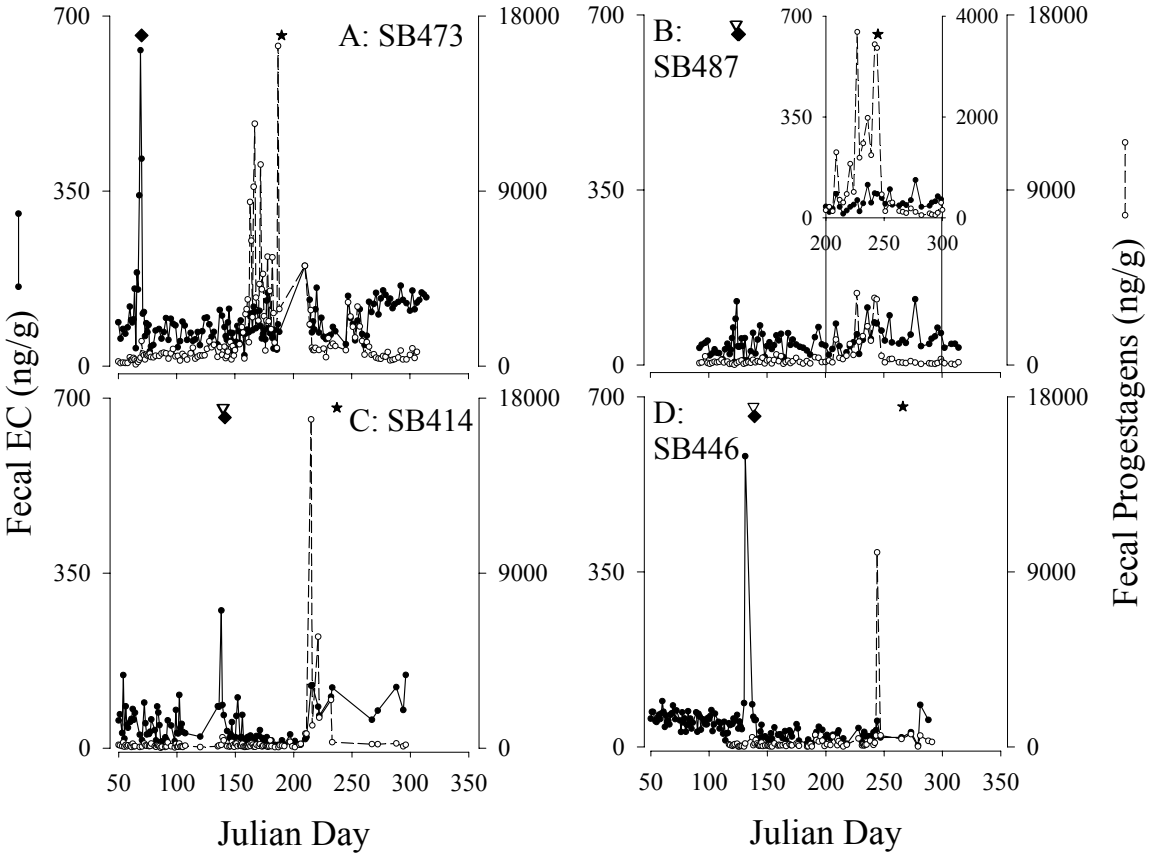

Figure 6. Representative profiles of fecal estrogen (closed circles, solid lines) and progestagen (open circles, dashed lines) metabolites of parturient females (A) SB473, (B) SB487. (C) SB414 and (D) SB446. Open triangles denote natural breeding, closed diamonds represent artificial insemination and closed stars denote parturition. All data are aligned to the Julian Day. Note fecal progestagen scale on inset graph of profile B.

individuals than they were for the two females (SB473, SB446; 1331.8 ± 208.8 ng/g; range, 15.9 – 16451.4 ng/g) that gave birth to a single cub.

Dates of mating or AI, gestation length, and parturition dates for the 8 parturient females for which behavioral and/or hormonal data were available were analyzed with respect to Julian Day (SB374, 2002, 2004; SB382, 2002; SB414, 2001, 2004; SB446, 2001, 2002; SB473, 2004; SB476, 2004; SB477, 2004; SB487, 2004). All mating and AI events occurred between Day 50 - 150 in all years (107.2 ± 9.1 d; range, 54 – 139 d). The mean primary rise in fecal progestagens lasted 80.5 ± 3.9 d (range, 61 – 104 d), whereas the secondary rise lasted 38.8 ± 3.0 d (range, 26 – 55 d). The mean luteal phase duration for parturient females was 121.5 ± 4.4 d (range, 99- 145 d), and mean parturition occurred on Day 228.7 ± 9.2 d (range, Day 191 – 273).

3.5. Comparison of fecal progestagens during parturient and nonparturient luteal phases

The dynamics of fecal progestagen excretion from the parturient and nonparturient females represented in Figures 5 and 6 were assessed to determine if differences existed between the two data sets. Fecal progestagen concentrations during the primary rise were greater ($P < 0.05$) in nonparturient (460.9 ± 28.0 ng/g; range, 60.8 - 1456.1 ng/g) than parturient (291.0 ± 20.9 ng/g; range, 15.9 – 1279.2 ng/g) females. However, no differences ($P > 0.05$) were detected in fecal progestagens during the secondary rise in parturient (3096.5 ± 455.3 ng/g; range, 101.4 – 16894.5 ng/g) and nonparturient females (3125.5 ± 339.4 ng/g; range, 149.7 – 12326.5 ng/g). The average increase in fecal progestagens from the primary to the secondary rise was not different (*P*

> 0.05) between parturient (11.7 ± 6.5-fold; range, 6.1 – 20.8-fold) and nonparturient (6.0 ± 2.5-fold; range, 2.9 – 9.1-fold) females. There were no differences in the length of the primary (parturient, 82.3 ± 8.4 d; range, 63 – 104 d; nonparturient, 95.5 ± 10.0 d; range, 74 – 122 d) and secondary (parturient, 36.8 ± 2.2 d; range, 31- 41 d; nonparturient, 42.3 ± 5.4 d; range, 28 – 52 d) intervals in parturient and nonparturient females.. The overall length of the luteal phase for parturient (119.0 ± 7.4 d; range, 99 – 135 d) and nonparturient (137.8 ± 12.8 d; range, 117 – 174 d) females were not different ($P > 0.05$).

Co-chromatographic profiles depicting fecal progestagens immunoreactivity after HPLC separation from two pregnant (SB414, 2001; SB446, 2001) and two pseudopregnant (SB473, 2002; SB452, 2003) females are presented in Figure 7. Correlation between the two data sets was significant ($r = 0.84$; $P < 0.05$). Large, broad immunoactive peaks (fractions 4 - 13) detected in both pregnant and pseudopregnant females co-eluted with pregnane-diol-glucuronide (PdG). Additionally, a broad band of immunoreactivity (fractions 63 – 80) in pregnant and pseudopregnant females co-eluted in the range of ^{3}H P4 (fraction 66). At least six additional unidentified immunoreactive peaks (fractions 20 – 60) were detected in both pregnant and pseudopregnant females.

3.6. Fecal progestagens in acyclic giant pandas

Representative profiles from females that exhibited an absence of the typical hormonal changes associated with follicular development or luteal activity are depicted in Figure 8. Acyclic fecal progestagen values (101.9 ± 4.5 ng/g; range, 4.6 – 1245.4 ng/g) were significantly lower ($P > 0.05$) than pre-luteal phase fecal progestagen concentrations

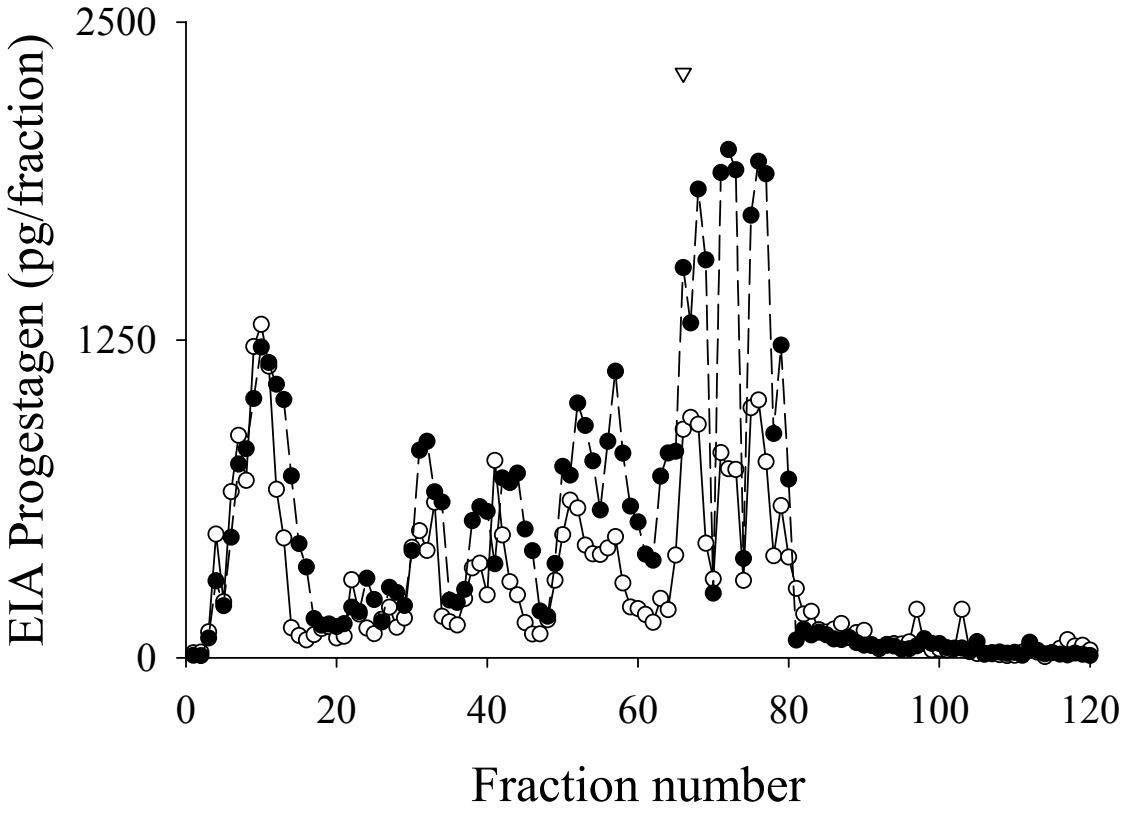

Figure 7. Co-chromatographic profiles depicting fecal progestagens immunoreactivity after HPLC separation from two pregnant (open circles, solid lines) and two pseudopregnant (closed squares, dashed lines) females. Triangle indicates peak elution of ^{3}H progesterone (4-Pregnen-3, 20-dione; fraction 66) that was added as a co-chromatographic marker to both fecal pools before HPLC analysis.

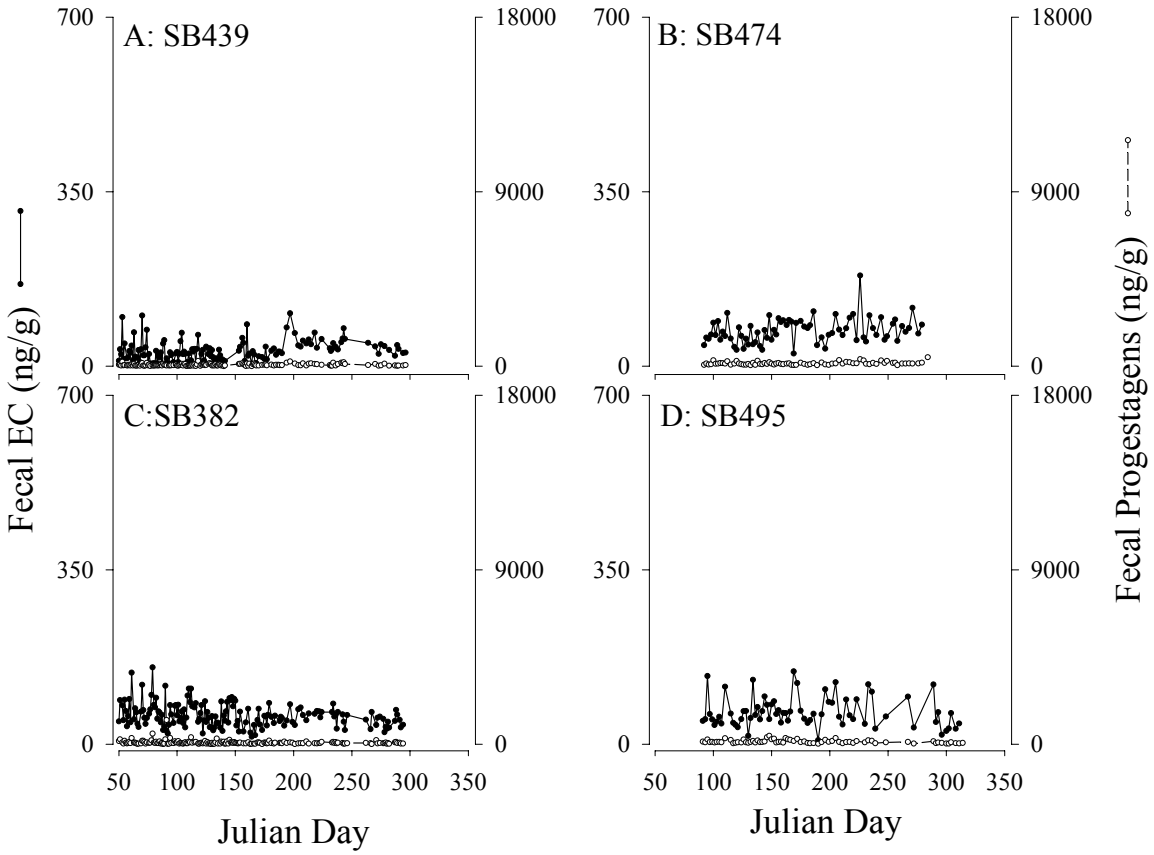

Figure 8. Representative profiles of fecal estrogen (closed circles, solid lines) and progestagen (open circles, dashed lines) metabolites of acyclic females (A) SB439, (B) SB474. (C) SB382 and (D) SB495. All data are aligned to the Julian Day. Note scales for fecal EC and progestagen concentrations and Julian Day are the same as those presented in Figures 5 and 6.

from the nonparturient (295.1 ± 21.2 ng/g; range, 43.6 – 1174.5 ng/g) and parturient (142.9 ± 10.5 ng/g; range, 7.7 – 446.8 ng/g) females. A total of eleven periods of acyclicity were documented by fecal hormone analyses. For all but one case the females were of reproductive age (5 - 12 y). Of the remaining ten periods of acyclicity, six females (including SB382, Fig. 8C) had given birth the previous year.

4. Discussion

We demonstrated, for the first time, that CL function in the female giant panda can be accurately monitored by noninvasive assessments of fecal progestagens, and we defined two distinct luteal phase intervals characterized by a primary and secondary rise in luteal progestagen excretion. The primary rise in fecal progestagens occurred immediately following estrus, even in animals that were not mated or artificially inseminated, which suggested that CL formation is obligate following ovulation in giant pandas. The shorter secondary rise in fecal progestagens was more consistent in duration than the primary rise, and analogous to what has been documented in other species that experience delayed implantation. These data are consistent with the biphasic trend in urinary progestagen metabolites excretion documented following estrus in giant pandas (Bonney et al., 1982; Hodges et al., 1984; Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Narushima et al., 2003; Dehnhard et al., 2006; Steinman et al., 2006). We confirmed that fecal progestagens were ineffective for distinguishing pregnancy status, but the hypothesis that this species experiences obligate pseudopregnancy and delayed implantation was strongly supported.

Pseudopregnancy traditionally describes females that fail to conceive (i.e., no gamete fertilization) but still exhibit the physiologic and behavioral changes normally associated pregnancy (Erskine, 1998). In 2002, female SB473 (represented in Fig. 4A) experienced her first estrus, was not bred or artificially inseminated, had no physical contact with a male, but still exhibited a typical biphasic fecal progestagen excretion profile. Although this particular case represented a true pseudopregnancy, all giant panda females that ovulated exhibit identical steroid excretory patterns, regardless of pregnancy status. Historically, cyclic female giant pandas that did not give birth have been classified as pseudopregnant, but we suspect that this underestimates the incidence of failed pregnancies or embryonic loss (Steinman et al., 2006). This suspicion is supported by one report (Sutherland-Smith et al., 2004) that documented two fetuses via ultrasound in a pregnant giant panda that gave birth to a single offspring. In the European badger, another species that experiences delayed implantation, pre-implantation embryonic loss and post-implantation fetal loss are well documented (Cresswell et al., 1992; Yamaguchi et al., 2006). More work is clearly needed to establish the incidence of pregnancy loss in giant pandas, but until reliable methods for pregnancy diagnosis are developed for this species, we suggest that females that fail to give birth following ovulation, breeding and/or AI be designated nonparturient rather than pseudopregnant.

Fecal progestagen increased immediately following the EC peak, which confirmed ovulation in all nonparturient and parturient females. The duration of the initial increase in progestagen excretion (63 – 122 d) was more variable than that of the secondary rise (28 – 52 d) for all cyclic females. This trend was very similar to what has been reported in other Carnivora that experience delayed implantation, including the

mink (Allais and Martinet, 1978; Papke et al., 1980), European badger (Canivenc and Bonnin, 1981; Yamaguchi et al., 2006), ferret (Enders, 1952) and spotted skunk (Mead, 1981), where progestagens were initially increased after ovulation, but progestagen concentrations were lower and more variable than during the secondary rise interval. Furthermore, the shorter secondary rise interval in these species corresponded with post implantation fetal development (Enders, 1952; Allais and Martinet, 1978; Foresman and Mead, 1978; Papke et al., 1980; Canivenc and Bonnin, 1981; Mead, 1981; Yamaguchi et al., 2006). In the American black bear, morphological studies revealed that corpora lutea form rapidly after ovulation and change very little until the implantation of the conceptus into the uterine wall (Wimsatt, 1963). We speculate that the presence of a conceptus has little impact on CL function, which explains the absence of luteal phase differences in parturient and nonparturient giant pandas. And while our endocrine data lends considerable credence to the theory that obligate delayed implantation occurs in the giant panda, more studies, including the use of serial ultrasonography in parturient giant pandas, are required for confirmation.

The gestation length derived from this study (121.5 ± 4.4 d; range, 99 - 145 d) is within the 85 to 185 d previously reported (Zhu et al., 2001). Our systematic efforts to document the excretory dynamics of the luteal phase, including during the primary and secondary rise intervals, revealed that there were no substantive differences between parturient and nonparturient females. Within the Order Carnivora, progestagen production in parturient and nonparturient females are indistinguishable in the dog (*Canis lupus;* Smith and McDonald, 1974; Concannon et al., 1975),red wolf (*Canis rufus;* Walker et al., 2002), maned wolf (*Chrysocyon brachyurus;* Velloso et al., 1998;

Songsasen et al., 2006), bush dog (*Speothos venaticus;* DeMatteo et al., 2006), wolverine (*Gulo gulo;* Mead et al., 1993), black-footed ferret (*Mustela nigripes;* Brown, 1997), and dwarf mongoose (*Helogale parvula*; Creel et al., 1991). In addition to exhibiting indistinguishable luteal progestagen excretion patterns, there were no differences in the milieu of immunoreactive progestagen metabolites detected following co-chromatographic HPLC analysis. Collectively, our data support the hypothesis that the lifespan and function of the CL during parturient and nonparturient cycles are indistinguishable and that the giant panda experiences pseudopregnancy.

Weak estrus has been reported to be a commonly observed phenomenon that is an impediment to the captive breeding success of giant pandas (Shuling et al., 1997). In dairy cattle, weak estrus (or silent ovulation) occurs when a female ovulates but does not display overt estrual behaviors (Allrich, 1994). In the present study, hormonal evaluations suggested that all females that had been classified as having exhibited weak estrus were non-ovulatory. While it is possible that increased fecal EC excretion during a presumptive follicular phase was missed due to infrequent sampling regimens, our conclusion is supported by the absence of concomitantly increased fecal progestagen excretion, which would have confirmed that ovulation occurred. These data indicate that many females classified as having experience weak estrus were actually anestrual, which suggests that the incidence of this phenomenon may be underestimated in giant pandas.

After pregnancy in female SB473 (Fig. 6A), she did not cycle the following breeding season while nursing her cub. Lactational anestrus is common in other Ursidae, including the brown bear (Hensel et al., 1969; Craighead et al., 1976), polar bear (*Ursus martimus;* Ramsay and Stirling, 1988), and American black bears (Wimsatt, 1963;

Johnston et al., 1994), and typically results in alternate year cubing. Although six of the ten adult females that experienced anestrus had given birth the previous year, cubs in China are typically pulled from the dam before the next breeding season in an attempt to achieve annual cub production. In cases of infanticide, female brown bears experienced estrus in the first breeding season after parturition (Bellemian et al., 2006), which suggested that recovery from the lactational block is rapid. While it is assumed that giant pandas recover quickly from lactational suppression, noninvasive endocrine monitoring could be used to document the latency to ovulation following cessation of nursing.

Overall, we have demonstrated the usefulness of monitoring fecal progestagens for assessing CL function in captive giant pandas, but these methods have great promise for establishing reproductive-endocrine norms in free-living giant pandas. Periodic fecal collection (as little as 2 – 3 samples/wk) from known individuals would be sufficient to provide significant insight into luteal activity of wild pandas, and when combined with assessments of fecal glucocorticoid analyses, these methods may provide important objective information about reproduction and well-being in wild populations of giant pandas.

