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Methods of estrus detection and correlates of the reproductive cycle in the sun bear (*Helarctos malayanus*)

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Abstract

The objective was to explore multiple methods for detecting and characterizing the reproductive cycle of the sun bear (*Helarctos malayanus*). Thirteen *H. m. euryspilus* females, loaned from the Malaysian government to US zoos, were used. Fecal metabolite concentrations of estrogen and progesterone were compared to vaginal cytology, changes in genital appearance, and behavior (videotapes and zookeeper observations). Cytology and video behavior were characterized during five hormonally defined states: high, low, and baseline progesterone, estrus, and high estrogen. Among states, there were significant differences in cytology and behavior. Sexual, affiliative, and stereotypic behaviors were highest during estrus, whereas affiliative and social behaviors were lowest during high progesterone. In this captive breeding population, 30.8% of females cycled two or three times a year, 30.8% cycled once a year, and 38.5% did not cycle during this study. Inter-estrus intervals were (mean ± SEM) 115.7 ± 6.3 d (range, 101–131). Spearman rank correlations were significant between both ordinal sexual and affiliative behaviors and vulva swelling and color. Sexual behavior was significantly positively correlated with superficial and keratinized cells, but negatively correlated with parabasal and basophilic cells in cycling females (opposite pattern for appetitive behavior). In conclusion, data for cytology, vulva changes and behavior were consistent with, and complementary to, hormonal data; collectively, they delineated estrus and identified specific reproductive types.

Keywords: Sun bear; Estrus; Vaginal cytology; Fecal hormone concentrations; Reproductive behavior

1. Introduction

Sun bears (*Helarctos malayanus*) are distributed throughout the low and tropical forests of Southeast Asia [1]. However, their range is shrinking, with further decline and fragmentation anticipated [2]. The subspecies *H. m. euryspilus* is found only on the island of Borneo and is notably smaller than their mainland counterparts [3,4]. The sun bear is listed by Convention on International Trade in Endangered Species (CITES) as Appendix I and falls under the IUCN category "Vulnerable" [5]. Captive breeding efforts in North American zoos have met with limited success, leading to a steady decline in the captive population [6].

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Very few studies of female sun bear reproductive biology have been conducted. Schwarzenberger et al [7,8] studied hormone profiles of captive female sun bears in European zoos; however, their behavioral data were limited to the timing of mating [7,8]. They also presented hormone metabolite data on two free-ranging females [8]. Onuma et al [9,10] examined bears that were captive *in situ*. They collected fecal samples and immobilized a female to obtain vaginal smears on two occasions (1 y apart) [10]. Thus, these studies have focused almost exclusively on fecal hormone metabolite concentrations, and to our knowledge there are no reports that systematically assess multiple behavioral and biological measures of the reproductive cycle of this species.

Urinary and fecal hormone metabolite assays are well-established means to assess reproductive status in a diversity of mammalian species, and have the added advantage of being non-invasive [11–15]. Therefore, they often serve as a standard against which other techniques for detecting estrogenic cues may be measured or compared. Moreover, these hormone assays provided information regarding both the follicular and luteal phases of the reproductive cycle. A rise in estrogens (follicular phase) culminating in a peak value, followed by a rise in progestins (luteal phase), indicates that ovulation occurred, followed by an active CL producing progestins [16].

Hormonal concentrations are also reflected in the appearance of cells of the vaginal epithelium. Cytology has been used to elucidate phases of the estrous cycle and general timing of ovulation in domestic [17,18] and exotic [19–21] species. A series of smears is required for accurate estimation of cycle state [22,23]. Operant conditioning to gain the animal's cooperation for repeated reproductive data collection has become increasingly common [24] and has been used successfully to collect vaginal smear data on bears [20]. Further, when the animals are in a position for vaginal swabbing, visual assessment of the vulva can be done and retrospectively related to endocrine status [25–27].

The progressive maturation of cells through the layers of the vaginal epithelia results in the ratio of parabasals:intermediates:superficial cells known as the Maturation Index (MI). Shifts in the MI correspond to cycle stage changes from follicular phase to pregnancy [22,28]. Papanicolaou (PAP) staining can aid in further delineating the reproductive cycle by monitoring shifts among basophilic (blue), acidophilic (pink), and keratinized (yellow) epithelial cells in some species [19,20,29]. Similar to the MI, this continuum of color changes reflects circulating hormone metabolite concentrations, for example, with keratinized cells corresponding to very high estrogen and estrus [22].

Finally, behavioral assessment can play a crucial role in identifying and understanding reproductive issues or problems faced by species in captivity [30]. Several studies reported giant panda *(Ailuropoda melanoleuca)* reproductive behavior in detail [31–34]. Incorporating caretaker observations adds other potential cycle-associated behavioral cues, e.g. changes in appetite, activity levels, and voiding patterns.

The goals of the present study were to examine and inter-relate multiple methods for characterizing the reproductive cycle of the sun bear, describe the states of the cycle, and facilitate detection of estrus in this species. The present study drew from methodology developed for the giant panda [20,26,32]. Specifically, we assessed: 1) hormone metabolites from feces; 2) vaginal cytology and vulva appearance; and 3) observer and keeper-collected behavioral data. We also sought to categorize the reproductive potential of the entire US zoo founder population of *H. m. euryspilus* by assessing whether or not each female had estrous cycles. Our longterm objective was to promote improved reproductive management of this species, both in participating zoos and in *in situ* facilities.

2. Methods

2.1. Subjects

Thirteen female wild-caught, former-pet, Bornean sun bears on loan to eight US zoos from the Malaysian Government were used (Table 1). These animals represented the entire US captive female founder population of *H. m. euryspilus* sun bears when the study was conducted (2001–2006). Age estimates ranged from ~2–19 y (at the start of the study). One of the San Diego Zoo females (No. 665) became pregnant during the study; data collected during pregnancy were not included in this study. Based on the intensive monitoring of all subjects in this study, we are reasonably confident that no additional, undocumented breedings and unsuccessful pregnancies occurred. All bears were exhibited and socially housed in semi-naturalistic enclosures. Ten of the 13 females were housed with a male for at least part of the study period (Table 1). Social and other potential influences on the sun bear's reproductive cycle will be addressed in another publication.

Table 1
Data regarding 13 sun bear females