CHAPTER 4

Non-invasive endocrine measures of gonadal and adrenal function in the male giant panda (*Ailuropoda melanoleuca*)

Abstract

The purpose of this study was to establish fecal androgen and glucocorticoid (GC) excretion as a valid measure of adrenal and gonadal function in the male giant panda. The physiological validity of fecal GC was demonstrated by a 15-fold (2,133.6 ng/g) increase in hormone excretion (baseline, 143.6 ± 15.7 ng/g) 10 h after the exogenous administration of a pharmacological dose (2 IU/kg, i.m.) of andreocorticotropic hormone (ACTH). Fecal GC concentrations peaked (3,479.6 ng/g) 12 h post-ACTH administration and declined to baseline 8 h later (20 h post-ACTH). Matched urinary and fecal androgen ($r = 0.61$) and GC ($r = 0.53$) were strongly correlated with one another in single male that was assessed over a 2-year interval. Longitudinal fecal androgen and GC excretory profiles in male giant pandas housed at North American and Chinese facilities revealed similar excretory profiles. In general, fecal androgens increased ($P > 0.05$) 2.3-fold (252.9 ± 15.9 ng/g; range, 205.5 – 294.1 ng/g; $P < 0.05$) above baseline concentrations (112.0 ± 12.6 ng/g; range, 78.8 – 156.3 ng/g) coincident with the onset of

the 5-month annual breeding season. In all males (n = 5), androgen metabolite concentrations declined and were basal by the end of the breeding season (June). Fecal GC measures generally tracked androgen excretion patterns (r = 0.53 – 0.76), with mean peak GC excretion (362.6 ± 23.1 ng/g; 302.5 – 431.5 ng/g) representing a 2.1 fold increase ($P < 0.05$) over baseline concentrations (173.8 ± 24.1 ng/g; range, 122.3 – 233.3 ng/g). Fecal androgen and GC in a single male tracked during the transition from subadult (3 years of age) to sexual maturity (6 years of age) were excreted in parallel. In this male basal fecal androgen values were positively correlated with age (r = 0.93; $P < 0.05$) and increased 88% ($P < 0.05$) from age 5 (70.4 ± 23 ng/g) to 6 (132.8 ± 5.0 ng/g) years. Similarly, baseline fecal GC concentrations were positively correlated with age (r = 0.82; $P < 0.05$) and increased 66% ($P < 0.05$) form age 5 (139.0 ± 4.7 ng/g) to 6 (231.3 ± 7.1 ng/g) years. Collectively, these data demonstrate that validity of non-invasive methods for quantifying gonadal and adrenal steroid metabolites in the feces of the male giant panda. These methods represent an effective and efficient means for studying the biology and well-being of this unique endangered species.

1. Introduction

The breeding season of the giant panda (*Ailuropoda melanoleuca*) generally occurs sometime between February and June, with peak breeding activities in April (see review, Steinman et al., 2006). In the wild, female giant pandas have been observed mating with more than one male during behavioral estrus, often with males fighting one another for breeding access to the female (Schaller et al., 1985; Zhu et al., 2001). Under

managed conditions, male giant pandas are capable of breeding and fertilizing multiple females during a single breeding season (Xie and Gipps, 2007). Increased urinary androgen (Bonney et al., 1982; Snyder et al., 2004; MacDonald et al., 2006) and glucocorticoid (GC) excretion (Owen et al., 2005; MacDonald et al., 2006) in males have corresponded with known reproductive intervals. Although urine allows for noninvasive assessment of gonadal and adrenal function, there are limitations to this approach. For example, giant pandas are often housed in large enclosures on soil substrate, making consistent urine collection difficult. Additionally, urinary monitoring of reproductive and adrenal activities in free-ranging giant pandas would pose significant challenges, and in most cases would be impractical. Fecal hormone monitoring techniques have tremendous potential for overcoming these obstacles, and for facilitating further research on giant panda reproduction and well-being under *ex situ* and *in situ* conditions.

Fecal steroid monitoring is being used to evaluate reproductive status in female giant pandas and has been effective in tracking reproductive activity in other Ursidae, including the spectacled (*Tremarctos ornatus*; Dehnhard et al., 2006), sloth (*Melursus ursinus*; Young et al., 2004), sun (*Helarctos malayanus*; Onuma et al., 2002; Schwarzenberger et al., 2004; Hesterman et al., 2005), brown (*Ursus arctos*; Ishikawa et al., 2002; Ishikawa et al., 2003; von der Ohe et al., 2004; Dehnhard et al., 2006), and Asiatic black (*Ursus thibetanus*; Young et al., 2004) bears. Although urine has been useful in facilitating our understanding of basic adrenal and testis function in the male giant panda, fecal collection is a viable alternative to blood and urine collection that permits simplified, long-term monitoring of hormonal changes (Monfort, 2003). It is estimated that only 58% of the captive adult giant panda population successfully breed

through natural mating (Zhang et al., 2006). Additional reproductive-endocrine work is needed to help elucidate the reasons for this poor reproductive success in the *ex situ* population.

Modern zoo management practices are designed to ensure animal well-being and minimize exposure to stressors. However, several studies have demonstrated that some zoo-maintained species and/or individuals may experience stress with the potential for adversely impacting normal physiology and behaviors (see review, Carlstead, 1996). Acute stress, marked by short-term activation of the adrenal gland, can be beneficial for survival, but chronic adrenal stimulation can induce a variety of deleterious physiological and behavioral effects (see review, Pottinger, 1999). Considering the widely variable reproductive fitness of captive male giant pandas (Howard et al., 2006), studies on the potential that stress may play in poor reproductive performance are warranted. Seasonal fluctuations in urinary GC excretion have previously been described in the giant panda (Owen et al., 2005; MacDonald et al., 2006), as have responses to noise stressors (Owen et al., 2004; Powell et al., 2006), but fecal GC metabolites have not been validated for assessing adrenal activity in giant pandas. Adrenal challenges with the purpose of artificially stimulating the adrenal cortices, are a common approach for demonstrating physiological validity of using excreted GC measures as an index of adrenal function (Monfort et al., 1998; Goymann et al., 1999; Wasser et al., 2000; Young et al., 2001; Touma and Palme, 2005). The general objective of this study was to validate fecal measures for assessing reproductive status and well-being in male giant pandas. More specifically, we sought to: (1) determine excretory route and time course of GC excretion following exogenous ACTH administration; (2) compare two approaches for extracting

fecal hormone metabolites; (3) validate enzyme immunoassays (EIA) for quantifying fecal androgen and GC metabolites; (4) examine correspondence between urinary and fecal androgen and GC measures; and (5) evaluate androgen and GC metabolites excretion within and among males at multiple *ex situ* facilities, as well as pubertal development in a single male.

2. Methods

2.1. Study animals and facilities

2.1.1. North America

Smithsonian's National Zoological Park (SNZP; 39˚N, 77˚W) managed one male SB458 (DOB 8/27/97) and Zoo Atlanta (ZA; 33˚N, 84˚W) housed male SB461 (DOB 9/9/97) throughout the course of this study (2001 – 2005). Water was provided to the animals *ad libitum* and the pandas were fed a freshly cut bamboo diet (>75%) that was supplemented with fruit and a diet biscuit that was high in fiber, vitamins, minerals and amino acids. Each animal was housed in an enclosure that consisted of an indoor (50 - 100 m^2) and outdoor (100 – 300 m^2) area. Although managed within auditory, olfactory and visual proximity of a female conspecific, physical contact between males and females were primarily limited to periods of behavioral estrus, with only the SNZP allowed intermittent contact throughout the year.

2.1.2. China

The China Conservation and Research Center for the Giant Panda at the Wolong Nature Reserve (Wolong; 31˚N, 103˚E) houses 25 adult male giant pandas, of which three (SB308, SB394, SB399) reproductively active, healthy adult individuals (age range, 8 – 17 y) were used for the present study. Individuals were fed a diet that primarily consisted of freshly cut bamboo supplemented with a high-fiber biscuit, with water available *ad libitum*. Indoor (30 – 60 m^2) and outdoor (100 – 300 m^2) areas were part of each enclosure. Physical interaction with estrual females was permitted during the breeding season (February – June) to allow for mating opportunities, however outside of this period males were housed individually and limited to auditory, visual and olfactory contact with females.

2.2. Sample collection and processing

2.2.1. Urine

Urine samples were collected fresh in the morning three to seven days a week and stored frozen (-20˚C) in capped, plastic specimen tubes (12 x 75mm) until analysis. All collected urine samples were free from contact with feces and standing water, to avoid cross contamination and dilution. Urinary hormone concentrations were indexed with creatinine (Cr; Taussky, 1954) to account for variations in water excretion and expressed as hormone mass per mg of Cr (ng/mg Cr). In brief, urine samples were diluted (1:10 in BSA-free phosphate buffer) and added (0.05ml) to a microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) along with Cr standard (0.00625 to 0.1 mg/ml, Sigma; St. Louis, MO) in duplicate. To each standard and sample, dH_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(25ºC) for 30 min prior to optical density (OD; reading filter 490 nm, reference 620 nm) assessment on a microplate spectrophotometer (Dynex MRX; Dynex Technologies, Chantilly, VA). Urine samples that were too dilute (< 0.1 mg Cr/ml) were not included in additional hormone analyses, and resulted in the exclusion of ~10% of urine samples.

2.2.2. Feces

Freshly voided feces (< 1 h post-excretion) were collected each morning (1 – 7 d/wk) and stored frozen (-20˚C) in sealable plastic bags until processing. Prior to hormone extraction, fecal samples were free dried (Lyophilizer, Labconco, Kansas City, MO), crushed to a powder, and extracted using previously described methods (Chapter 2). A subset of fecal samples collected during the ACTH challenge trial (see below) were used to compare hormone profiles generated from the extraction of undried (0.5 g feces) versus dried (0.1 g feces) feces. The extraction procedure followed methods previously described previously (Brown et al., 1994; Wasser et al., 1994; Monfort et al.1997) and fecal extractants were reconstituted in phosphate buffer (1 ml) and then stored frozen (-20˚C) until analysis.

2.2.3. ACTH challenge

Exogenous adrenocorticotropic hormone (2 IU/kg synthetic ACTH; Cortrosyn, Wedgewood Pharmacy, Swedesboro, NJ) was administered to SB458 by intramuscular injection at 0800 h on October 1, 2001. To minimize handling stress, SB458 was pre-

conditioned to cooperatively participate in examinations that included blood draws and injections. Blood was collected 1 h before and 1 h after adrenal stimulation. Urinary and fecal samples were collected daily for 3-d before ACTH administration and to establish baseline pre-treatment GC concentrations. An additional 17 fecal samples were collected throughout the 24-h period following ACTH administration and urines were collected at 13.8 h and 23.3 h post-ACTH.

2.3. Endocrine analyses

2.3.1. Cortisol EIA

A single antibody Cortisol EIA (Munro and Lasley, 1988; Young et al., 2001) was used to analyze GC metabolite concentrations in serum, urine and feces. Microtiter plates (96 well, Nunc-Immuno, Maxisorp; Fisher Scientific, PA) were allowed to adsorb cortisol antibody (R4866, C. Munro, University of California, Davis, CA) for 12 to 18 h (4°C) before the addition of 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, MO). Plates were incubated at room temperature (25°C; 1 h) after the addition (0.05 ml) of an enzyme-linked F (C. Munro, University of California, Davis, CA). Following incubation, unbound components were removed with wash solution and a chromagen solution (ABTS in citrate buffer; Sigma Chemical Co., St. Louis, MO) was added (0.1 ml) to all wells. When optimal OD was reached (1.00 OD), the resultant color change was quantified on a microtiter plate reader (Dynex MRX; Dynex Tehnologies, VA). Interassay CV for two

internal controls (n = 108 assays) were 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 EIA. Significant recoveries ($P < 0.05$) were demonstrated when unlabeled standard was added to urine (r = 0.99; y = 1.15x - 4.2) and fecal extracts (r = 1.00; y = 0.90x - 3.38).

2.3.2. Androgen EIA

Androgen metabolite concentrations in diluted urine and fecal extracts were quantified with a single antibody testosterone (T) EIA (Munro and Lasley, 1988; Dloniak et al., 2004). Polyclonal anti-T (R156/7; C. Munro, University of California, Davis, CA) was diluted (1:7500), added (0.05 ml) to microtiter plates (96 well, Nunc-Immuno, Maxisorp; Fisher Scientific, PA) and allowed to set (12 to 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; Sterloids, RI), unadsorbed antiserum was removed with wash solution. Enzyme conjugated T (0.05 ml; C. Munro, University of California, Davis, CA) was then added to each well containing standard or sample and incubated (2 h; 25°C) before unbound components were removed. A chromagen solution was then added (0.1 ml) to each well and was allowed to incubate (~ 30 min) before optimal densities were determined (maximum binding = 1.00 OD; reading filter 405 nm; reference filter 540 nm) on a microtiter plate reader (Dynex MRX; Dynex Tehnologies). Interassay coefficients of variation (CV) for two 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 = 1.00; y = 1.25x - 3.85) and fecal extracts (r = 0.99; y = 0.94x - 0.01) to unaltered standards demonstrated significant recovery ($P < 0.05$).

2.3.3. High pressure liquid chromatography (HPLC)

Fecal androgen and GC metabolites were analyzed by reverse phase HPLC (Varian ProStar; Varian Analytical Instruments, MA) with polarity gradients as previously described (Staley et al., 2007). For androgen metabolite identification, fecal extracts from six samples containing elevated androgen concentrations were pooled, evaporated to dryness, reconstituted in 0.3 ml of phosphate-buffered saline (PBS; 0.01 M $NaPO_4$, 0.14 NaCl, 0.5 % BSA, 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 co-chromatographic markers. The pooled sample (0.3 ml PBS) for GC metabolite separation was obtained from the combination of six 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 radio-labeled markers. Retention times of radioactive markers were determined by combining 0.1 ml of each HPLC fraction with 3 ml scintillation cocktail and radioactivity quantified on a radioactive beta counter (Beckman Instruments Inc., Fullerton, CA). Residual fractions were dried under air, resuspended in 0.3 ml assay buffer, assayed, and immunoactive peaks were compared to the retention times of radiolabelled markers.

2.4. Statistical analysis

Baseline fecal and urinary 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 two standard deviations (SD) were removed until no values exceeded mean + 2SD. 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. Associations between and among data sets were assessed using Pearson's correlation (parametric) or Spearman's correlation (nonparametric) tests. Comparisons between data sets were conducted for both baseline mean and overall mean (all sample hormone concentrations in the collection period) values. Parametric data were evaluated using a Student's *t*-test or one-way analysis of variance (ANOVA). Nonparametric data sets were evaluated using the Mann-Whitney test or Kruskal-Wallis one-way ANOVA. Baseline iterations were conducted using Microsoft Excel 2007 (Microsoft, Inc., Seattle, WA) and all other statistical tests were conducted using SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA).

3. Results

3.1. ACTH Challenge

Serum, urine and fecal GC concentrations in SB458 before and after ACTH administration are depicted Figure 9. Serum GC concentrations increased 11-fold (12.8 ng/ml) over baseline concentrations (1.1 ng/ml) within 1 h post-ACTH administration. The first post-ACTH fecal sample (~10 h post-ACTH) was increased 14.9-fold (2,133.6 ng/g) above pre-ACTH GC concentrations (143.6 ± 15.7 ng/g; range, 100.6 – 216.1 ng/g). Peak fecal GCs (3,479.6 ng/g) were detected ~12 h post-ACTH, remained elevated for an additional 7 h before declining to baseline concentrations 20 h post-ACTH administration. GC concentrations derived from the samples collected within 1 d (< 24 h) of the ACTH challenge (1580.1 ± 294.6 ng/g; range, 148.9 – 3479.6 ng/g) were higher than pre-ACTH GC values. Fecal GCs in dried and undried samples paralleled one another ($r = 0.98$; $P < 0.05$) but hormone concentrations in dried samples were approximately 6.5-fold higher. Only two urine samples were collected in the 24 h post ACTH administration; the first (13.8 h post-ACTH) represented a 5.6-fold (711.1 ng/mg Cr) increase above pre-ACTH concentrations (128.7 ± 11.9 ng/mg Cr; range, 91.4 – 176.3 ng/mg Cr). The second sample was collected 23 h after ACTH administration, had a value (56.1 ng/mg Cr) that was within the range of pre-ACTH urinary GC concentrations.

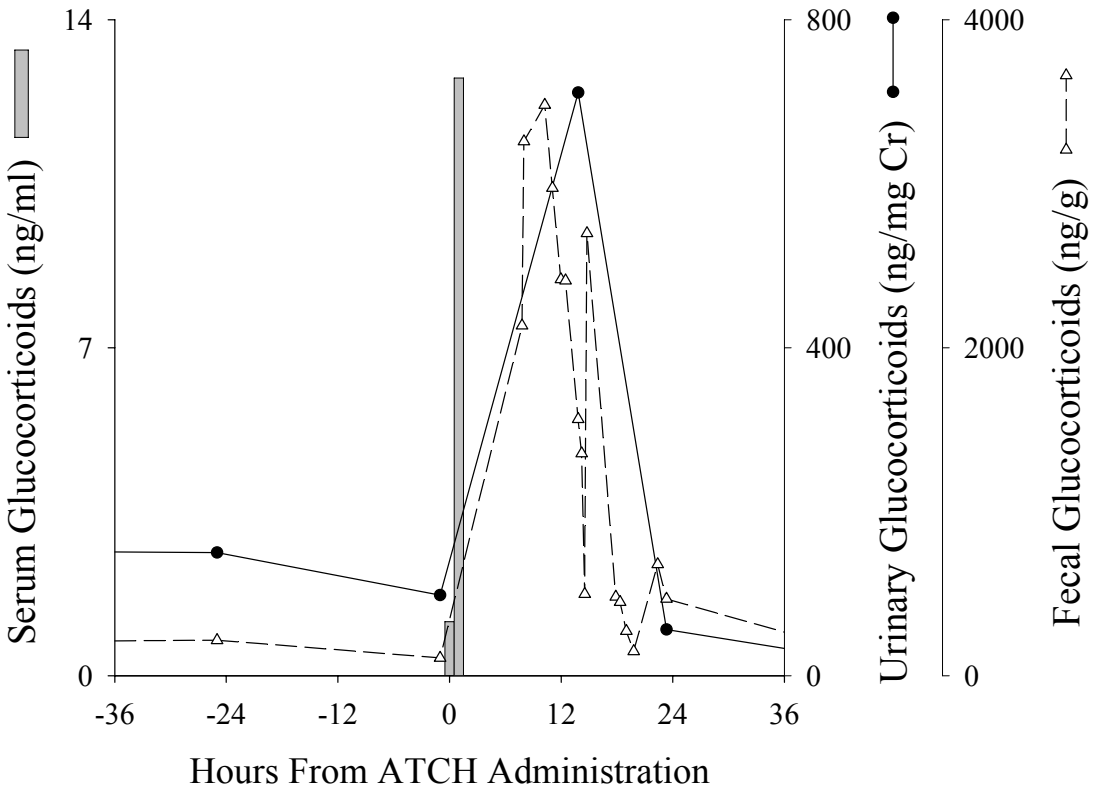

Figure 9. Serum (Grey bars), urinary (closed circles, solid line) and fecal (open triangles, dashed line) GC concentrations before and after exogenous ACTH administration in SB458. Data are aligned to the hour time of ACTH administration.

3.2. HPLC

Immunoactivity from fecal androgen peaks quantified following HPLC separation co-eluted with ^{3}H-testosterone and ^{3}H-androstenedione at fractions 33 and 43, constituting 11.0% and 2.9% of total immunoactivity, respectively. An additional five, unidentified immunoactive peaks were detected by androgen EIA. The largest unidentified immunoactive peak (fractions 9 – 13) represented 38.0% of total immunoactivity, with the additional peaks occurring at fractions 15 (10.8%), 19 (7.4%), 25 (15.6%) and 56 (2.2%).

Five distinct immunoactive peaks were identified by cortisol EIA following HPLC. The largest immunoactive peak (fractions 46 – 52; 52.2% total immunoactivity) co-eluted with ^{3}H corticosterone (fraction 48). The second most prominent immunoactive fraction (fraction 42; 12.8%) co-eluted with ^{3}H cortisol, while the additional three unidentified immunoactive peaks occurred at fractions 18, 39 and 57, representing 6.5%, 5.8% and 4.1% of total immunoactivity, respectively. No immunoactivity co-eluted with ^{3}H desoxycorticosterone (fraction 53).

3.3. Matched urinary and fecal endocrine measures

Matched urinary and fecal androgen and GC excretory patterns are presented in Figure 10. Androgen measures between urine and feces (SB458) revealed similar temporal excretory patterns (Fig. 10A; $r = 0.61$; $P < 0.05$), as were urinary and fecal GC values (Fig.10B; $r = 0.53$; $P < 0.05$). Additionally, there was good correspondence

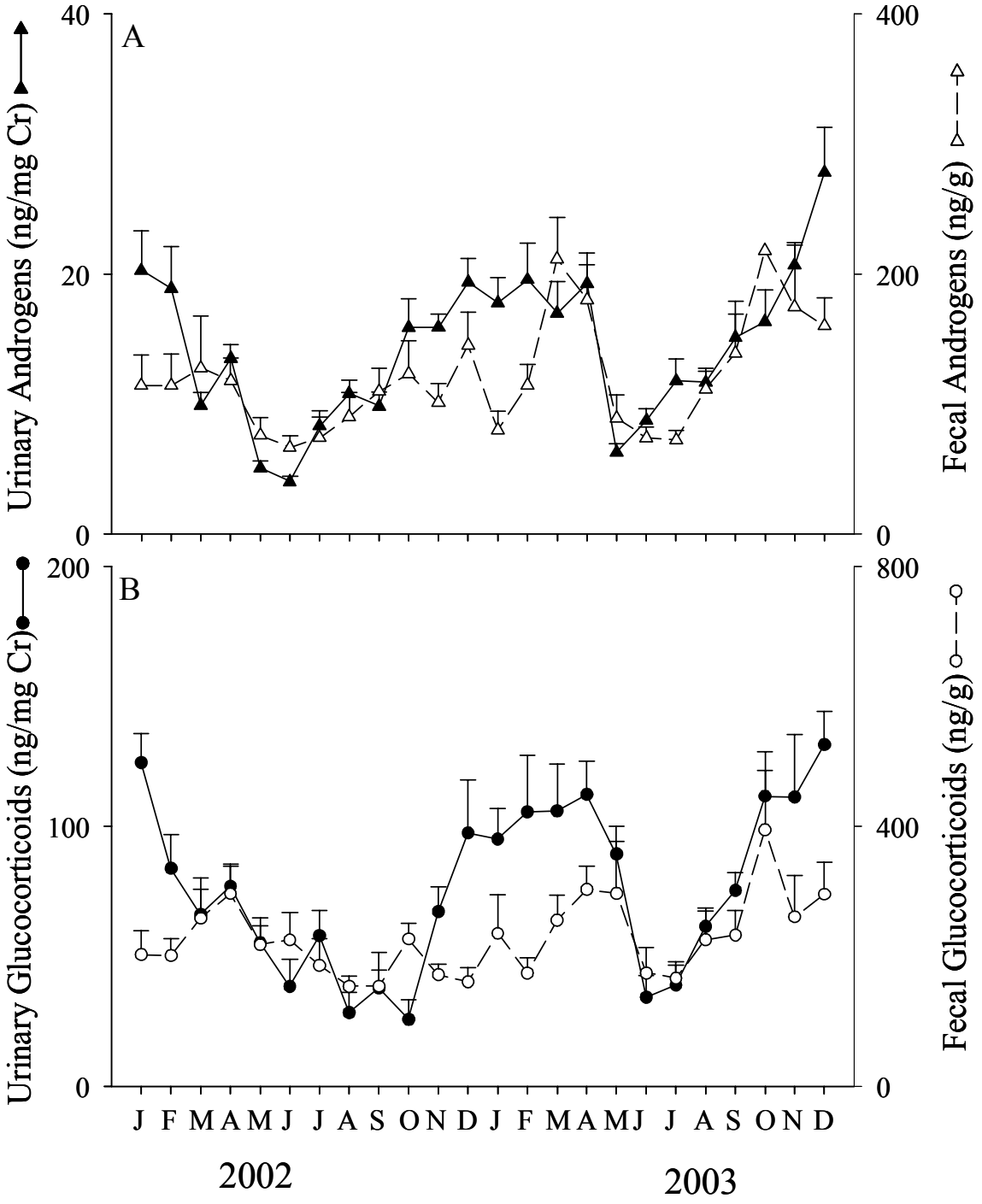

Figure 10. Matched monthly (± SE) urinary (closed triangles, solid line) and fecal (open triangles, dashed line) androgen metabolites (A) and monthly (± SE) urinary (closed circles, solid line) and fecal (open circles, dashed line) GC metabolites (B) for 24 months. Data are aligned to the month of the year.

between androgen and GC excretion in the urine ($r = 0.74$; $P < 0.05$) and feces ($r = 0.62$; $P < 0.05$).

3.4. North American male seasonality

Androgen and GC metabolite concentrations were quantified over two successive years (2004 – 2005) in two adult (SB458, SB461) males housed in North American facilities. Because there were no within-male differences in annual androgen and GC concentrations among years (all tests $P > 0.05$), hormone data for both years were combined and averaged by month for each individual (± SE; Fig. 11). For SB458, temporal trends in fecal androgen and GC excretion were similar ($r = 0.74$; $P < 0.05$) Fecal androgens were elevated ($P < 0.05$) above baseline (156.3 ± 4.1 ng/g) during February (243.5 ± 29.6 ng/g), March (260.2 ± 25.9 ng/g), September (229.8 ± 30.9 ng/g), October (256.5 ± 21.9 ng/g), November (255.6 ± 26.4 ng/g) and December (298.9 ± 35.6 ng/g). The seasonal trend in the fecal GC excretion was less pronounced, and GC concentrations only exceeded ($P < 0.05$) baseline concentrations (233.3 ± 5.1 ng/g) during October (403.8 ± 31.2 ng/g) and December (459.1 ± 96.8 ng/g). Likewise, temporal trends in fecal androgen and GC excretion were similar ($r = 0.61$; $P < 0.05$) in SB461 (Fig. 11B). However, fecal androgen concentrations were elevated ($P < 0.05$) above baseline (111.8 ± 2.4 ng/g) in February (184.9 ± 15.2 ng/g) and March (226.1 ± 14.0 ng/g). Fecal GC concentrations only exceeded ($P < 0.05$) baseline (159.4 ± 2.8 ng/g) in March (326.5 ± 40.8 ng/g).

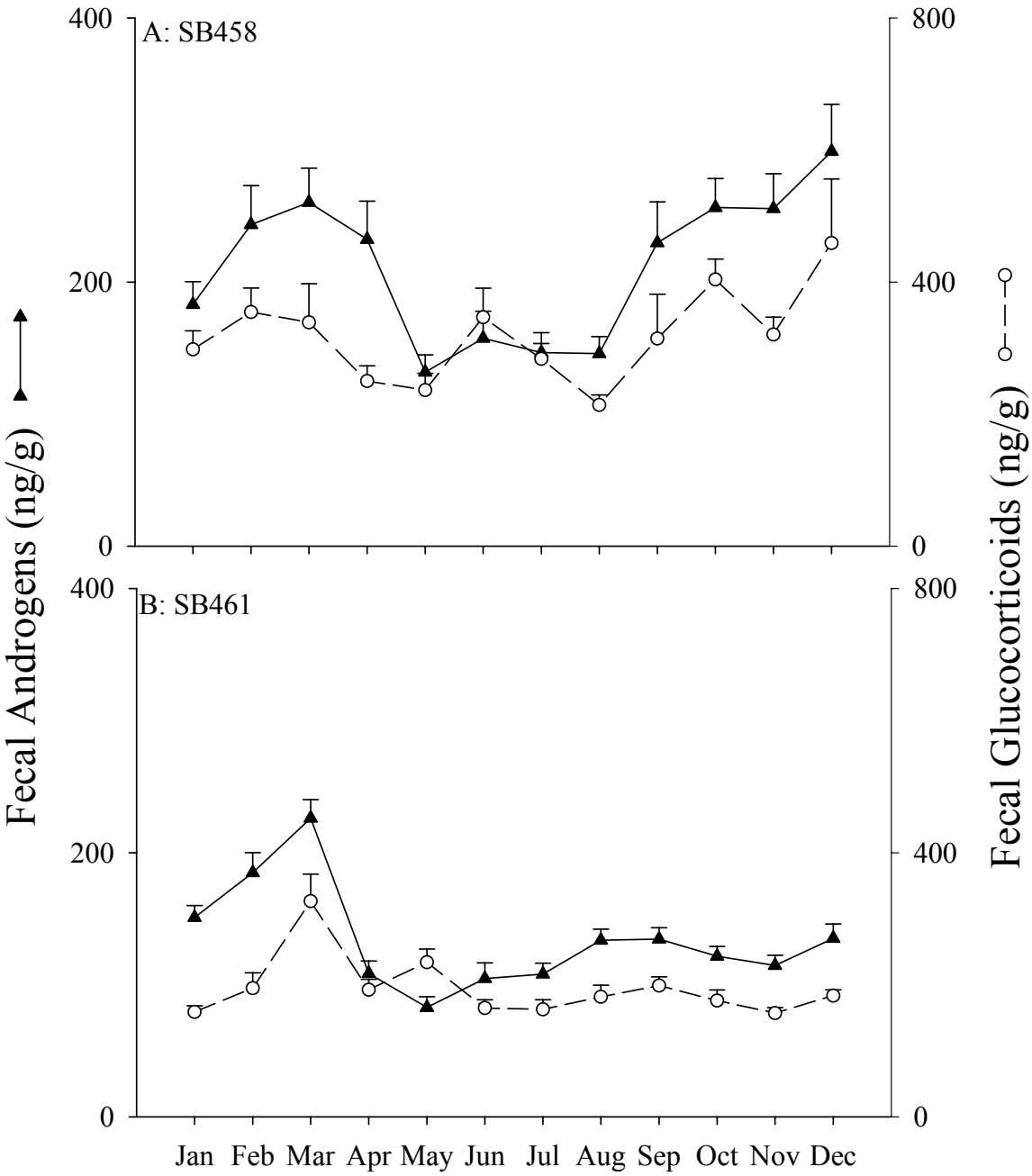

Figure 11. Seasonal trends in fecal androgen (closed triangles, solid line) and GC (open circles, dashed line) excretion averaged by month (± SE) over a 2-year interval (2004, 2005) in SB458 (A) and SB461 (B). Data are aligned by the month of the year.

Between-male differences ($P < 0.05$) in overall androgen (SB458, 209.1 ± 7.3 ng/g; SB461, 133.6 ± 3.5 ng/g) and GC (SB458, 314.4 ± 12.6 ng/g; SB461, 194.4 ± 5.8 ng/g) excretion concentrations were evident, whereby SB458 excreted approximately 1.6-fold more androgen and GC than SB461. Additionally, baseline androgen and GC values from SB458 were greater ($P < 0.05$) than basal androgen and GC measures from male SB461.

3.5. Wolong male seasonality

Fecal androgen and GC metabolite profiles of three representative males from Wolong are presented in Figure 12. Androgen and GC measures for male SB308 (Fig. 12A) were significantly correlated ($r = 0.76$; $P < 0.05$) throughout the collection period. Androgen concentrations were significantly elevated ($P < 0.05$) above baseline (78.8 ± 2.9 ng/g) during February (294.1 ± 87.9 ng/g), however GC values exceeded ($P < 0.05$) baseline (122.3 ± 4.3 ng/g) throughout the breeding season (February, 440.5 ± 86.0 ng/g; March, 341.6 ± 72.1 ng/g; April, 295.2 ± 68.9 ng/g; May, 187.9 ± 35.8 ng/g; June, 247.5 ± 42.0 ng/g). The correlation between androgen and GC excretory patterns for male SB 394 (Fig. 12B) was positive ($r = 0.53$) but not significant ($P > 0.05$). Fecal androgen metabolites were greater than ($P < 0.05$) basal values (112.8 ± 7.5 ng/g) for the first two months of the breeding season (February, 209.4 ± 17.9 ng/g; March, 256.7 ± 36.8 ng/g), and two months during autumn (September, 238.6 ± 41.2 ng/g; November, 207.1 ± 41.3 ng/g). Fecal GCs were elevated above ($P < 0.05$) baseline (228.0 ± 8.3 ng/g) for the

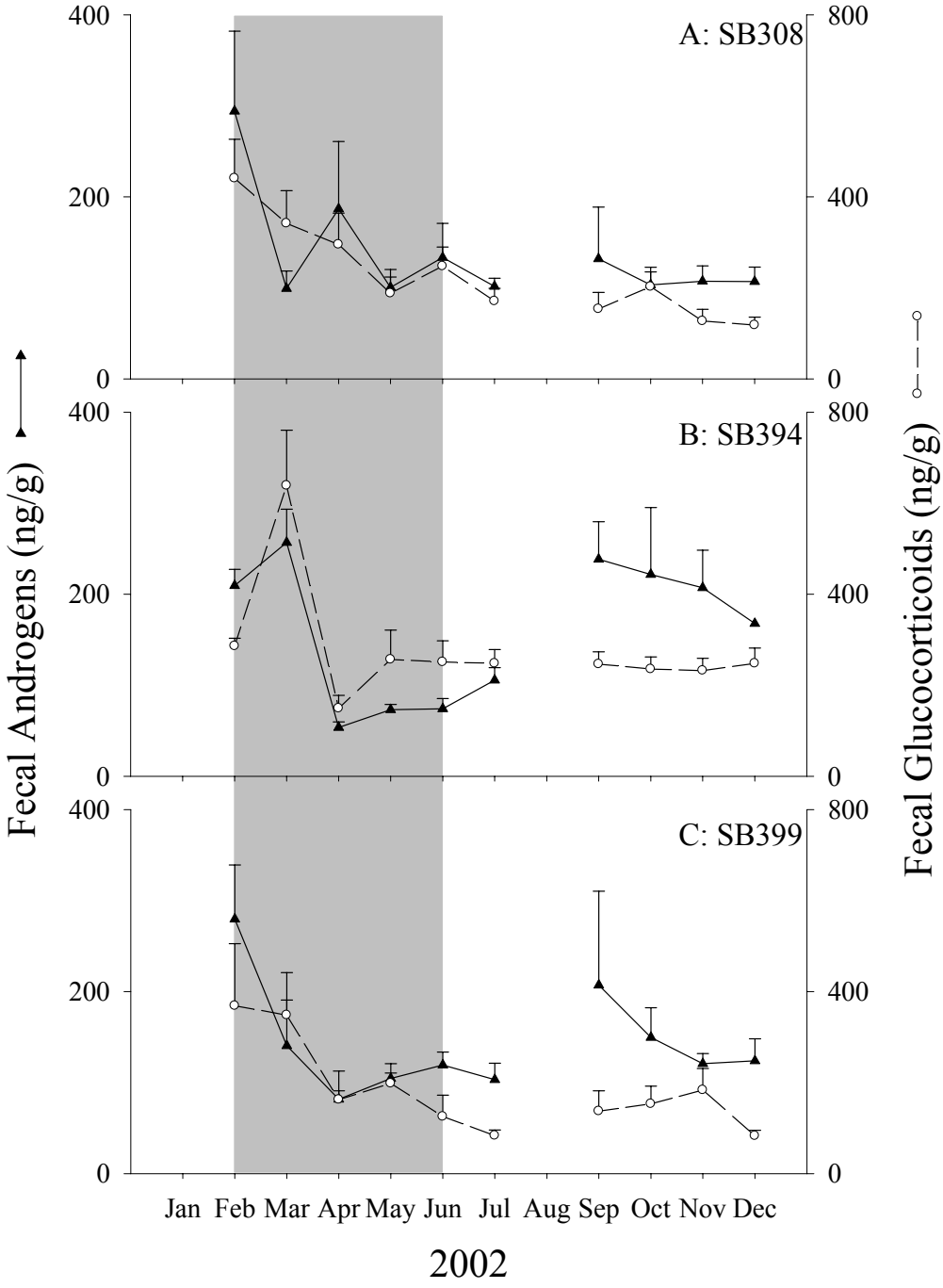

Figure 12. Representative fecal androgen (closed triangles, solid line) and GC (open circles, dashed line) excretion profiles averaged by month (± SE) in SB308 (A), SB394 (B) and SB399 (C). Shaded area represents the known interval of the giant panda breeding season. Data are aligned to the month of the year.

same two months that androgens were increased (February, 286.0 ± 17.3 ng/g; March, 638.7 ± 121.8 ng/g), but were basal in all other months. Although trends in androgen and GC excretion for male SB399 (Fig. 12C) appeared similar, they were not significantly correlated ($r = 0.66$; $P > 0.05$). Androgen values were greater than ($P < 0.05$) baseline (100.5 ± 5.0 ng/g) only during February (279.7 ± 59.4 ng/g), however GC monthly concentrations were greater than ($P < 0.05$) baseline (125.8 ± 6.2 ng/g) during February (368.8 ± 136.6 ng/g), March (348.2 ± 93.7 ng/g) and May (198.3 ± 22.9 ng/g).

Baseline fecal androgen concentrations of male SB399 were not different ($P > 0.05$) from those of SB308 or SB394; however baseline androgens in SB308 and SB394 differed ($P < 0.05$). Overall androgen concentrations for all three males (SB308, 134.1 ± 14.3 ng/g; SB394, 159.0 ± 14.2 ng/g; SB399, 143.5 ± 16.4 ng/g) were not different ($P > 0.05$). Overall GC concentrations (271.7 ± 16.2 ng/g) in male SB394 were greater than ($P < 0.05$) overall values from the other two males (SB308, 215.3 ± 16.3 ng/g; SB399, 186.9 ± 21.0 ng/g), which were not different ($P > 0.05$) from one another. Similarly, baseline GC values in SB308 and SB399 were not different ($P > 0.05$), but were significantly lower ($P < 0.05$) than basal GCs in SB394.

3.6. Male Puberty

SB458 arrived at the SNZP as a sub-adult in December 2000. Over five successive years, fecal androgen and GC metabolites were quantified to document hormonal changes associated with puberty (Table 2). Overall and baseline androgen concentrations increased significantly ($P < 0.05$; ~71 and 32% increase, respectively)

Table 2. Comparison of overall and baseline fecal androgen and glucocorticoid concentrations (mean ± SE ng/g) over successive years in male SB458

Age	Androgen Conc.		Glucocorticoid Conc.	
	Overall	Baseline	Overall	Baseline
3	61.5 ± 3.6[a]	46.8 ± 2.1[a]	170.1 ± 7.7[a]	145.2 ± 4.1[a]
4	104.9 ± 6.2[b]	61.7 ± 2.6[b]	228.9 ± 13.3[b]	137.2 ± 4.3[a]
5	113.2 ± 5.8[b]	70.4 ± 2.3[b]	213.4 ± 11.9[ab]	139.0 ± 4.7[a]
6	192.5 ± 9.8[c]	132.8 ± 5.0[c]	296.0 ± 14.1[c]	231.3 ± 7.1[b]
7	182.5 ± 9.9[c]	130.0 ± 4.3[c]	297.7 ± 19.4[c]	219.0 ± 7.6[b]
Correlation coefficient (r)[d]	0.94[e]	0.93[e]	0.92[e]	0.82

Within column means with different superscripts are significantly different ($P < 0.05$)

[d] Linear regression between age (dependent) and mean hormone concentration (independent)

[e] $P < 0.05$

from 3 to 4 years of age, and no significant changes in year-to-year hormonal measures was detected between 4 to 5 years of age. There was a 1.9-fold and 1.7-fold increase (both, $P < 0.05$) in baseline and overall androgen excretion between 5 and 6 years of age. No differences ($P > 0.05$) were detected in baseline or overall androgen excretion between 6 and 7 years of age.

Although overall GC values increased (~35%) from 3 to 4 years of age ($P < 0.05$), no differences ($P > 0.05$) were detected in baseline or overall GC excretion between 3 and 5 years of age. As with the fecal androgen measures, significant increases ($P < 0.05$) occurred in baseline (1.6 fold) and overall (1.4 fold) GC excretion between 5 and 6 years of age.

4. Discussion

This study validated fecal androgen and GC measures for assessing longitudinal patterns of gonadal and adrenal function in the male giant panda. The physiological validity of fecal androgens was demonstrated by strong correlations between matched urinary and fecal measures across multiple reproductive intervals, whereas GC validity was confirmed by demonstrating a cause-and-effect relationship between the administration of exogenous ACTH and the appearance of GC metabolites in excreta. Additionally, this was the first study to examine the longitudinal endocrine patterns associated with pubertal development in a single male giant panda.

The ACTH challenge was also important for confirming that the excretion lag times (i.e., time from adrenal activation to the appearance of a detectable increase in

excreted GC) for both urine and feces was less than 24 h. This is the first study to demonstrate that the excretory lag time in feces (~10 h) was shorter than for urine (~13 h), which likely reflects the unusually rapid gut transit time (6 – 7 h; Dierenfeld et al., 1982; Edwards et al., 2006b) documented for this species. The short excretory lag time in giant pandas in both urine and feces suggest that short term fluctuations in adrenal activity might be missed with a sampling regimen similar to the one used in the present study (1 – 7 d/wk) it is likely that prolonged or chronic stress would be detectable in animals sampled longitudinally for extended intervals.

While both measures provide a valid index of adrenal function, each method has its advantages and disadvantages. For example, fecal sampling is simpler and more reliable because giant pandas defecate more frequently than they urinate. However, fecal processing is more laborious, and therefore more expensive, and turnaround time is longer compared to urinary measures. Urine has the disadvantage of being easily diluted with water post-urination, which can make the sample unsuitable for hormonal assessments. Our study revealed that fecal drying – a prolonged process that typically requires lyophilizing samples – can be avoided thereby reducing processing time and turnaround time, which may facilitate the use of fecal methods in China, where access to large capacity lyophilizers is limited. Overall, these data demonstrated the physiological validity of urinary and fecal GC measures for assessing adrenal status in the giant panda.

Circannual variations in fecal androgen excretion in the present study were similar to previous reports of seasonal androgen activity in the giant panda (MacDonald et al., 2006) and other Ursidae, including the black (*Ursus americanus*, McMillin et al., 1976; Palmer et al., 1988; Garshelis and Hellgren, 1994); polar (*Ursus maritimus*, Palmer

et al., 1988) and brown (Tsubota and Kanagawa, 1989) bear. In general, fecal androgens peaked early (February) and declined to baseline values by the close of the breeding season (June). Further, androgen metabolite concentrations were increased months before (as early as September) the breeding season in two males (SB394; SB458). Although this trend was not present in all males, androgens were shown to be increased before the breeding season in the brown bear (Tsubota and Kanagawa, 1989) and American black bear (Palmer et al., 1988). Increased androgen production before the breeding season is believed to not only promote spermatogenesis but also play a role in metabolic function during torpor (Nelson et al., 1978; Garshelis and Hellgren, 1994). However, as the giant panda does not experience torpor, it is unlikely that androgens would play the same metabolic role of the brown and American black bear. Despite an increase in autumnal androgen excretion in SB458, reproductive behaviors (e.g., increased activity, vocalizations and scent marking) were not evident until a female was housed in close proximity. Thus, while increased androgen production is clearly a prerequisite for testicular activity and spermatogenesis, we speculate that androgens alone only facilitate the expression of normal sexual behaviors, and that additional stimuli (e.g., olfactory and auditory cues, female presence etc.) are necessary for evoking the full repertoire of sexual behaviors necessary for reproductive success.

We compared baseline androgen excretion among successful and unsuccessful breeding males to search for clues as to why 42% of the male giant pandas in the *ex situ* population have not bred successfully (Zhang et al., 2006). The oldest male in our study (~17 y) excreted the lowest baseline androgen concentrations (SB308; 78.8 ± 2.9), yet he had sired more offspring (33) than any other male in the *ex situ* population (Xie and

Gipps, 2007). In contrast, SB399 had never bred successfully, yet he excreted baseline androgen concentrations that were similar to other breeding males. Likewise, SB458 excreted the highest baseline androgen concentrations of any male in the study, but had never successfully mated. Lower androgen concentrations in aged (> 8 y) American black bears are believed to be associated more with an inability to compete for mates rather than reproductive senescence (Garshelis and Hellgren, 1994). Overall, we found no differences in baseline fecal androgen excretion between non-breeding and breeding males, which is similar to findings based on urinary GC excretion in male giant pandas (MacDonald, 2006). We speculate that differences in baseline androgen concentrations may not be ideal for among-male comparisons because hormone excretion among males can vary due to individual-animal differences in metabolic, excretion and/or hormone clearance rates. More work is needed to determine if the expression of a longitudinal "seasonal" pattern in androgen excretion may be a better predictor of fertility and/or potential reproductive success in male giant pandas.

Seasonal changes in GCs are believed to be related to underlying changes in reproductive behavior, energy mobilization and exposure to seasonal stressors; however the interplay of these mechanisms and seasonal adrenal action are not well understood (see review, Romero, 2002). In the present study, an annual rhythm in fecal GC excretion was exhibited in all males examined and generally correlated with changes in androgen excretion, which was consistent with previously published patterns in male giant pandas derived using urinary GC monitoring (Owen et al., 2005; MacDonald et al., 2006). Generally, fecal GC excretion peaked during the early months of the breeding season (February - March), and declined thereafter to baseline concentrations by summer.

Increased GC during or just prior to the breeding season is a common feature in many mammals (see review, Romero, 2002), including the wolf (*Canis lupus*, Sands and Creel, 2004), northern muriqui (*Brachyteles arachnoids*, Strier et al., 1999), tufted capuchin monkey (*Cebus paella nigritus*, Lynch et al., 2002), and squirrel monkey (*Saimiri boliviensis boliviensis*, Schiml et al., 1996) where elevated GCs were thought to be associated with increased inter-male competition for breeding opportunities. In particular, the squirrel monkey exhibited increased adrenal activity associated with increased fat metabolism during the breeding season, which enhanced their ability to successfully compete with other males for mating opportunities (Schiml et al., 1996). Cortisol was increased during the reproductive season in the arctic ground squirrel (Boonstra et al., 2001a; Boonstra et al., 2001b), which presumably increased energy mobilization for successful reproduction. Captive male giant pandas have been reported to exhibit increased activity patterns and reduced appetite while pursuing estrual females (see review, Snyder et al., 2004), and wild male giant panda engage in ferocious battles with other males for the opportunity to mate with females in estrus (Schaller et al., 1985). While more work is needed to confirm our speculation, our data suggests that GC production in giant panda males may serve to bolster metabolism during the breeding season, which may ultimately augment reproductive fitness.

While it is difficult to separate the impacts of co-varying factors, we also demonstrated a clear, positive correspondence between fecal GC and androgen excretion, which suggests that these hormones may act synergistically to enhance reproductive success. Whereas GC were "chronically" elevated during the breeding season, these increases appeared to be adaptive and there was no evidence that GCs suppressed

gonadal steroid production, as can occur in animals that experience chronic stress (Bambino and Hsueh, 1981; Cumming et al., 1983; Fenske, 1997; Welsh Jr. et al., 1998; Sapolsky et al., 2000). The reproductive life history of the giant panda, taken in the context of GC and androgen production, suggests that androgens are critical for modulating testis function and sexual and aggressive behaviors, whereas GCs are key to unlocking the energy needed for giant panda males to successfully compete for mating opportunities and secure their reproductive fitness.

We documented, for the first time, the hormonal changes that occur in the male giant panda during the pubertal transition from subadult to adult. Androgen concentrations (both baseline and overall) increased steadily from 3 to 7 years of age, but our data suggests that pubertal onset occurred from 4 - 5 years of age, whereas sexual maturity was likely achieved at 6 years of age. These endocrine data fit well with studbook records that indicate that the youngest male to have mated successfully and sired a cub was 5.5 years of age (Xie and Gipps, 2007). The onset of sexual behaviors has been documented in a 5 year old male (Kleiman et al., 1979) and sperm has been collected from males as young as 5.5 years of age (Howard et al., 2006), although it was of poor quality compared to older males. Similar ages of sexual maturation have been described for the grizzly bear (*Ursus arctos horribilis*, White Jr. et al., 1998; White Jr. et al., 2005) and American black bear (Garshelis and Helgren, 1994;). In grizzly bears, males are considered adult at 5.5 years of age, when spermatozoa accumulate in the seminiferous and epididymal tubules (White Jr. et al., 1998). In the American black bear serum testosterone concentrations, mate pairings and weight were greatest in males ≥ 4 years (Garshelis and Helgren, 1994). Our study documented puberty in a single male,

however additional studies are underway to investigate the hormonal milieu, gamete biology and behavior in additional giant panda males across the peri-pubertal interval.

Finally, our study revealed the potential for adapting fecal steroid monitoring to the study of wild giant pandas. The ability to store and process undried feces will greatly simplify sample processing, making these methods readily adaptable under field conditions. Integrating androgen and GC monitoring with behavioral and health assessments, ecological monitoring and evaluations of human-animal conflicts has tremendous potential for studying the factors needed to ensure the long-term survival of extant wild giant pandas, as well as animals that may be reintroduced in the future.

From this study we were able to conclude that: (1) adrenal action in response to a pharmacological stimulation is rapidly assessed (< 10 h) in fecal GC values and is relatively short lived (< 24 h); (2) testosterone and F EIAs were validated for quantifying fecal androgen and GC metabolites in the feces of the male giant panda. Additionally, strong correlations between matched urinary and fecal androgen and GC profiles demonstrated biological validation; (3) seasonal rhythms in fecal androgen and GC excretion were remarkably similar among the males in this study. Generally, androgen and GC values were at their highest at the onset of the breeding season and declined to baseline range by the end of the season and remained low until the next breeding season neared; (4) for the first time sexual maturation was hormonally described for one male. Androgen and GC values increased to adult concentrations at age 6.

CHAPTER 5

Non-invasive endocrine measures of adrenal function in the female giant panda (*Ailuropoda melanoleuca*)

Abstract

Reproductive hormone dynamics are well described in the female giant panda; yet adrenal glucocorticoid (GC) measures have only been assessed in five females, with only one study reporting longitudinal variation in GC excretion. Acute stress, marked by short-term activation of the adrenal gland, can be beneficial for survival, but chronic adrenal stimulation can induce a variety of deleterious physiological and behavioral effects, including a reduction in reproductive fitness. Fecal GC in 17 adult female giant pandas were evaluated with respect to estrus, reproductive seasonality, parturient and nonparturient luteal phases, lactation and acyclicity. Periestrual fecal EC and GC measures were compared in five females, with four demonstrating a positive ($P < 0.05$) correlation ($r = 0.57 – 0.92$) between the two measures, suggesting that GC may play a facilitating role in the hormonal milieu associated with estrus. Among the reproductive states, fecal GC values for both nonparturient (495.9 ± 100.7 ng/g) and parturient (654.1 ± 106.5 ng/g) females highest ($P < 0.05$) during the periestrus interval . Further,

nonparturient females excreted lower ($P < 0.05$) GC concentrations during the secondary period of the luteal phase (334.8 ± 24.8 ng/g) than nonparturient females (470.4 ± 54.0 ng/g), suggesting possible physiological differences between the two subsets of females Although fecal GC concentrations in cyclic nonparturient females were not different across all seasons ($P > 0.05$), seasonal differences were found in acyclic, nonlactational females (winter, 302.1 ± 33.4 ng/g; spring, 212.7 ± 18.1 ng/g; summer, 214.3 ± 14.8 ng/g; autumn, 155.1 ± 9.0 ng/g). Overall, fecal GC concentrations in cyclic and acyclic females were similar, which suggests that stress may not be the primary cause of reproductive inactivity in these females. These data are important for demonstrating that reproductive status and seasonal factors modulate adrenal function in the female giant panda.

1. Introduction

Gonadal steroid dynamics throughout the estrous cycle of the giant panda are well described (Bonney et al., 1982; Hodges et al., 1984; Murata et al., 1986; Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; Meyer et al., 1997; Lindburg et al., 2001; Durrant et al., 2002; McGeehan et al., 2002; Czekala et al., 2003; Narushima et al., 2003; Dehnhard et al., 2006; Steinman et al., 2006), however adrenal steroids have only been documented in five females (Owen et al., 2004; Owen et al., 2005; Liu et al., 2006; Powell et al., 2006), with only one study reporting seasonal variation in adrenal function (Owen et al., 2005). Although adrenal glucocorticoids (GC) are produced in response to stressors (Brann Maresh, 1991; Sapolsky et al., 2000;

Charmandari et al., 2005), GC are also an important part of the hormonal mélange that regulates normal reproductive function (Brann and Maresh, 1991).

The female giant panda possess a unique combination of reproductive characteristics, including being seasonally monoestrus, experiencing a delay in embryonic implantation and exhibiting pseudopregnancy in the absence of fertilization (see review, Steinman et al., 2006). The breeding season of the giant panda is generally confined to the months of February - June (Monfort et al., 1989; Czekala et al., 1998; Lindburg et al., 2001; Czekala et al., 2003; Steinman et al., 2006), during which time spontaneous ovulation (Monfort et al., 1989; Lindburg et al., 2001; Czekala et al., 2003, Steinman et al., 2006; Chapter 2) is followed by an obligate, biphasic luteal phase. The luteal phase consists of a primary rise (74 - 122 d) and a subsequent secondary rise (40 - 50 d) that corresponds to the period of fetal development in a pregnant giant panda females (Hodges et al., 1984; Chaunduri et al., 1988; Masui et al., 1989; Monfort et al., 1989; Mainka et al., 1990; McGeehan et al., 2002; Czekala et al., 2003; Narushima et al., 2003; Steinman et al., 2006; Chapter 3). Progestagen profiles between pregnant and nonpregnant females are indistinguishable, indicating that pseudopregnancy may be obligate in nonpregnant giant pandas (Monfort et al., 1989; Mainka et al., 1990; Narushima et al., 2003; Steinman et al., 2006; Chapter 3).

The mammalian stress response has evolved as an adaptive mechanism for coping with perturbations to homeostasis (Sapolsky et al., 2000; Möstl and Palme, 2002). Acute stress, marked by short-term activation of the adrenal gland, can be beneficial for survival, but chronic adrenal stimulation can induce a variety of deleterious physiological and behavioral effects, including a reduction in reproductive fitness. What is not well

appreciated, however, is that normal activities of the reproductive lifecycle, including courtship and mating, can also trigger an acute stress response (Möstl and Palme, 2002). Acute stress can stimulate or augment reproductive function in estrogen-primed females (Brann and Maresh, 1991), whereas chronic stress can suppress gonadotropin and steroid hormone secretion, thereby altering normal reproductive physiology (Brann and Maresh, 1991; Liptrap, 1993; Dobson and Smith, 1995; Ferin, 1999; Tilbrook et al., 2000).

Currently only four studies have investigated GC metabolite excretion in the female giant panda. Two studies (Owen et al., 2004; Powell et al., 2006) primarily focused on giant panda adrenal response to noise stressors. Both studies found that noise stressors posed no significant detriment to health and well-being of the studied individuals (females, $n = 2$; males, $n = 2$). A single report detailing temporal fecal GC excretion patterns in three adult females found no correspondence between increased stereotypic behaviors and elevated GC concentrations (Liu et al, 2006). However this report did not examine the relationship between mating, estrogen and GC excretion. The endangered status of the giant panda makes captive propagation a high priority for the species, thus estrual females are usually subjected to multiple breedings and/or artificially inseminated (AI) to ensure successful fertilization (Howard et al., 2006). We do not know if repeated breeding and/or AI attempts induce a stress response in estrual females. Further, the common occurrence of acyclicity in captive females may pose an impediment to growing a genetically, self-sustaining population (Shuling et al., 1997; Chapter 2). In other Ursidae, including the brown (*Ursus arctos*; Hensel et al., 1969; Craighead et al., 1976), polar (*Ursus martimus*; Ramsay and Stirling, 1988), and American black (*Ursus americanus*; Wimsatt, 1963; Johnston et al., 1994) bears,

lactating females are typically acyclic. At giant panda breeding facilities, however, cubs are often prematurely removed from their mother in an effort to mitigate the lactational block on estrus and to encourage females to re-enter estrous during the subsequent reproductive season. Although captive husbandry in giant pandas is of the highest quality (Swaisgood et al., 2003; Swaisgood et al., 2006b), and under constant improvement, the effects of stress on reproductive success and well-being have not been thoroughly evaluated (Swaisgood et al., 2006a).

Fecal GC have been assessed in diverse taxa (Millspaugh and Washburn, 2004; Wasser et al., 2000), and is preferable to blood sampling because it obviates the need for stress-inducing animal handling (Harlow et al, 1987; Wasser et al., 2000; Möstl and Palme, 2002; Millspaugh and Washburn, 2004). We have previously validated fecal ovarian steroids in the female and fecal androgens and GC in male giant pandas (Chapter 2, 3, 4). In this study, fecal GC in 17 adult females were evaluated with respect to estrus, reproductive seasonality, parturient and nonparturient luteal phases, lactation and acyclicity. Specifically, this study was designed to: (1) document fecal GC excretion during the periestrual interval and examine the impact of natural breeding and AI on GC excretion; (2) evaluate GC excretion across the reproductive cycle of parturient and nonparturient females; (3) determine if fecal GC are excreted in a seasonal pattern in nonparturient cyclic females; (4) compare fecal GC excretion in acyclic and cyclic females; and (5) document adrenal activity in a single lactating female monitored for one complete year.

2. Methods

2.1. Study animals and facilities

2.1.1. North America

Both Smithsonian's National Zoological Park (SNZP; 39˚N, 77˚W) and Zoo Atlanta (ZA; 33˚N, 84˚W) maintained one adult female and one adult male giant panda throughout the course of this study (2001 – 2006). The females housed at SNZP (Studbook [SB]473; DOB; 22 Jul., 1998) and ZA (SB452; DOB; 25 Aug.,1997) were in good health throughout the study. Each female had fresh water available *ad libitum* and a diet that consisted primarily of fresh bamboo (≥ 75%), with fruit and a high-fiber biscuit as supplements. Females had access to outdoor yards (100 – 300 m^2) and indoor enclosures (50 - 100 m^2) that included heat in the winter and air-conditioning in the summer. Outside of behavioral estrus, females were generally managed separately from their conspecific male, however enclosures allowed for olfactory and auditory contact and visual communication between individuals at all other times. However during behavioral estrus, male-female physical interaction was allowed to permit natural mating. Successful and unsuccessful mating attempts were recorded, and when deemed appropriate, AI was conducted.

2.1.2. China

The China Conservation and Research Centre for the Giant Panda at the Wolong Nature Reserve (Wolong Breeding Centre; 31˚N, 103˚E) maintains adult male ($n > 25$) and female ($n > 40$) giant pandas. For this study, fecal samples were collected from 15 reproductively active, healthy adult females (mean age, 8.8 y; age range, 3 – 16 y). All females were fed a diet that consisted primarily of bamboo, with fruit and high-fiber biscuit supplements, and had water *ad libitum*. Females were maintained as singletons in and indoor-outdoor enclosure (indoor area,30 – 60 m^2;outdoor enclosure, 100 – 300 m^2). Male-female action was permitted during behavioral estrus to allow breeding, but females had of olfactory, auditory and visual contact with conspecific males.

2.2. Fecal sample collection and processing

Freshly voided feces (< 1 h post-excretion) were collected each morning (1 – 7 d/wk) and stored frozen (-20˚C) in sealable plastic bags until processing. Prior to hormone extraction, fecal samples were freeze dried (Lyophilizer, Labconco, Kansas City, MO), crushed to a powder, extracted (Brown et al., 1994; Wasser et al., 1994; Monfort et al.1997), reconstituted in phosphate buffer (1 ml) and then stored frozen (-20˚C) until analysis. Tritiated (^3H) cortisol added to a subset of fecal samples prior to extraction yielded a 84.9 % (± 3.8 %) recovery.

2.3. Endocrine analyses

2.3.1. Cortisol EIA

Fecal GC metabolite concentrations were determined using a single antibody cortisol EIA (Munro and Lasley, 1988; Young et al., 2001). Cortisol antibody (R4866, C. Munro, University of California, Davis, CA) was added (0.05 ml) to microtiter plates (96 well, Nunc-Immuno, Maxisorp; Fisher Scientific, PA), and allowed to adsorb for 12 to 18 h (4°C). Diluted fecal samples were added (equivalent to, 0.5 – 1.0 µl) induplicate and cortisol standards (0.05 ml; range 0.08 – 1,000 ng/ml; 17-Hydroxycorticosterone; Sigma-Aldrich, MO) in triplicate, to the antibody coated plates. Each well received 0.05 ml of enzyme (horse-radish peroxidase [HRP]) conjugated F (C. Munro, University of California, Davis, CA) and incubated at room temperature (25°C; 1 h) before unbound components were removed with wash solution. A chromagen solution (ABTS in citrate buffer; Sigma Chemical Co., St. Louis, MO) was added (0.1 ml) to all wells. Plates were read on a microtiter plate reader (Dynex MRX; Dynex Tehnologies, VA) when optimal optical density (OD) was reached (1.00 OD). Inter-assay CV for two internal controls (n = 88 assays) were 12.8% (mean binding 29.3%) and 13.3% (mean binding 70.4%), and intra-assay CV was < 10%. Both urine and feces demonstrated parallel displacement with the cortisol EIA. Significant recoveries ($P < 0.05$) were demonstrated when unlabeled standard was added to fecal extracts ($r = 1.00$; $y = 0.99x - 3.48$).

2.3.2. Estrogen conjugate (EC) EIA

Estrogen concentrations were quantified in fecal extracts with a single antibody estrogen conjugate (EC) EIA (Stabenfeldt et al., 1991). EC antibody (R583; C. Munro, University of California, Davis, CA) was added (0.05ml) to microtiter plates and set at 4°C for12 to 18 h. Diluted fecal extracts (equivalent to 0.5 – 5.0 µl) and standards (39 – 10,000 pg/ml; 1,3,5(10)-estratrien-17-one 3-sulfate [E1S]; Sigma-Aldrich, MO) were added (0.05 ml) in duplicate and triplicate, respectively, with 0.05 ml of enzyme estrogen-HRP (C. Munro, University of California) to the antibody coated plates. After 2 h incubation (25°C), unbound components were removed and chromagen solution was added (0.1 ml) to each well. When optimal OD was reached (1.00 OD) the plates were read on a microplate reader. Inter-assay coefficients of variation (CV) for two internal controls (n = 10 assays) were 13.5% (mean binding, 41.2%) and 7.4% (mean binding, 72.3%), and intra-assay CV was < 10%.

2.4. Data analysis

For the periestrual analysis, fecal GC concentrations were aligned to day from peak fecal EC for each individual. For reproductive stage analyses, fecal GC concentrations were partitioned by individual into pre-periestrus (50-d interval preceding periestrus onset), periestrus (interval of increased fecal estrogen concentrations associated with behavioral estrus), a biphasic luteal phase (1˚, 2˚ rise intervals, Chapter 3), and a post-luteal interval (50-d post-interval following the end of the parturient or nonparturient luteal phase). Seasonal analysis included only mature (≥ 4 y; Kleiman et al., 1979;

Schaller et al., 1985; Steinman et al., 2006), acyclic and cyclic nonparturient females. Cyclic parturient females were excluded from these analyses because of the possible effect that cub-rearing and lactation may have on seasonal adrenal function. Females were classified as being acyclic based on reproductive endocrine evaluations (data not presented). For seasonal analyses, individual female fecal GC concentrations were divided into winter (21 Dec. – 20 Mar.), spring (21 Mar. – 20 Jun.), summer (21 Jun. – 19 Sep.) and autumn (20 Sep. – 20 Dec.). Between reproductive stage and season comparisons of mean fecal GC concentrations were determined by Holm-Sidak one-way repeated measures analysis of variance. Within-reproductive stage and seasonal evaluations of mean fecal GC values were conducted using a Mann-Whitney test. One female was lactational for a year and her GC data were presented separately from the other acyclic, nonlactating females. Within-season comparisons of the lactational female GC data and those of the cyclic and acyclic females were conducted using a Kruskal-Wallis one-way ANOVA. Correlations between parturient and nonparturient, and cyclic an acyclic females were determined by Pearson correlation coefficient. Relation between standard and fecal extract with added standard during validation was determined through linear regression. All data were tested for normality (Kolmogorov-Smirnov test) prior to additional statistical tests and all statistics were performed at alpha = 0.05. Statistical analyses were conducted in SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA).

3. Results

3.1. Periestrual GC excretion

Fecal GC and EC profiles of periestrual events from five female giant pandas (SB414, SB504, SB452, SB544, SB473) are presented in Figure 13 with hormone excretion aligned to day of fecal EC peak (Day 0). Fecal GC concentrations in samples spanning the interval from the first to the last day of mating and/or AI (543.5 ± 114.5 ng/g; range, 160.6 – 1305.5 ng/g) were not different ($P > 0.05$) from GC concentration outside of this time period (393.0 ± 32.9 ng/g; range, 57.9 – 1992.7 ng/g) for all females in the presented data set. The first of three mate introductions for female SB414 (Fig. 13A) commenced the day after the fecal EC peak with no evidence of increased were GC excretion observed in this female who became pregnant and gave birth after ~114 d gestation. Fecal GC excretion was greater ($P < 0.05$) in the 20-d pre-ovulatory interval (839.7 ± 182.4 ng/g) than during the subsequent 20-d post-ovulatory period (361.1 ± 47.1 ng/g), and there was no correlation between fecal EC and GC measures ($r = 0.13$; $P > 0.05$). Female 504 (Fig. 13B) mated five times over successive days begin on Day 0 but did not give birth. Fecal GC increased on the first day of breeding (1,602.2 ng/g) coincident with peak EC, and both measures were strongly correlated ($r = 0.95$; $P < 0.05$). Incomplete sampling during the 20-d period preceding peak EC excretion prevented comparisons to the 20-d post-ovulatory interval. Mate introductions for female SB452 (Fig. 13C) occurred on Day 0 and Day 1, but because copulation was unsuccessful, AI was conducted on Day 1 and Day 2. Fecal GC increased on Day 0 but

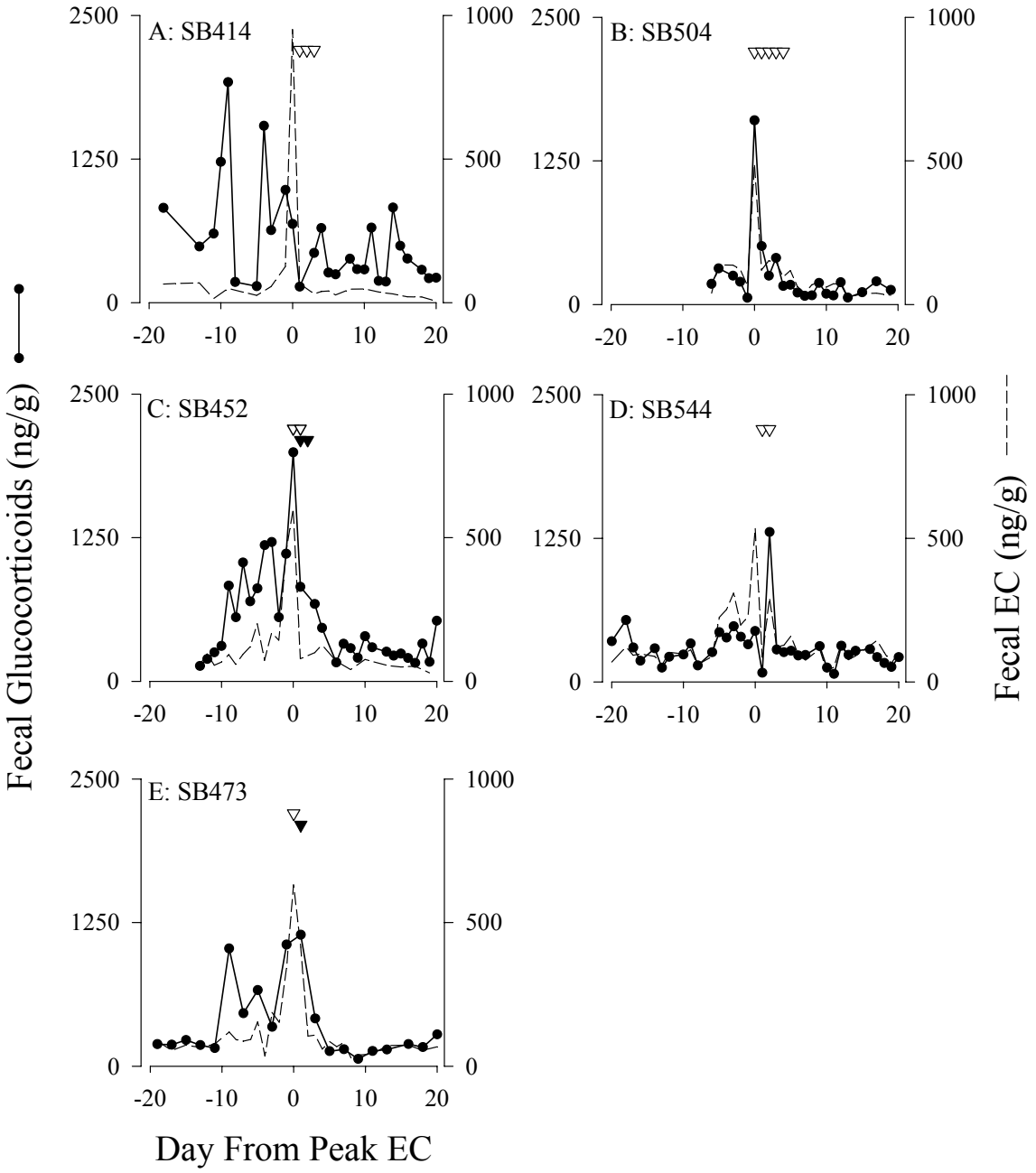

Figure 13: Periestrual fecal GC (closed circles, solid line) and EC (dashed line) excretion are depicted for five adult female giant pandas (A) SB414, (B) SB504. (C) SB452, (D) SB544 and (E) SB473. Open triangles denote days of mate introduction and closed triangles mark days of when AI was performed. Data are aligned to day of peak fecal EC.

not on subsequent days and the luteal phase in this female concluded without parturition. Fecal EC and GC were positively correlated ($r = 0.77$; $P < 0.05$) and GC values were greater ($P < 0.05$) during the 20-d pre-ovulatory interval (776.1 ± 136.1 ng/g) than during the 20-d post-ovulatory interval (337.2 ± 45.4 ng/g). Natural breeding for female SB544 (Fig. 13D) occurred on Day 1 and Day 2 with increased fecal GC detected on Day 2 (1,305.5 ng/g). Fecal GC and EC correlated with one another ($r = 0.57$; $P < 0.05$) in this nonparturient female, but fecal GC concentrations during the 20-d before (303.8 ± 28.5 ng/g) and after (277.8 ± 63.0 ng/g) the fecal EC peak were not different ($P > 0.05$). Natural mating was unsuccessful on Day 0 in female SB473 (Fig. 13E), so AI was conducted on Day 1, the female conceived and gave birth after ~121 d gestation. The highest GC value (1,143.2 ng/g) was measured on Day 1and GC excretion was significantly correlated with fecal EC ($r = 0.84$; $P < 0.05$). No differences ($P > 0.05$) were detected in fecal GC excreted 20-d before (478.2 ± 119.3 ng/g) and after (281.2 ± 100.6 ng/g) the fecal EC peak.

3.3. GC excretion in parturient and nonparturient females

Mean (+ SE) fecal GC concentrations from nonparturient ($n = 7$) and parturient ($n = 10$) females segregated into different reproductive stages are presented in Figure 14. Only the periestrual interval (495.9 ± 100.7 ng/g) was different ($P < 0.05$) from the other reproductive stages (pre-periestrus 307.1 ± 63.4 ng/g; primary rise, 249.1 ± 30.2 ng/g; secondary rise, 334.8 ± 24.8 ng/g; post-luteal 257.6 ± 33.2 ng/g) in the nonparturient females. For the parturient females, the pre-periestrus interval (327.1 ± 77.7 ng/g) and

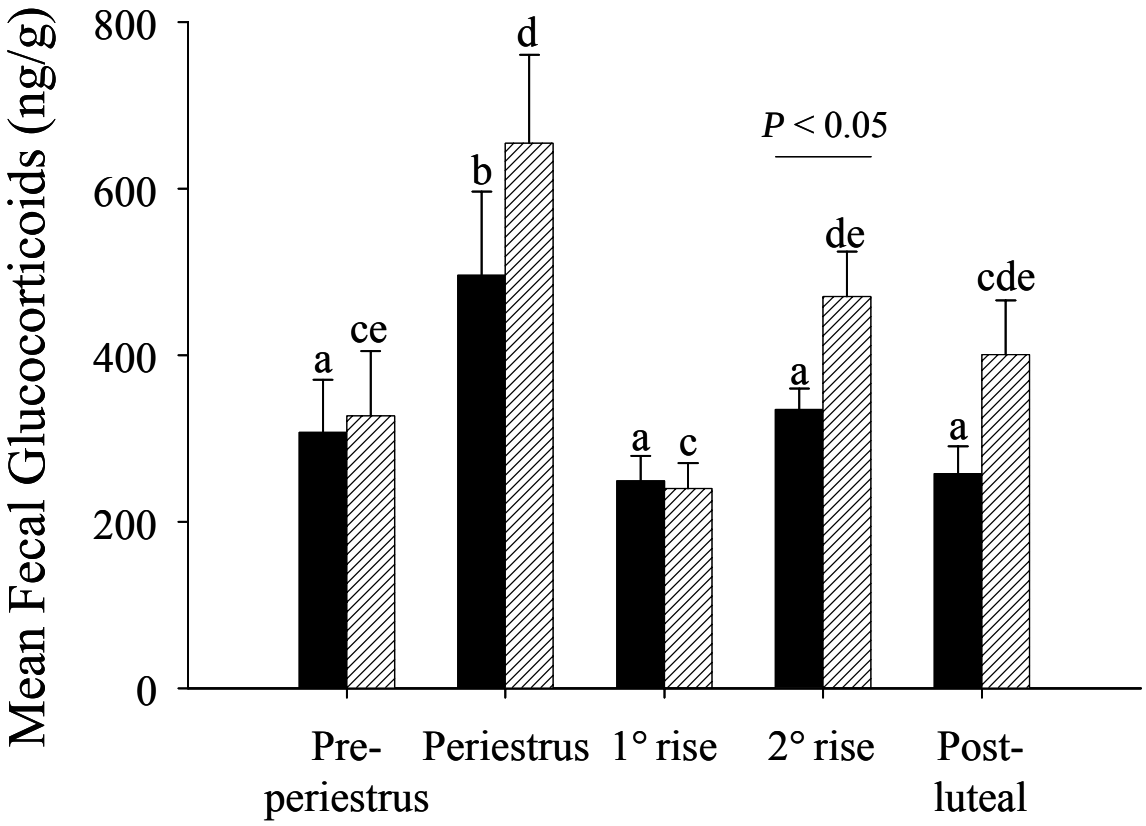

Figure 14: Mean (+ SE) fecal GC concentrations across reproductive stages are presented for nonparturient (n = 7; solid bars) and parturient (n = 10; hatched bars) female giant pandas. Bars with different scripts differ ($P < 0.05$; Holm-Sidak one-way repeated measures ANOVA) among the reproductive states for nonparturient (a, b) and parturient (c, d, e) females. P-values denote differences ($P < 0.05$; Mann-Whitney test) within reproductive stage for comparisons between nonparturient and parturient females.

the primary rise (239.6 ± 30.9 ng/g) GC values were similar ($P > 0.05$), but lower ($P < 0.05$) than the periestrual period (654.1 ± 106.5 ng/g). An increase ($P < 0.05$) in GC concentrations occurred between primary and secondary (470.4 ± 54.0 ng/g) rise intervals; there were no differences ($P > 0.05$) among post-luteal (400.6 ± 64.8 ng/g) values and the other reproductive stages. Comparisons of fecal GC excretion within reproductive stages between nonparturient and parturient females revealed only one difference ($P < 0.05$): nonparturient females excreted lower concentrations of GC during the secondary rise interval. Fecal GC trends across the reproductive stages were similar ($r = 0.91$; $P < 0.05$) for nonparturient and parturient females.

3.3. Seasonality of GC excretion in cyclic and acyclic females

Mean (+ SE) seasonal fecal GC values for cyclic nonparturient ($n = 10$) and acyclic ($n = 6$) females are shown in Figure 15. There were no seasonal differences ($P > 0.05$) among the cyclic nonparturient females (winter, 337.8 ± 87.6 ng/g; spring, 278.6 ± 41.1 ng/g; summer, 296.2 ± 25.8 ng/g; autumn, 226.2 ± 30.0 ng/g). Fecal GC concentrations for acyclic females were highest ($P < 0.05$) in winter (302.1 ± 33.4 ng/g) compared with spring (212.7 ± 18.1 ng/g), summer (214.3 ± 14.8 ng/g) and autumn (155.1 ± 9.0 ng/g). There were no differences ($P > 0.05$) in GC excretion between spring and summer; however autumnal GC values were the lowest ($P < 0.05$) among all of the seasons. The only within-seasonal difference ($P < 0.05$) in GC values between cyclic and acyclic females occurred during the summer; however all other seasons were not different ($P > 0.05$). Additionally, seasonal trends in GC excretion between cyclic and acyclic

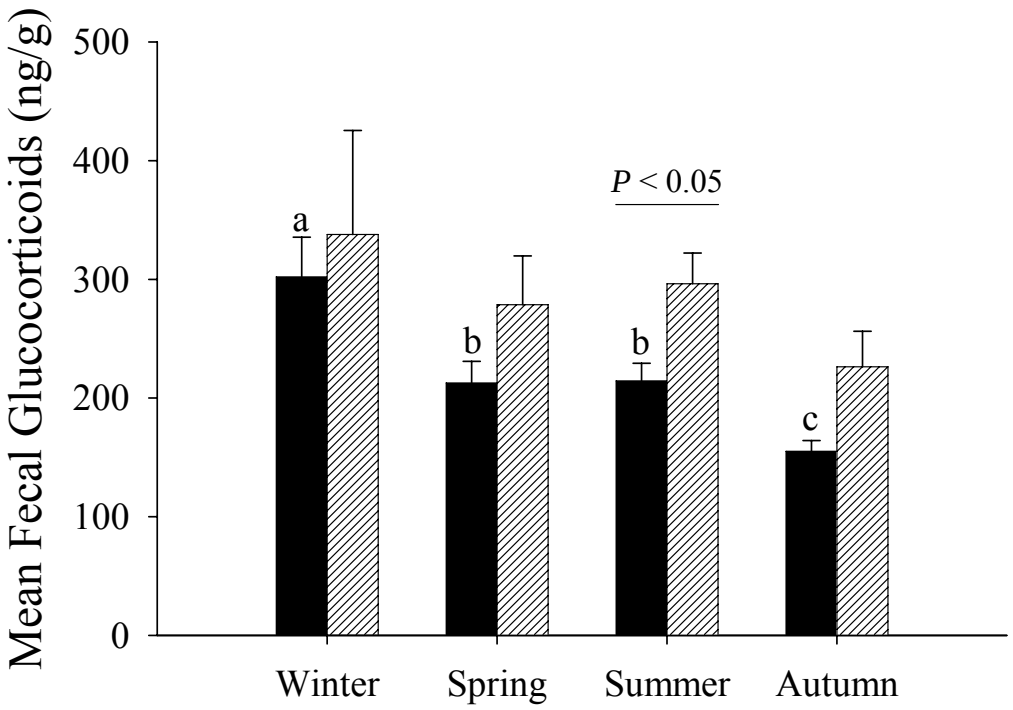

Figure 15: Mean (+ SE) seasonal fecal GC concentrations for acyclic (n = 6; solid bars) and cyclic (n = 10; hatched bars) female giant pandas. Bars with different scripts denote seasonal differences ($P < 0.05$; Holm-Sidak one-way repeated measures ANOVA) in acyclic females (a, b, c). P-values denote differences ($P < 0.05$; Mann-Whitney test) within season for comparisons between acyclic and cyclic females.

females were strongly correlated ($r = 0.97$, $P < 0.05$).

Only one female (SB473; not represented in Fig. 15) nursed a cub throughout a year during the course of this study, and during that year she did not experience an estrous cycle. Seasonal fecal GC measures for this lactating female did not differ ($P > 0.05$) between winter (315.7 ± 40.6 ng/g)and spring (299.9 ± 24.1 ng/g). Additionally, no difference ($P > 0.05$) was determined between summer (394.1 ± 43.0 ng/g) and autumn (466.5 ± 53.2 ng/g). However, both winter and spring were lower ($P < 0.05$) than summer and autumn. Although winter, spring and summer fecal GC values for the lactating female were not different ($P < 0.05$) from the cyclic and acyclic females represented in Figure 3; GC concentrations during autumn were higher ($P < 0.05$) in the lactating female than the cyclic and acyclic females. Additionally there was no seasonal relation in GC trends between the lactional female and the cyclic ($r = -0.73$; $P > 0.05$) or acyclic ($r = -0.74$; $P > 0.05$) females.

4. Discussion

This study provided the first in-depth analysis of adrenal activity associated with seasonality, estrus, luteal function (parturient and nonparturient) and lactation in a large number of female giant pandas. These data are important for demonstrating that, in general, current regimes for breeding and or AI of periestrual females do not illicit an adrenal response, and hence physiological stress. We demonstrated strong correspondence between fecal GC and EC excretion during the periestrual interval, which

suggests that adrenal activation represents and adaptive mechanism for facilitating reproductive fitness.

Seasonal GC excretion was documented in nonlactational acyclic females but not in cyclic nonparturient females. The lack of seasonal GC excretion in cyclic females contrasted with a previous study that documented seasonal urinary GC excretion in a single female giant panda (Owen et al., 2005). While seasonal GC patterns were not evident, we found a strong association between reproductive stage and GC concentrations, particularly during the periestrual interval. Furthermore, we uncovered differences in fecal GC between parturient and nonparturient females during the secondary rise stage of the luteal phase. While seasonality may play a role in modulating adrenal function, this impact was not evident in the present study, but may have been masked by the strong effect of reproductive stage on adrenal function. We also documented that fecal GC during lactational anestrus only differed from nonlactating acyclic and nonparturient cyclic females during the autumn, when the nursing cub was approximately 1 year of age.

We predicted that fecal GC would be elevated coincident with breeding and/or AI, yet increased GC excretion after breeding was observed in only one of five females studied (Fig. 13D). Although acute stress can induce only a transient increase in fecal GC (i.e., < 24 h; Chapter 4), each female was bred or artificially inseminated at least twice, making it unlikely that an acute elevation in fecal GC was missed in every case.

Fecal GC were consistently higher during the periestrual interval for both parturient and nonparturient females compared to all other reproductive stages. In the sow, acute stress during the follicular phase did not affect follicular development or

ovulation (Turner et al., 2005) and repeated exposure to males during estrus not only increased cortisol secretion but also stimulated sexual behavior (Turner et al., 1998). Unexpectedly, a strong correlation was found between EC and GC measures in four of the five female giant pandas in the present study. Although little information exists on the relationship between adrenal and ovarian activity during estrus in wildlife, considerable information on domestic species suggests that GC are part of the hormonal milieu that facilitates ovulation (Brann and Maresh, 1991; Ferin, 1999). Increased serum cortisol in estrus dairy cows was associated with peak estradiol concentrations and estrus behaviors (Lyimo et al., 2000). Conversely, pharmacological dosages of exogenous ACTH administered to estrual cows interfered with ovulation by blocking the ovulatory LH surge (Stoebel and Moberg, 1981). For the giant panda, we speculate that increased GC excretion coincident with the pre-ovulatory EC peak may reflect the increased activity and agitation observed in estrual females, and that such "stress" may actually be adaptive in facilitating the expression of an appropriate behavioral milieu and/or for stimulating ovulation. The absence of spikes in CG associated with natural mating and/or the physical manipulations associated with AI suggests that these disturbances do not induce adrenal activation, and by extrapolation, physiological stress. More work is needed to understand the potential role that adrenal function plays in modulating ovarian function and reproductive fitness in the giant panda.

We discovered a significant increase in fecal GC coincident with the secondary rise stage of the luteal phase – the presumptive time of implantation and fetal development – in parturient, but not nonparturient female giant pandas. The giant panda undergoes obligate pseudopregnancy and hormone profiles between parturient and

nonparturient females are indistinguishable (Monfort et al., 1989; Mainka et al., 1990; Narushima et al., 2003; Steinman et al., 2006; Chapter 3). While more work is needed to confirm this, we hypothesize that GC may serve as a maker for embryonic development, and hence could have potential as a means for diagnosing pregnancy in giant pandas. While data on fecal GC excretion during pregnancy in wildlife is sparse, pregnant blue foxes (*Alopex lagopus*) excreted significantly higher concentrations of fecal GC than pseudopregnant females (Sansen et al., 2005). Additionally, increased GC secretion during pregnancy is a common feature in a host of mammalian species, including the rat (*Rattus norvegicus*, Atkinson and Waddell, 1995), cow (*Bos Taurus*, Smith et al., 1973), ewe (*Ovis aries*, Banchero et al., 2006) and human (Lindsay and Neiman, 2005). Although the direct role in stimulating GC secretion and the physiological role are as of yet completely defined; it is believed that GC acts synergistically with gonadal steroids in meeting the metabolic needs of colostrum synthesis (Banchero et al., 2006) and in increasing uterine blood flow to the developing fetus (Chang and Zhang, 2008). Thereby it is possible that the increase in GC in the parturient giant pandas in this study may be associated with fetal development, however additional longitudinal studies on known pregnant and pseudopregnant (i.e., non-bred or documented nonpregnant via ultrasound) giant pandas will need to be conducted to further determine if fecal GC might provide an effective means for tracking pregnancy in this species.

Fecal GC values during the post-partum interval in giant pandas were similar to GC concentrations during the secondary rise stage of the luteal phase. Although these data contrast those other wildlife, including the harp seal (*Phoca groenlandica*, Engelhardt and Ferguson, 1980), yellow-pine chipmunk (*Tamias amoenus,* Kenagy and

Place, 2000), Japanese macaque (*Macaca fuscata*, Kondo et al., 2003) and spotted hyena (*Crocuta crocuta,* Goymann et al., 2001), whereby lactating females secreted higher GC concentrations than non-lactating females, they are not entirely unique. For example, there were no differences in pregnant and lactating GC concentrations the ring-tailed lemur (*Lemur catta*, Cavigelli et al., 1999), chacma baboon (*Papio hamadryas ursinus,* Weingrill et al., 2004) and arctic ground squirrel (*Spermophilis parryii*, Boonstra et al., 2001a). Because giant panda cubs born in captivity are often weaned prematurely (i.e., <1 y) little information is available on the biology of normal, undisturbed lactation, which can last up to 2 y in wild pandas. A seasonal examination of GC excretion in one female giant panda (SB473) that nursed a cub for more than a year revealed maximal GC excretion during autumn, about the time the cub was over a year of age. In the sow, metabolic demands of lactation are highest before weaning and suckling induces cortisol secretion to meet those energy demands (see review, Quesnel and Prunier, 1995). Further, in the cow and rodent species, GC values are increased during lactation due to decreased nutrient stores (Neville, 2006). In the giant panda, nutrition derived from bamboo is minimal (Edwards et al., 2006b), suggesting that insufficient fat stores may be attributed to increased concentrations of GC during lactation. Although it can not be delineated that the autumnal GC increase in the lactational female in our study was solely attributed to increased lactational demands of the cub a >1 year of age, the GC data suggest that seasonal adrenal function is attenuated by factors associated with cub rearing.

With the exception of a single female (SB473), lactational anestrus could not explain why several females were acyclic during the first reproductive season post-cub

removal. Longitudinal GC assessments did not suggest that chronic stress was a factor in suppressing reproduction in these females. There is little information about the idiopathic acyclicity in wildlife species, but serum cortisol concentrations did not explain this phenomenon in female African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants, which was attributed to hyperprolactinemia (Brown et al., 2004). In the present study ($n = 6$), acyclicity was transient as all these females gave birth one to five years (mean, 2.0 ± 0.6 y) after experiencing the period of acyclicity (Xie and Gipps, 2007). However, acyclicity can be attributed to other factors not investigated in this study. In the gilt (*Sus scrofa scrofa*), insufficient or depleted nutrient stores following weaning prolongs anestrus (Armstrong and Britt, 1987). If the time from weaning to the start of the next breeding season is insufficient for female giant pandas to replenish the nutrient stores then it is possible that follicular development may be inhibited and the female would be acyclic for that year. Yet, given the transient nature of infertility in the giant panda, acyclicity may not be detrimental to achieving reproductive targets in the *ex situ* population, however determining the factors that lead to reproductive inactivity may aid in better management of the species.

In the male giant panda androgens and GC parallel one another with peak activity during the two seasons in which the breeding activity is greatest (winter, spring) (Owen et al., 2005; MacDonald et al., 2006; Chapter 4). In contrast, the reproductive stages of the female span all seasons, which may influence the association of GC values in females with seasons. For example, periestrus, the reproductive stage in which GC excretion was highest in all cyclic females, generally occurs in winter and spring, thereby incorporating those data into the respective seasons would likely increase the seasonal GC mean.

Whereas anestrus, the period of reproductive inactivity in all cyclic females between the end of the luteal phase and the start of the next breeding season has no associated reproductive metabolic costs, may start as early as July or as late as October. Further seasonal GC fluctuations observed in free-living animals have been associated with seasonal variations in environment, including diet and weather (Romero, 2002; Huber et al., 2003; Millspaugh and Washburn, 2004). This exposure to pronounced environemental changes with the seasons may explain why seasonal GC variation was observed in a the free-living American black bear (Harlow et al., 1990) and not in population of captive giant pandas we studied. Exposure to seasonal environmental fluctuations are somewhat mitigated in captivity, where giant pandas have heated indoor enclosures during winter and access to air-conditioned or other cooling mechanisms (misters, sprayers) in the summer, in addition to a constant food supply. Considering that both season and reproductive stage can have significant effects on energy usage (Schneider, 2004), which will affect adrenal function (Romero, 2002), factors associate with reproductive stage and season must be considered when evaluating GC measures to determine adrenal activity in the female.

Although fecal GC concentrations did not vary seasonally in cyclic nonparturient females (n = 10), which contrasts results of a previous study that examined urinary GC excretion in a single female (Owen et al., 2005), seasonal differences in GC excretion in acyclic nonlactational females were detected. Gonadal function is involved in increasing adrenal action; however without that reproductive mechanism in place, seasonal modulation of adrenal function in the acyclic females is more likely to become evident. Yet it is important to note that although adrenal function was affected by reproductive

stage in the cyclic females there was a strong correlation in seasonal GC excretion the acyclic and cyclic females, suggesting that season and reproductive stage affect GC production.

Utilization of fecal GC measures to track adrenal function in the female giant panda has tremendous potential to improve management and understand biology the species. Fecal GC assessments would be of particular value in studying adrenal activity in free-living giant pandas where animals may be adversely impacted by humans. Additionally, wild populations are exposed to greater extremes in environment and food availability. Correlating these factors with adrenal function would be an important tool to determine physiological response of giant pandas to stochastic events, such as bamboo die-offs (Reid et al., 1989). However it is important that future assessments of fecal GC concentrations consider annual rhythms and reproductive state (e.g., estrual, luteal, anestrual, lactational) to ensure valid comparisons (Millspaugh and Washburn, 2004), but also consider and account for inter-female variability. Further, reliable assessments of adrenal function and stress response can only be obtained from longitudinally collected samples from known individuals to ensure accurate assessment of temporal trends in GC excretion. Although the data present in this paper provide new and valuable information on adrenal function in the female giant panda they are not definitive. Additional studies on ex situ and in situ population will allow for better management and conservation of this rare species.

CHAPTER 6

Future directions and application of non-invasive endocrine monitoring of the endangered giant panda

1. Current directions

The captive population of giant panda has nearly doubled in the past ten years to a current population of more than 220 individuals. The importance of this growth however is that it is largely attributed to improved management from information gained on basic biological information on the species (Ellis et al., 2006a). The first year in which the captive population was comprised of more captive-born individuals than wild-born was 1997. Up until that point, captive population growth was attributed to obtaining pandas from the wild. Since 1997, the number of captive-born giant pandas has grown to represent > 75% of the *ex situ* population (Xie and Gipps, 2007). An improved understanding of the reproductive biology of this species was a major factor in growing the captive population (Zhang et al., 2006). A basic understanding of the brief window of fertility females experience each year has helped aid in propagation efforts; however other reproductive phenomena, such as delayed implantation, pseudopregnancy and acyclicity had remained largely unknown. The data presented in this study are a

significant contribution to the scholarly knowledge of these reproductive phenomena for the female giant panda. Progestagen dynamics of the luteal phase remain similar for parturient and nonparturient females, however for the first time a significant hormonal difference was found in the excreted glucocorticoids during the second period of the luteal phase. Further, the study of male reproductive biology provided the first account of the hormonal transition of a juvenile male into sexual maturity. However of additional value, fecal endocrine measures in the giant panda were developed and validated, a technique that has great potential to monitor and study aspects of the wild giant panda that are largely unknown.

2. *In situ* studies

The knowledge of giant panda reproductive biology is based on relatively few sources, the primary one is *The Giant Pandas of Wolong* (Schaller et al., 1985). Although Schaller et al. (1985) provided detailed behavioral information on giant panda breeding for a handful of individuals, quantifiable physiological information is absent. Until now, non-invasive endocrine measures were almost exclusively done by analyzing gonadal steroid metabolites in the urine (see review, Steinman et al., 2006). Although this approach has been met with some success in captivity, where daily access to undiluted, uncontaminated urine on a clean substrate is not a problem, urine collection would be nearly impossible for monitoring giant pandas in the wild. Accounts of finding large amounts of giant pandas on a regular (nearly daily) basis are common for those that enter giant panda habitat, even if observations of the individuals that left the feces are

uncommon (pers. comm., McShea, W.). Therefore there are frequent opportunities to collect feces and evaluate hormonal concentrations in those samples. However, the greatest value in tracking endocrine parameters of giant pandas in the wild is attributing fecal collections to known individuals. The primary means to assure that samples are coming from a specific individual is to follow an animal via radio-telemetry. Initially met with skepticism, radio-collaring giant pandas have proven not to cause stress (Durnin et al., 2000) and is currently being employed to track released individuals in Wolong. However, if radio-collaring is not practical, microsatellite DNA analysis of exfoliated intestinal cells that surround the feces is another means of identifying a fecal sample to a specific individual (David et al., 2006).

Once longitudinally collected feces from known animals are analyzed for reproductive and adrenal hormone metabolites, questions about the biology of wild giant pandas can be addressed, such as: (i) are estrual events confined to a shorter breeding season? (ii) what is the duration of the luteal phase? (iii) is acyclicity common in nonlactating females? (iv) is the age of the onset of puberty in wild males and females different from captive counterparts? (v) are seasonal adrenal GC measures more dynamic in males and females? (vi) how does adrenal activity correlate with weather and food availability? (vii) are there differences in the reproductive and or adrenal biology between populations proximal to human disturbance and those that are further removed from anthropogenic influences? (viii) are there differences in androgen concentrations between males of different ages and how does that relate to breeding success? (ix) do autumnal estruses occur in the wild or is it an phenomenon only in captive management?

3. *Ex situ* studies

Although the captive population is no substitute for the wild population, it can serve as a base from which to further grow our understanding about the reproductive and adrenal physiology of the species. Further studies should investigate (i) the minimum lag time between weaning and the ability of a female to cycle again; (ii) the differences in adrenal function between parturient and truly pseudopregnant females during the secondary rise in luteal progestagens; (iii) the influence of nutritional factors on adrenal function; (iv) the uniformity of the age of the onset of puberty in all females and males; (iv) monitor glucose levels in association with adrenal and gonadal steroid metabolite measures in females to determine; (v) the role and influence of glucocorticoids during estrus. Additionally, concurrent with this study, is another study investigating the reproductive biology of the male giant pandas housed at the Chengdu Research Base. The male reproductive study is not only utilizing fecal monitoring of androgen metabolites, but also correlating those values with sperm and seminal parameters, behavior and age.

4. Endocrine monitoring in China

A comprehensive knowledge of every biological aspect of the giant panda is without significant value unless China – the people and government – are willing to conserve the species. Fortunately, there is considerable interest in China to ensure long-term survival of the giant panda (Ellis et al., 2006a). This study was undertaken in partial

fulfillment of an obligation the Smithsonian's National Zoological Park has with the Chinese State Forestry Administration and the United States Fish and Wildlife service. Housing giant pandas in the United States is a privilege for a zoo, and in return for that privilege, housing institutions must contribute to *in situ* as well as *ex situ* conservation. Considering that the future of the giant panda is ultimately in the hands of the Chinese we initiated this endocrine study with the understanding that the techniques and methods we developed would eventually be carried-out in China, where our methods would have the greatest conservation impact. Deciding to utilize the feces to study the reproductive and adrenal physiology of the giant panda was an obvious choice. Fecal hormone monitoring would be an invaluable tool for tracking gonadal and adrenal activity in free-living giant pandas. Further, we sought to employ analysis techniques that would be user friendly, inexpensive, portable and not require any hazardous materials. Enzyme immunoassays (EIA) have emerged as the ideal technique to monitor endocrinological parameters in wildlife. Therefore, developing EIA assessment of fecal hormones was the best approach to monitor endocrine dynamics of giant pandas that would also be pragmatic for facilities in China.

During the course of this study, I had the privilege of training three Chinese colleagues at the Smithsonian's National Zoological Park Conservation and Research Center (CRC) on giant panda fecal hormone monitoring techniques. Particularly, one Chinese colleague, a veterinarian from the Chengdu Research Base, spent six months training with me at the CRC in 2004. Although the biochemistry and physiology behind reproductive and adrenal endocrinology are fairly easy to pick-up, honing the techniques and skills take time. Following her training at the CRC, I traveled with her back to China

to help set-up a fecal processing and EIA laboratory at the Chengdu Research Base. The laboratory has since proven invaluable in monitoring reproductive hormone changes associated with estrus to aid in timed breedings and artificial inseminations. Additionally the laboratories have acted a spring-board for newly developed and future endocrine studies carried out by the Chengdu Research Base on the giant panda, tiger, red panda and Asiatic black bear.

5. An integrative approach

Endocrinology is only one discipline involved in the giant panda conservation program currently underway by North American Zoos. The larger conservation program for the giant pandas involves varying disciplines that include, but not limited to: genetics, population ecology, geographic information systems, behavioral biology, gamete biology, nutrition, habitat ecology, husbandry and veterinary medicine. An interdisciplinary approach however is necessary for species conservation programs to be successful. Further, the multidisciplinary team involved in giant panda conservation must also work cooperatively with other stakeholders that include, national and international government agencies, non-governmental organizations and the public. The popularity of giant pandas has not only made them an ambassador for conservation, but also makes them an umbrella species, affording species it shares habitat with protection. However, the popularity has also put conservation efforts for the species under a spotlight. Success or failure of giant panda conservation will affect conservation efforts for other species (Ellis et al., 2006a). Fortunately, considerable strides have been made and are continually

being made in a growing a genetically self-sustaining captive population, increasing the size, number and connectivity of protected habitat and a better understanding of the basic biology of the species.

REFERENCES

REFERENCES

Akingbemi, B.T., Ge, R.S., Hardy, M.P., 1998. Leydig cells. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, Ca, pp. 1021-1033.

Albrecht, E.D., Pepe, G.J., 1998. Placental steroidogenesis in primate pregnancy. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, pp. 889-898.

Allais, C., Martinet, L., 1978. Relation between daylight ratio, plasma progesterone levels and timing of nidation in mink (*Mustela vison*). Journal of Reproduction and Fertility 54, 133-136.

Allrich, R.D., 1994. Endocrine and neural control of estrus in dairy cows. Journal of Dairy Science 77, 2738-2744.

Asa, C.S., Valdespino, C., 1998. Canid reproductive biology: an integration of proximate mechanisms and ultimate causes. American Zoologist 38, 251-259.

Armstrong, J.D., Britt, J.H., 1987. Nutritionally-induced anestrus in gilts: metabolic and endocrine changes associated with cessation and resumption of estrous cycles. Journal of Animal Sciences 65, 508-523.

Atkinson, H.C., Waddell, B.J., 1995. The hypothalamic-pituitary-adrenal axis in rat pregnancy and lactation: circadian variation and interrelationship of plasma adrenocorticotropin and corticosterone. Endocrinology 136, 512-520.

Bales, K.L., French, J.A., McWilliams, J., Lake, R.A., Dietz, J.M., 2006. Effects of social status, age, and season on androgen and cortisol levels in wild male golden lion tamarins (*Leontopithecus rosalia*). Hormones and Behavior 49, 88-95.

Ballou, J.D., Miller, P.S., Xie, Z., Wei, R., Zhang, H., Zhang, A., Huang, S., Sun, S., David, V.A., O'Brien, S.J., Traylor-Holzer, K., Seal, U.S., Wildt, D.E., 2006. Analysis of demographic and genetic trends for developing a captive breeding masterplan for the giant panda. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U.K., pp. 495-519.

Bambino, T.H., Hsueh, A.J., 1981. Direct inhibitory effect of glucocorticoid upon testicular luteinizing hormone receptors and steroidogenesis in vivo and in vitro. Endocrinology 108, 2142-2148.

Banchero, G.E., Clariget, R.P., Bencini, R., Lindsay, D.R., Milton, T.J.B., Martin, G.B., 2006. Endocrine and metabolic factors involved in the effect of nutrition on the production of colostrum in female sheep. Reproduction Nutrition Development 46, 447-460.

Baragona, K., 2001. Comment on: The pandas' habitat at Wolong Nature Reserve. Science 293, 603.

Bellemian, E., Swenson, J.E., Taberlet, P., 2006. Mating strategies in relation to sexually selected infanticide in a non-social carnivore: the brown bear. Ethology 112, 238-246.

Berger, J., Testa, J.W., Roffe, T., Monfort, S.L., 1999. Conservation endocrinology: a noninvasive tool to understand relationships between carnivore colonization and ecological carrying capacity. Conservation Biology 13, 980-989.

Bernard, R.T.F., Cumming, G.S., 1997. African bats: evolution of reproductive patterns and delays. The Quarterly Review of Biology 72, 253-274.

Blatchley, F.R., Donovan, B.T., 1976. Progesterone secretion during pregnancy and pseudopregnancy in the ferret. Journal of Reproduction and Fertility 46, 455-456.

Bonney, R.C., Wood, D.J., Kleiman, D.G., 1982. Endocrine correlates of behavioural oestrus in the female giant panda (*Ailuropoda melanoleuca*) and associated hormonal changes in the male. Journal of Reproduction and Fertility 64, 209-215.

Boone, W.R., Keck, B.B., Catlin, J.C., Casey, K.J., Boone, E.T., Dye, P.S., Schuett, R.J., Tsubota, T., Bahr, J.M., 2004. Evidence that bears are induced ovulators. Theriogenology 61, 1163-1169.

Boone, W.R., Richardson, M.E., Greer, J.A., 2003. Breeding behavior of the American black bear *Ursus americanus*. Theriogenology 60, 289-297.

Boonstra, R., 2005. Equipped for life: the adaptive role of the stress axis in male mammals. Journal of Mammalogy 86, 236-247.

Boonstra, R., Hubbs, A.H., Lacey, E.A., McColl, C.J., 2001a. Seasonal changes in glucocorticoid and testosterone concentrations in free-living arctic ground squirrels from the boreal forest of the Yukon. Canadian Journal of Zoology 79, 49-58.

Boonstra, R., McColl, C.J., Karels, T.J., 2001b. Reproduction at all costs: the adaptive stress response of male arctic ground squirrels. Ecology 82, 1930-1946

Bousfield, G.R., 1998. LH (luteinizing hormone). In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 1034-1055.

Brann, D.W., Mahesh, V.B., 1991. Role of corticosteroids in female reproduction. FASEB Journal 5, 2691-2698.

Brooks, T., Bruner, A.G., Bruner, J., DaFonesca, G.A.B., Liu, R., Sung, W., Yan, X., 2001. Comment on: The pandas' habitat at Wolong Nature Reserve. Science 293, 603.

Brown, J.L., 1997. Fecal steroid profiles in black-footed ferrets exposed to natural photoperiod. Journal of Wildlife Management 61, 1428-1436.

Brown, J.L., Walker, S.L., Moeller, T., 2004. Comparative endocrinology of cycling and non-cycling Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. General and Comparative Endocrinology 136, 360-370.

Brown, J.L., Wasser, S.K., Wildt, D.E., Graham, L., 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction 51, 776-786.

Brown, J.L., Wildt, D.E., 1997. Assessing reproductive status in wild felids by non-invasive faecal steroid monitoring. International Zoo Yearbook 35, 173-191.

Brown, J.L., Wildt, D.E., Graham, L.H., Byers, A.P., Collins, L., Barrett, S., Howard, J., 1995. Natural versus chorionic gonadotropin-induced ovarian responses in the clouded leopard (*Neofelis nebulosa*) assessed by fecal steroid analysis. Biology of Reproduction 53, 93-102.

Brown, J.L., Wildt, D.E., Wielebnowski, N., Goodrowe, K.L., Graham, L.H., Wells, S., Howard, J.G., 1996. Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. Journal of Reproduction and Fertility 106, 337-346.

Canivenc, R., Bonnin, M., 1979. Delayed implantation is under environmental control in the badger (*Meles meles L.*). Nature 278, 849-850.

Canivenc, R., Bonnin, M., 1981. Environmental control of delayed implantation in the European badger, (*Meles meles*). Journal of Reproduction and Fertility, Supplement 29, 25-33.

Carlstead, K., 1996. Effects of captivity on the behavior of wild animals. In: Kleiman, D.G., Allen, M.E., Thompson, K.V., Lumpkin, S. (Eds.), Wild mammals in captivity. University of Chicago Press, Chicago, IL, pp. 317-333.

Carlstead, K., Brown, J.L., Strawn, W., 1993. Behavioral and physiological correlates of stress in laboratory cats. Applied Animal Behaviour Science 38, 143-158.

Catton, C., 1990. Pandas. Facts On File Publications. New York, New York.

Cavigelli, S.A., 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemurs catta*. Animal Behaviour 57, 935-944.

Chang, K., Zhang, L., 2008. Steroid hormones and uterine vascular adaptation to pregnancy. Reproductive Sciences 15, 336-348.

Chang, K.-R., Mao, F.C., Yang, C.-C., Chan, F.-T., 2004. Seasonal changes in reproductive hormones of female Formosan black bears (*Ursus thibetanus formosanus*) in captivity. Endemic Species Research 6, 27-34.

Charmandari, E., Constantine, T., Chrousos, G., 2005. Endocrinology of the stress response. Annual Review of Physiology 67, 259-284.

Chaunduri, M., Kleiman, D.G., Wildt, D.E., Bush, M., Frank, E.S., Thau, R.B., 1988. Urinary steroid concentrations during natural and gonadotrophin-induced oestrus and pregnancy in the giant panda (*Ailuropoda melanoleuca*). Journal of Reproduction and Fertility 84, 23-28.

Chorn, J., Hoffman, R.S., 1978. *Ailuropoda melanoleuca*. Mammalian Species 110, 1-6.

Conaway, C.H., 1971. Ecological adaptation and mammalian reproduction. Biology of Reproduction 4, 239-247.

Concannon, P.W., Hansel, W., Visek, B., 1975. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. Biology of Reproduction 13, 112-119.

Concannon, P.W., McCann, J.P., Temple, M., 1989. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. Journal of Reproduction and Fertility 39, 3-25.

Conn, P.M., Jennes, L., Janovick, J.A., 1998. GnRH (gonadotropin-releasing hormone). In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 464-478.

Craighead, J.J., Craighead, F.C.J., Sumner, J., 1976. Reproductive cycles and rates in the grizzly bear, *Ursus arctos horribilis*, of the Yellowstone ecosystem. Third International Conference on Bears, pp. 337-356.

Creel, S., Creel, N.M., Mills, M.G.L., Monfort, S.L., 1997. Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. Behavioral Ecology 8, 298-306.

Creel, S.C., Monfort, S.L., Wildt, D.E., Waser, P.M., 1991. Spontaneous lactation is an adaptive result of pseudopregnancy. Nature 351, 660-662.

Cresswell, W.J., Harris, S., Cheeseman, C.L., Mallinson, P.J., 1992. To breed or not to breed: an analysis of the social and density-dependent constraints on the fecundity of female badgers (*Meles meles*). Philosophical Transactions: Biological Sciences 338, 393-407.

Cumming, D.C., Quigely, M.E., Yen, S.S.C., 1983. Acute suppression of circulating testosterone levels by cortisol in men. Journal of Clinical Endocrinology and Metabolism 57, 671-673.

Czekala, N., Lindburg, D., Durrant, B., Swaisgood, R., He, T., Tang, C., 1998. The estrogen profile, vaginal cytology, and behavior of a giant panda female during estrus. In: Zhang, A.a.G.H. (Ed.), Proceedings of the international symposium on the protection of the giant panda (*Ailuropoda melanoleuca*). Sichuan Publishing House of Science and Technology, Chengdu, China, pp. 111-113.

Czekala, N., McGeehan, L., Steinman, K., Xuebing, L., Gual-Sil, F., 2003. Endocrine monitoring and its application to the management of the giant panda. Zoo Biology 22, 389-400.

Daniel Jr., J.C., 1981. Delayed implantation in the northern fur seal (*Callorhinus ursinus*) and other pinnipeds. Journal of Reproduction and Fertility, Supplement 29, 35-50.

David, V.A., Sun, S., Zhang, Z., Shen, F., Zhang, G., Zhang, H., Xie, Z., Zhang, Y.P., Ryder, O.A., Ellis, S., Wildt, D.E., Zhang, A., O'Brien, S.J., 2006. Parentage assessment among captive giant pandas in China. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U.K.

Dehnhard, M., Hildebrandt, T., Knauf, T., Jewgenow, K., Kolter, L., Goritz, F., 2006. Comparative endocrine investigations in three bear species based on urinary steroid metabolites and volatiles. Theriogenology 66, 1755-1761.

DeMatteo, K.E., Porton, I.J., Kleiman, D.G., Asa, C.S., 2006. The effect of the male bush dog (*Speothos venaticus*) on the female reproductive cycle. Journal of Mammalogy 87, 723-732.

Dierenfeld, E.S., Hintz, H.F., Robertson, J.B., van Soest, P.J., Oftedal, O.T., 1982. Utilization of bamboo by the giant panda. Journal of Nutrition 112, 636-641.

Dloniak, S.M., French, J.A., Place, N.J., Weldele, M.L., Glickman, S.E., Holekamp, K.E., 2004. Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*). General and Comparative Endocrinology 114, 340-348.

Dobson, H., Smith, R.E., 1995. Stress and reproduction in farm animals. Journal of Reproduction and Fertility, Supplement 49, 451-461.

Durnin, M.E., Swaisgood, R.R., Czekala, N., Hemin, Z., 2000. The effect of radio collars on stress related behavior and hormones on the giant panda (*Ailuropoda melanoleuca*). Panda 2000 International Conference: Conservation Priorities for the New Millennium, San Diego, CA.

Durrant, B., Czekala, N., Olson, M., Anderson, A., Amodeo, D., Campos-Morales, R., Gual-Sill, F., Ramos-Garza, J., 2002. Papanicolaou staining of exfoliated vaginal epithelial cells facilitates the prediction of ovulation in the giant panda. Theriogenology 57, 1855-1864.

Durrant, B., Olson, M.A., Amodeo, D., Anderson, A., Russ, K.D., Campos-Morales, R., Gual-Sil, F., Garza, J.R., 2003. Vaginal cytology and vulvar swelling as indicators of impending estrus and ovulation in the giant panda (*Ailuropoda melanoleuca*). Zoo Biology 22, 313-321.

Durrant, B.S., Ravida, N., Spady, T., Cheng, A., 2006. New technologies for the study of carnivore reproduction. Theriogenology 66, 1729-1736.

Edwards, M.S., Wei, R., Hawes, J., Sutherland-Smith, M., Tang, C., Li, D., Hu, D., Zhang, G., 2006a. The neonatal giant panda: hand-rearing and medical management. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U.K., pp. 315-333.

Edwards, M.S., Zhang, G., Wei, R., Liu, X., 2006b. Nutrition and dietary husbandry. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U.K., pp. 101-158.

Ellis, S., Pan, W., Xie, Z., Wildt, D.E., 2006a. The giant panda as a social, biological and conservation phenomenon. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U.K., pp. 1-16.

Ellis, S., Snyder, R.J., Zhang, Q., Wei, R., Zhong, W., Lam, M., Sims, R., 2006b. Life histories and behavioural traits as predictors of breeding success. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U. K., pp. 87-100.

Enders, R.K., 1952. Reproduction in the mink (*Mustela vison*). Proceedings of the American Philosophical Society 96, 691-755.

Englehard, G.H., Ferguson, J.M., 1980. Adaptive hormone changes in harp seals, *Phoca groenlandica*, and grey seals, *Halicoerus grypus*, during the post natal period. General and Comparative Endocrinology 40, 434-445.

Erskine, M.S., 1998. Pseudopregnancy. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 120-124.

Fenske, M., 1997. Role of cortisol in the ACTH-induced suppression of testicular steroidogenesis in guinea pigs. Journal of Endocrinology 154, 407-414.

Ferguson, S.H., McLoughlin, P.D., 2000. Effect of energy availability, seasonality, and geographic range on bear life history. Ecography 23, 193-200.

Ferin, M., 1999. Stess and the reproductive cycle. The Journal of Clinical Endocrinology and Metabolism 84, 1768-1774.

Foley, C.A.H., Papageorge, S., Wasser, S.K., 2001. Noninvasive stress and reproductive measures of social and ecological pressures in free-ranging African elephants. Conservation Biology 15, 1134-1142.

Foresman, K., Mead, R., 1978. Luteal control of nidation in the ferret (*Mustela putorius*). Biology of Reproduction 18, 490-496.

Foresman, K.R., Daniel, J.C., 1983. Plasma progesterone concentrations in pregnant and non-pregnant black bears (*Ursus americanus*). Journal of Reproduction and Fertility 68, 235-239.

Fuchs, A.-R., Fields, M.J., 1998. Parturition, nonhuman mammals. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 703-716.

Funk, C.R., DeMayo, F.J., 1998. Progesterone actions on reproductive tract. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 6-16.

Gao, H.B., Tong, M.H., Hiu, Y.Q., Guo, Q.S., Ge, R.S., Hardy, M.P., 2002. Glucocorticoid induces apoptosis in rat leydig cells. Endocrinology 143, 130-138.

Garshelis, D.L., 2006. On the allure of noninvasive genetic sampling - putting a face to the name. Ursus 17, 109-123.

Garshelis, D.L., Hellgren, E.C., 1994. Variation in reproductive biology of male black bears. Journal of Mammalogy 75, 175-188.

Goymann, W., East, M.L., Wachter, B., Honer, O.P., Möstl, E., Van't Hof, T.J., Hofer, H., 2001. Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyenas. Proceedings of the Royal Society of London, Biology 268, 2453-2459.

Graham, L., Schwarzenberger, F., Mostel, E., Galama, W., Savage, A., 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biology 20, 227-236.

Graham, L.H., Goodrowe, K.L., Raeside, J.I., Liptrap, R.M., 1995. Non-invasive monitoring of ovarian function in several felid species by measurement of fecal etsradiol-17β and progestins. Zoo Biology 14, 223-237.

Hamlett, G.W.D., 1935. Delayed implantation and discontinuous development in the mammal. The Quarterly Review of Biology 10, 432-447.

Harlow, H.J., Beck, T.D.I., Walters, L.M., Greenhouse, S.S., 1990. Seasonal serum glucose, progesterone, and cortisol levels of black bears (*Ursus americanus*). Canadian Journal of Zoology 68, 183-187.

Hellgren, E.C., Vaughan, M.R., Gwazdauskas, F.C., Williams, B., Scalon, P.F., Kirkpatrick, R.L., 1991. Endocrine and electrophoretic profiles during pregnancy and nonpregnancy in captive female black bears. Canadian Journal of Zoology 69, 892-898.

Hensel, R.J., Troyer, W.A., Erickson, A.W., 1969. Reproduction in the female brown bear. Journal of Wildlife Management 33, 357-365.

Herrero, S., Hamer, D., 1977. Courtship and copulation of a pair of grizzly bears, with comments on reproductive plasticity and strategy. Journal of Mammalogy 58, 441-444.

Hess, R.A., 1998. Spermatogenesis, overview. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 539-545.

Hesterman, H., Wasser, S.K., Cockrem, J.F., 2005. Longitudinal monitoring of fecal testosterone in male Malayan sun bears (*U. malayanus*). Zoo Biology 24, 403-417.

Hinshelwood, M.M., 1998. Steroidogenesis, overview. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 644-653.

Hodges, J.K., Bevam, D.J., Celm, M., Hearn, J.P., Jones, D.M., Kleiman, D.G., Knight, J.A., Moore, H.D.M., 1984. Aspects of the reproductive endocrinology of the female giant panda (*Ailuropoda melanoleuca*) in captivity with special reference to the detection of ovulation and pregnancy. The Journal of the Zoological Society of London 203, 253-267.

Howard, J., Huang, Y., Wang, P., Li, D., Zhang, G., Hou, R., Zhang, Z., Durrant, B.S., Spindler, R.E., Zhang, H., Zhang, A., Wildt, D.E., 2006a. Role and efficiency of artificial insemination and genome resource banking. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U.K., pp. 469-494.

Howard, J., Kersey, D.C., Aitken-Palmer, C., Monfort, S.L., Wildt, D.E., 2008. Capacity of the giant panda to give birth after a single intrauterine insemination using precise ovulation detection. Society for the Study of Reproduction, 41st Annual Meeting. Society for the Study of Reproduction, Inc, Kailua-Kona, Hawaii.

Howard, J.G., Zhang, Z., Li, D., Huang, Y., Li, G., Zhang, M., Ye, Z., Zhang, J.P., Huang, S., Spindler, R.E., Zhang, H., Wildt, D.E., 2006b. Male reproductive biology in giant pandas in breeding programmes in China. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, UK, pp. 159-197.

Howland, B.E., Sanford, L.M., Palmer, W.M., 1985. Changes in serum levels of LH, FSH, prolactin, testosterone, and cortisol associated with season and mating in male pygmy goats. Journal of Andrology 6, 89-96.

Huang, Y., Wang, P.Y., Zhang, H.M., 2001. Efficiency of artificial insemination in giant pandas at the Wolong Breeding Center. Proceedings: American Society of Andrology, Journal of Andrology.

Huber, S., Palme, R., Arnold, W., 2003. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). General and Comparative Endocrinology 130, 48-54.

Ishikawa, A., Kikuchi, S., Katagiri, S., Sakamoto, H., Takahashi, Y., 2002. Efficiency of fecal steroid hormone measurement for assessing reproductive function in the Hokkaido brown Bear (*Ursus arctos yesoensis*). Japanese Journal of Veterinary Research 50, 17-27.

Ishikawa, A., Sakamoto, H., Katagiri, S., Takahashi, Y., 2003. Changes in sexual behavior and fecal steroid hormone concentrations during the breeding season in female Hokkaido brown bears (*Ursus arctos yesoensis*) under captive condition. Journal of Veterinary Medical Science 65, 99-102.

Johnson, L., Falk, G.U., Spoede, G.E., 1998. Male reproductive system, nonhuman mammals. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, Ca, pp. 49-60.

Johnston, L.A., Donoghue, A.M., Igo, W., Simmons, L.G., Wildt, D.E., Rieffenberger, J., 1994. Oocyte recovery and maturation in the American black bear (*Ursus americanus*): a model for endangered ursids. the Journal of Experimental Zoology 269, 53-61.

Juengel, J.L., McIntush, E.W., Niswender, G.D., 1998. Corpus luteum. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 703-709.

Kenagy, G.J., Place, N.J., 2000. Seasonal changes in plasma glucocorticosteroids of free-living female yellow-pine chipmunks: effects of reproduction and capture and handling. General and Comparative Endocrinology 117, 189-199.

Khan, M.Z., Altman, J., Isani, S.S., Yu, J., 2002. A matter of time: evaluating the storage of fecal samples for steroid analysis. General and Comparative Endocrinology 128, 57-64.

Kiso, Y., Yamauchi, S., 1984. Histochemical study on hydroxysteroid dehydrongenases in the trophoblast of the dog placenta. Japan Journal of Veterinary Science 46, 219-223.

Kleiman, D.G., 1984. Social and reproductive behavior of the giant panda (*Ailuropoda melanoleuca*). Proceedings of the International Symposium on Giant Panda, pp. 45-58.

Kleiman, D.G., Karesh, W.B., Chu, P.R., 1979. Behavioural changes associated with oestrus in the giant panda Ailuropoda melanoleuca with comments on female proceptive behaviour. International Zoo Yearbook 19, 217-223.

Kondo, M., Kishi, H., Kojima, C., Jin, W., Suzuki, J., Shimizu, K., Itoh, M., Ohkura, S., Tsukamura, H., Maeda, K.I., Watanabe, G., Taya, K., 2003. Lactation-associated infertility in Japanese monkeys (*Macaca fuscata*) during the breeding season. Zoo Biology 22, 65-76.

Kontoleon, A., Swanson, T., 2003. The willingness to pay for property rights for the giant panda: can a charismatic species be an instrument for nature conservation? Land Economics 79, 483-499.

Kubokawa, K., Ishii, S., Tajima, H., Saitou, K., Tanabe, K., 1992. Analysis of sex steroids in the feces of giant pandas. Zoological Science 9, 1017-1023.

Lacy, R.C., 1993. VORTEX: A computer simulation model for population viability analysis. Wildlife Research 20, 45-65.

Lacy, R.C., 1995. Clarification of genetic terms and their use in the management of captive populations. Zoo Biology 14, 565-578.

Lindburg, D.G., Czekala, N.M., Swaisgood, R.R., 2001. Hormonal and behavioral relationships during estrus in the giant panda. Zoo Biology 20, 537-543.

Lindenfors, P., Dalen, L., Angerbjorn, A., 2003. The monophyletic origin of delayed implantation in carnivores and it's implications. Evolution 57, 1952-1956.

Lindsay, J.R., Neiman, L.K., 2005. The hypothalamic-pituitary-adrenal axis in pregnancy: challenges in disease detection and treatment. Endocrine Reviews 26, 775-799.

Lindzey, J., Korach, K.S., 1998. Estrogen action on the female reproductive tract. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 79-86.

Liptrap, R.M., 1993. Stress and reproduction in domestic animals. Annals of the New York Academy of Sciences 697, 275-284.

Liu, J., Chen, Y., Guo, L., Gu, B., Liu, H., Hou, A., Liu, X., Sun, L., Liu, D., 2006. Stereotypic behavior and fecal cortisol level in captive giant pandas in relation to environmental enrichment. Zoo Biology 25, 445-459.

Lopes, F.L., Desmarais, J.A., Murphy, B.D., 2004. Embryonic diapause and its regulation. Reproduction 128, 669-678.

Lumpkin, S., Seidensticker, J., 2002. Smithsonian book of giant pandas. Smithsonian Institution Press. Washington, D.C.

Lyimo, Z.C., Nielen, M., Ouweltjes, W., Kruip, T.A.M., van Eerdenburg, F.J.C.M., 2000. Relationship among estradiol, cortisol and intensity of estrous behavior in dairy cattle. Theriogenology 53, 1783-1795.

Lynch, J.W., Ziegler, T.E., Strier, K.B., 2002. Individual and seasonal variation in fecal testosterone and cortisol levels of wild male tufted capuchin monkeys, *Cebus apella nigritus*. Hormones and Behavior 41, 275-287.

MacDonald, E., Czekala, N., Gual-Sil, F., Nakao, T., 2006. Urinary testosterone and cortisol metabolites in male giant pandas *Ailuropoda melanoleuca* in relation to breeding, housing, and season. Acta Zoologica Sinica 52, 242-249.

Mainka, S.A., Cooper, R.M., Mao, L., Guanlu, Z., 1990. Urinary hormones in two juvenile female giant pandas (*Ailuropoda melanoleuca*). Journal of Zoo and Wildlife Medicine 21, 334-341.

Masui, M., Hiramatsu, H., Nose, N., Nakazato, R., Sagawa, Y., Tajima, H., Saito, K., 1989. Successful artificial insemination in the giant panda (Ailuropoda melanoleuca) at Ueno Zoo. Zoo Biology 8, 17-26.

McCracken, J.A., 1998. Luteolysis. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, pp. 1083-1094.

McGeehan, L., Li, X., Jackintell, L., Huang, S., Wang, A., Czekala, N.M., 2002. Hormonal and behavioral correlates of estrus in captive giant pandas. Zoo Biology 21, 449-466.

McLachlan, R.I., Wreford, N.G., O'Donnell, L., de Krester, D.M., Robertson, D.M., 1996. The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. Journal of Endocrinology 148, 1-9.

McMillin, J.M., Seal, U.S., Rogers, L., Erickson, A.W., 1976. Annual testosterone rhythm in the black bear (*Ursus americanus*). Biology of Reproduction 15, 163-167.

Mead, R.A., 1981. Delayed implantation in mustelids, with special emphasis on the spotted skunk. Journal of Reproduction and Fertility, Supplement 29, 11-24.

Mead, R.A., 1989. The physiology and evolution of delayed implantation in carnivores. In: Gittleman, J.L. (Ed.), Carnivore behavior, ecology and evolution. Cornell University Press, Ithica, NY, pp. 437-464.

Mead, R.A., 1993. Embryonic diapause in vertebrates. Journal of Experimental Zoology 266, 629-641.

Mead, R.A., Bowles, M., Starypan, G., Jones, M., 1993. Evidence for pseudopregnancy and induced ovulation in captive wolverines (*Gulo gulo*). Zoo Biology 12, 353-358.

Mead, R.A., Rector, M., Starypan, G., Neirinckx, S., Jones, M., DonCarlos, M.N., 1991. Reproductive biology of captive wolverines. Journal of Mammalogy 72, 807-814.

Meyer, H.H.D., Rohleder, M., Streich, W.J., Goltenboth, R., Ochs, A., 1997. Sexual steroids profiles and ovarian activities of the female panda Yan Yan in the Zoo of Berlin. Berliner und Münchener Tierärztliche Wochenschrift 110, 143-147.

Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology 138, 189-199.

Ministry of Forestry, 1992. National conservation project for the giant panda and its habitat. Ministry of Forestry, Beijing, China.

Moberg, G.P., 1991. How behavioral stress disrupts the endocrine control of reproduction in domestic animals. Journal of Dairy Science 74, 304-311.

Monfort, S.L., 2003. Non-invasive endocrine measures of reproduction and stress in wild populations. In: Wildt, D.E., Holdt, W.V., Pickard, A.R. (Eds.), Reproduction and integrated conservation science. Cambridge University Press, Cambridge, UK.

Monfort, S.L., Arthur, N.P., Wildt, D.E., 1991. Monitoring ovarian function and pregnancy by evaluating excretion of urinary oestrogens in semi-free-ranging Prezwalski's horses (*Equus prezwalskii*). Journal of Reproduction and Fertility 91, 155-164.

Monfort, S.L., Brown, J.L., Wildt, D.E., 1993a. Episodic and seasonal rhythms of cortisol secretion in male Eld's deer (*Cervus eldi thamin*). Journal of Endocrinology 138, 41-49.

Monfort, S.L., Brown, J.L., Wood, T.C., Wemmer, C., Vargas, A., Williamson, L.R., Wildt, D.E., 1993b. Seasonal patterns of basal and GnRH-induced LH, FSH and testosterone secretion in Eld's deer stags (*Cervus eldi thamin*). Journal of Reproduction and Fertility 98, 481-488.

Monfort, S.L., Dahl, K.D., Czekala, N.M., Stevens, L., Bush, M., Wildt, D.E., 1989. Monitoring ovarian function and pregnancy in the giant panda (*Ailuropoda melanoleuca*) by evaluating urinary bioactive FSH and steroid metabolites. Journal of Reproduction and Fertility 85, 203-212.

Monfort, S.L., Harvey, E., Geutrs, L., Simmons, H.A., Williamson, L.R., Wildt, D.E., 1995. Urinary 3α, 17β-androstanediol glucuronide is a measure of androgenic status in Eld's deer stags (*Cervus eldi thamin*). Biology of Reproduction 53, 700-706.

Monfort, S.L., Mashburn, K.L., Brewer, B.A., Creel, S.R., 1998. Evaluating adrenal activity in African wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. Journal of Zoo and Wildlife Medicine 29, 129-133.

Monfort, S.L., Schwartz, C.C., Wasser, S.K., 1993c. Monitoring reproduction in captive moose using urinary and fecal steroid metabolites. Journal of Wildlife Management 57, 400-407.

Monfort, S.L., Wasser, S.K., Mashburn, K.L., Burke, M., Brewer, B.A., Creel, S.R., 1997. Steroid metabolism and validation of noninvasive endocrine monitoring in the African wild dog (*Lycaon pictus*). Zoo Biology 16, 533-548.

Moreira, N., Monterio-Filho, E.L.A., Moraes, W., Swanson, W.F., Graham, L.H., Pasquali, O.L., Gomes, M.L.F., Morais, R.N., Wildt, D.E., Brown, J.L., 2001. Reproductive steroid hormones and ovarian activity in felids of the Leopardus genus. Zoo Biology 20, 103-116.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domestic Animal Endocrinology 23, 67-74.

Munck, A., Guyre, P.M., Holbrook, N.J., 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Reviews 5, 25-44.

Munro, C.J., Lasley, B.L., 1988. Non-radiometric methods for immunoassay of steroid hormones. In: Albertson, B.D., Haseltine, F.D. (Eds.), Non-radiometric assays: technology and application in polypeptide and steroid hormone detection. Allan Liss Corporation, New York, pp. 289-329.

Murata, K., Tanioka, M., Murakami, N., 1986. The relationship between the pattern of urinary oestrogen and behavioural changes in the giant panda *Ailuropoda melanoleuca*. International Zoo Yearbook 24/25, 274-279.

Narushima, E., Hayashi, Y., Hara, T., Nose, N., Komiya, T., 2003. Changes in urinary concentrations of total estrogen and pregnanediol in a female giant panda (*Ailuropoda melanoleuca*) from 1991 to 2000. Zoo Biology 22, 383-387.

Nelson, R.A., Wellik, D.L., McMillin, J.M., Palumbo, P.J., 1978. Role of testosterone in hibernating black bears. Physiologist 21, 84.

Nelson, R.J., Demas, G.E., 1996. Seasonal changes in immune function. The Quarterly Review of Biology 71, 511-548.

Neville, M.C., 2006. Lactation and its hormonal control. In : Knobil, E. (Ed.), Knobil and Neill's Physiology of Reproduction. Academic Press, St. Louis, MO, pp.2993-3054.

Norwitz, E.R., Schust, D.J., Fisher, S.J., 2001. Implantation and the survival of early pregnancy. New England Journal of Medicine 345, 1400-1408.

O'Brien, S.J., 1987. The ancestry of the giant panda. Scientific American 257, 102-107.

O'Brien, S.J., Nash, W.G., Wildt, D.E., Bush, M.E., Benveniste, R.E., 1985. A molecular solution to the riddle of the giant panda's phylogeny. Nature 317, 140-144.

Onclin, K., Verstegen, J., 1997. In vivo investigation of luteal function in dogs: effects of cabergoline, a dopamine antagonist, and prolactin on progesterone secretion during midpregnancy and diestrus. Domestic Animal Endocrinology 14, 25-38.

Onuma, M., Suzuki, M., Ohtaishi, N., 2001. Reproductive pattern of the sun bear (*Helarctos malayanus*) in Sarawak, Malaysia. Journal of Veterinary Medical Science 63, 293-297.

Onuma, M., Suzuki, M., Uchida, E., Niiyama, M., Ohtaishi, N., 2002. Annual changes in fecal estradiol-17β concentrations of the sun bear (*Helarctos malayanus*) in Sarawak, Malaysia. Journal of Veterinary Medical Science 64, 309-313.

Owen, M.A., Czekala, N.M., Swaisgood, R.R., Steinman, K., Lindburg, D.G., 2005. Seasonal and diurnal dynamics of glucocorticoids and behavior in giant pandas. Ursus 16, 208-221.

Owen, M.A., Swaisgood, R.R., Czekala, N.M., Steinman, K., Lindburg, D.G., 2004. Monitoring stress in captive giant pandas (*Ailuropoda melanoleuca*): behavioral and hormonal responses to ambient noise. Zoo Biology 23, 147-164.

Palmer, S.S., Nelson, R.A., Ramsay, M.A., Stirling, I., Bahr, J.M., 1988. Annual changes in serum sex steroid in male and female black (*Ursus americanus*) and polar (*Ursus maritimus*) bears. Biology of Reproduction 38, 1044-1050.

Papke, R.L., Concannon, P.W., Travis, H.F., Hansel, W., 1980. Control of luteal function and implantation in the mink by prolactin. Journal of Animal Science 50, 1102-1107.

Perry, R., 1969. The world of the giant panda. Bantam Books. New York, NY.

Plotsky, P.M., Cunningham, E.T.J., Widmaier, E.P., 1989. Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. Endocrine Reviews 10, 437-458.

Pottinger, T.G., 1999. The impact of stress on animal reproductive activities. In: Baum, P.H.M. (Ed.), Stress physiology in animals. CRC Press, Boca Raton, FL.

Powell, D.M., Carlstead, K., Tarous, L.R., Brown, J.L., Monfort, S.L., 2006. Effects of construction noise on behavior and cortisol levels in a pair of captive giant pandas (*Ailurpoda melanoleuca*). Zoo Biology 25, 391-408.

Predergast, B.J., Nelson, R.J., Zucker, I., 2002. Mammalian seasonal rhythms: behavior and neuroendocrine substrates. In: Pfaff, D., Arnold, A., Etgan, A., Fahrbach, S., Rubin, R. (Eds.), Hormones, Brain, and Behavior. Academic Press, San Diego, Ca., pp. 93-156.

Quensel, H., Prunier, A., 1995. Endocrine bases of lactional anoestrus in the sow. Reproduction Nutrition Development 35, 395-414.

Ramsay, M.A., Stirling, I., 1988. Reproductive biology and ecology of female polar bears. Journal of Zoology London 214, 601-634.

Reid, D.G., Gong, J., 1999. Giant panda conservation action plan. In: Servheen, C., Herrero, S., Peyton, B. (Eds.), Bears; status survey and conservation action plan. IUCN, Gland, Switzerland, pp. 241-254.

Reid, D.G., Jinchu, H., Sai, D., Wei, W., Yan, H., 1989. Giant panda *Ailuropoda melanoleuca* behaviour and carrying capacity following a bamboo die-off. Biological Conservation 49, 85-104.

Renfree, M.B., 1995. Monotreme and marsupial reproduction. Reproduction, Fertility and Development 7, 1003-1020.

Renfree, M.B., Calaby, J.H., 1981. Background to delayed implantation and embryonic diapause. Journal of Reproduction, Fertility and Development 29, 1-9.

Renfree, M.B., Shaw, G., 2000. Diapause. Annual Review of Physiology 62, 353-375.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology 128, 1-24.

Roser, J.F., 1998. Equine chorionic gonadotropin. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 29-37.

Sakkinen, H., Tornberg, J., Goddard, P.J., Eloranta, E., Ropstad, E., Saarela, S., 2004. The effect of blood sampling method on indicators of physiological stress in reindeer (*Rangifer tarandus tarandus*). Domestic Animal Endocrinology 26, 87-98.

Salesa, M.J., Anton, M., Peigne, S., Morales, J., 2006. Evidence of a false thumb in a fossil carnivore clarifies the evolution of pandas. Proceedings of the National Academy of Sciences 103, 379-382.

Sandell, M., 1990. The evolution of seasonal delayed implantation. The Quarterly Review of Biology 65, 23-42.

Sands, J., Creel, S., 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. Animal Behaviour 67, 387-396.

Sanson, G., Brown, J.L., Farstad, W., 2005. Non-invasive faecal steroid monitoring of ovarian and adrenal activity in farmed blue fox (*Alopex lagopus*) females during late pregnancy, parturition and lactation onset. Animal Reproduction Science 87, 309-319.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21, 55-89.

Sato, M., Tsubota, T., Komatsu, T., Watanabe, G., Taya, K., Murase, T., Kita, I., Kudo, T., 2001. Changes in sex steroids, gonadotropins, prolactin, and inhibin in pregnant and nonpregnant Japanese black bears (*Ursus thibetanus japonicus*). Biology of Reproduction 65, 1006-1013.

Schaller, G., Jinchu, H., Wenshi, P., Jing, Z., 1985. The giant pandas of Wolong. University of Chicago Press. Chicago, IL.

Schiml, P.A., Mendoza, S.P., Saltzman, W., Lyons, D.M., Mason, W.A., 1996. Seasonality in squirrel monkeys (*Saimiri scirureus*): social facilitation by females. Physiology and Behavior 60, 1105-1113.

Schneider, J.E., 2004. Energy balance and reproduction. Physiology and Behavior 81, 289-317.

Schoemaker, N.J., Wolfswinkel, J., Mol, J.A., Voorhout, G., Kik, M.J.L., Lumeij, J.T., Rijnberk, A., 2004. Urinary glucocorticoid excretion in the diagnosis of hyperadrenocorticism in ferrets. Domestic Animal Endocrinology 27, 13-24.

Schulz, L.C., Nelson, R.A., Pyter, L.M., Bahr, J.M., 2003. Induction of pseudopregnancy in the American black bear (*Ursus americanus*). Journal of Experimental Zoology 298A, 162-166.

Schwartz, M.K., Monfort, S.L., 2008. Genetic and endocrine tools for carnivore surveys. In: Long, R.A., MacKay, P., Ray, J.C., Zielinski, W.J. (Eds.), Noninvasive survey methods for North American carnivores. Island Press, Washington , D.C., pp. 228-250.

Schwarzenberger, F., Fredriksson, G., Schaller, K., Kolter, L., 2004. Fecal steroid analysis for monitoring reproduction in the sun bear (*Helarctos malayanus*). Theriogenology 62, 1677-1692.

Senger, P.L., 2003. Pathways to pregnancy and parturition, second edition. Current Conceptions, Inc. Pullman, WA.

Shuling, Z., Zhao, Q., Zhong, X., Wildt, D.E., Seal, U.S., 1997. Report of the giant panda captive management planning workshop, Chengdu, China. In: Shuling, Z., Zhao, Q., Zhong, X., Wildt, D.E., Seal, U.S. (Eds.), IUCN - World Conservation Union/SSC Conservation Breeding Specialist Group. IUCN, Apple Valley, MN, p. 266.

Smith, M.S., McDonald, L.E., 1974. Serum levels of luteinizing hormone and progesterone during the estrous cycle, pseudopregnancy and pregnancy in the dog. Endocrinology 94, 404-412.

Smith, V.G., Edgerton, L.A., Hafs, H.D., Convey, E.M., 1973. Bovine serum estrogens, progestins and glucocorticoids during late pregnancy, parturition and early lactation. Journal of Animal Science 36, 391-396.

Songsasen, N., Rodden, M., Brown, J.L., Wildt, D.E., 2006. Patterns of fecal gonadal hormone metabolites in the maned wolf (*Chrysocyon brachyurus*). Theriogenology 66, 1743-1750.

Spady, T.J., Lindburg, D.G., Durrant, B.S., 2007. Evolution of reproductive seasonality in bears. Mammal Review 37, 21-53.

Stabenfeldt, G.H., Daels, P.F., Munro, C.J., Kindahl, H., Huges, J.P., Lasley, B., 1991. An oestrogen conjugate enzyme immunoassay for monitoring pregnancy in the mare: limitations of the assay between days 40 and 70 of gestation. Journal of Reproduction and Fertility, Supplement 44, 37-44.

Staley, A.M., Blanco, J.M., Dufty Jr., A.M., Wildt, D.E., Monfort, S.L., 2007. Fecal steroid monitoring for assessing gonadal and adrenal activity in the golden eagle and peregrine falcon. Journal of Comparative Physiology B: Biochemical, Systemic , and Environmental Physiology 177, 609-622.

Steinetz, B.G., Brown, J.L., Roth, T.L., Czekala, N., 2005. Relaxin concentrations in serum and urine of endangered species: correlations with physiologic events and use as a marker of pregnancy. Annals of the New York Academy of Sciences 1041, 367–378.

Steinman, K., Monfort, S.L., McGeehan, L., Kersey, D., Gual-Sil, F., Snyder, R., Wang, P., Nakao, T., Czekala, N., 2006. Endocrinology of the giant panda and application of hormone technology to species management. In: Wildt, D.E., Zhang, A., Zhang, D., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, pp. 198-230.

Stoebel, D.P., Moberg, G.P., 1982. Effect of adrenocorticotropin and cortisol and luteinizing hormone surge and estrous behavior of cows. Journal of Dairy Science 65, 1016-1024.

Strier, K.B., Ziegler, T.E., Wittwer, D.J., 1999. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (*Brachyteles arachnoides*). Hormones and Behavior 35, 125-134.

Sutherland-Smith, M., Morris, P.J., Silverman, S., 2004. Pregnancy detection and fetal monitoring via ultrasound in a giant panda (*Ailuropoda melanoleuca*). Zoo Biology 23, 449-461.

Swaisgood, R.R., Ellis, S., Frothman, D.L., Sheperdson, D., 2003. Improving well-being for captive giant pandas: theoretical and practical issues. Zoo Biology 22, 347-354.

Swaisgood, R.R., Lindburg, D.G., Zhang, H., 2002. Discrimination of oestrus in giant pandas (*Ailuropoda melanoleuca*) via chemical cues in urine. Journal of Zoology 257, 381-386.

Swaisgood, R.R., Owen, M.A., Czekala, N.M., Mauroo, N., Hawk, K., Tang, J.C.L., 2006a. Evaluating stress and well-being in the giant panda: a system for monitoring. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, pp. 299-314.

Swaisgood, R.R., Zhang, G., Zhou, X., Zhang, H., 2006b. The science of behavioral management: creating biologically relevant living environments in captivity. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, pp. 274-298.

Talbot, S.L., Shields, G.F., 1996. A phylogeny of the bears (*Ursidae*) inferred from complete sequences of three mitochondrial genes. Molecular Phylogenetics and Evolution 5, 567-575.

Taussky, H.H., 1954. A microcolormetric determination of creatinine in urine by the Jaffe reaction. Journal of Biological Chemistry 208, 853-861.

Thom, M.D., Johnson, D.D.P., MacDonald, D.W., 2004. The evolution and maintenance of delayed implantation in the *mustelidae* (Mammalia: Carnivora). Evolution 58, 175-183.

Tilbrook, A.J., Turner, A.I., Clark, I.J., 2000. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Reviews of Reproduction 5, 105-113.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Annals of the New York Academy of Sciences 1046, 54-74.

Tsubota, T., Howell-Skalla, L., Boone, W.R., Garshelis, D.L., Bahr, J.M., 1998. Serum progesterone, estradiol, luteinizing hormone and prolactin profiles in the female black bear (*Usus americanus*). Animal Reproduction Science 53, 107-118.

Tsubota, T., Kanagawa, H., 1989. Annual changes in serum testosterone levels and spermatogenesis in the Hokkaido brown bear, *Ursus arctos yesoensis*. The Journal of the Mammalogical Society of Japan 14, 11-17.

Tsubota, T., Kanagawa, H., Yamamoto, K., Mano, T., Yamanaka, M., Kita, I., Tiba, T., 1992. Serum progesterone concentrations using P-EIA kit in captive and free-ranging Hokkaido brown bears (*Ursus arctos yesoensis*). The Journal of Veterinary Medical Science 54, 1-5.

Turner, A.I., Hemsworth, P.H., Hughes, P.E., Canny, B.J., Tilbrook, A.J., 1998. The effect of repeated boar exposure on cortisol secretion and reproduction in gilts. Animal Reproduction Science 51, 143-154.

Turner, A.I., Hemsworth, P.H., Tilbrook, A.J., 2005. Susceptibility of reproduction in female pigs to impairment by stress or elevation of cortisol. Domestic Animal Endocrinology 29, 398-410.

USFWS, 1998. Policy on giant panda permits. In: Interior, D.o. (Ed.). Federal Register, p. 24pgs.

Velloso, A.L., Wasser, S.K., Monfort, S.L., Dietz, J.M., 1998. Longitudinal fecal steroid excretion in maned wolves (*Chrysocyon brachyurus*). General and Comparative Endocrinology 112, 96-107.

Verhage, H.G., Beamer, N.B., Brenner, R.M., 1976. Plasma levels of estradiol and progesterone in the cat during polyestrus, pregnancy and pseudopregnancy. Biology of Reproduction 14, 579-585.

von der Ohe, C.G., Servheen, C., 2002. Measuring stress in mammals using fecal glucocorticoids: opportunities and challenges. Wildlife Society Bulletin 30, 1215-1225.

von der Ohe, C.G., Wasser, S.K., Hunt, K.E., Servheen, C., 2004. Factors associated with fecal glucocorticoids in Alaskan brown bears (*Ursus arctos horribilis*). Physiological and Biochemical Zoology 77, 313-320.

Walker, S.L., Waddell, W.T., Goodrowe, K.L., 2002. Reproductive endocrine patterns in captive female and male red wolves (*Canis rufus*) assessed by fecal and serum hormone analysis. Zoo Biology 21, 321-335.

Wasser, S.K., Davenport, B., Ramage, E.R., Hunt, K.E., Parker, M., Clarke, C., Stenhouse, G., 2004. Scat detection dogs in wildlife research and management: application to grizzly and black bears in the Yellowhead Ecosystem, Alberta, Canada. Canadian Journal of Zoology 82, 475-492.

Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, C.M., Bechert, U., Millspaugh, J.J., Larson, S., Monfort, S.L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. General and Comparative Endocrinology 120, 260-275.

Wasser, S.K., Monfort, S.L., Southers, J., Wildt, D.E., 1994. Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*). Journal of Reproduction and Fertility 101, 213-220.

Wasser, S.K., Monfort, S.L., Wildt, D.E., 1991. Rapid extraction of steroids for measuring reproductive cyclicity and early pregnancy in free-ranging yellow baboons (*Papio cynocephalus cynocephalus*). Journal of Reproduction and Fertility 92, 415-423.

Wasser, S.K., Papageorge, S., Foley, C., Brown, J.L., 1996. Excretory fate of estradiol and progesterone in the African Elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. General and Comparative Endocrinology 102, 255-262.

Weingrill, T., Gray, D.A., Barrett, L., Haenzi, S.P., 2004. Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproductive state and environmental factors. Hormones and Behavior 45, 259-269.

Weir, B.J., Rowlands, I.W., 1973. Reproductive strategies of mammals. Annual Review of Ecological Systems 4, 139-163.

Welsh Jr., T.H., Kemper-Green, C.N., Livingston, K.N., 1998. Stress and Reproduction. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, Ca, pp. 662-674.

White Jr., D., Berardinelli, J.G., Aune, K.E., 1998. Reproductive characteristics of the male grizzly bear in the continental United States. Ursus 10, 497-501.

White Jr., D., Berardinelli, J.G., Aune, K.E., 2005. Seasonal differences in spermatogenesis, testicular mass and serum testosterone concentrations in the grizzly bear. Ursus 16, 198-207.

Whitten, P.L., Brockman, D.K., Stavisky, R.C., 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearbook of Physical Anthropology 41, 1-23.

Wielebnowski, N., 2003. Stress and distress: evaluating their impact for the well-being of zoo animals. Journal of the American Veterinary Medical Association 223, 973-977.

Wildt, D.E., Ellis, S., Janssen, D., Buff, J., 2003. Toward more effective reproductive science for conservation. In: Holdt, W.V., Pickard, A.R., Rodger, J.C., Wildt, D.E. (Eds.), Reproductive science and integrated conservation. Cambridge University Press, Cambridge, UK.

Wimsatt, W.A., 1963. Delayed implantation in the Ursidae, with particular reference to the black bear (*Ursus americanus pallus*). In: Enders, A.C. (Ed.), Delayed Implantation. The University of Chicago Press, Chicago, Il, pp. 49-76.

Wyss, A., Flynn, J.J., 1993. A phylogenetic analysis and definition of the Carnivora. In: Szalay, F.S., Novacek, M.J., McKenna, M.C. (Eds.), Mammal Phylogeny. Springer Verlag, New York, pp. 32-52.

Xie, Z., Gipps, J., 2007. International Giant Panda (*Ailuropoda melanoleuca*) Studbook. Chinese Association of Zoological Gardens. Beijing, China.

Yamada, J.K., Durrant, B.S., 1989. Reproductive parameters of clouded leopards (*Neofelis nebulosa*). Zoo Biology 8, 223-231.

Yamaguchi, N., Dugdale, H.L., MacDonald, D.W., 2006. Female receptivity, embryonic diapause, and superfetation in the European badger (*Meles meles*): implications for the reproductive tactics of males and females. The Quarterly Review of Biology 81, 33-48.

Yiming, L., Zhongwei, G., Yang, Q., Yushan, W., Niemela, J., 2003. The implications of poaching for giant panda conservation. Biological Conservation 111, 125-136.

Yoshinaga, K., 1998. Estrogen secretion, regulation of. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, CA, pp. 108-118.

Young, A.J., Monfort, S.L., Clutton-Brock, T.H., 2008. The causes of physiological suppression among female meerkats: a role for subordinate restraint due to the threat of infanticide. Hormones and Behavior 53, 131-139.

Young, K.M., Brown, J.L., Goodrowe, K.L., 2001. Characterization of reproductive cycles and adrenal activity in the black-footed ferret (*Mustela nigripes*) by fecal hormone analysis. Zoo Biology 20, 517-536.

Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Monfort, S.L., Brown, J.L., 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. General and Comparative Endocrinology 137, 148-165.

Zhan, X., Li, M., Zhang, Z., Goossens, B., Chen, Y., Wang, H., Bruford, M.W., Wei, F., 2006. Molecular censusing doubles giant panda population estimate in a key nature reserve. Current Biology 16, 451-452.

Zhang, P., Shao, G., Zhao, G., Le Master, D.C., Parker, G.R., Dunning Jr., J.B., Li, Q., 2000. China's forest policy for the 21st Century. Science 288, 2135-2136.

Zhang, Y.-P., Ryder, O.A., 1993. Mitochondrial DNA sequence evolution in the *Arctoidae*. Proceedings of the National Academy of Sciences 90, 9556-9561.

Zhang, Y.-P., Ryder, O.A., 1994. Phylogenetic relationships of bears (the *Ursidae*) inferred from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 3, 351-359.

Zhang, Z., Zhang, A., Hou, R., Wang, J., Li, G., Fei, L., Wang, Q., Loeffler, K., Wildt, D.E., Maple, T.L., McManamon, R., Ellis, S., 2006. Historical perspective of breeding giant pandas ex situ and high priorities for the future. In: Wildt, D.E., Zhang, A., Zhang, H., Janssen, D.L., Ellis, S. (Eds.), Giant pandas: biology, veterinary medicine and management. Cambridge University Press, Cambridge, U. K., pp. 455-468.

Zhu, X., Lindburg, D.G., Pan, W., Froney, K.A., Wang, D., 2001. The reproductive strategy of giant pandas (*Ailuropoda melanoleuca*): infant growth and development and mother-infant relationships. Journal of Zoology 253, 141-155.

Zirkin, B.R., 1998. Spermatogenesis, hormonal control of. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, San Diego, Ca, pp. 556-563.

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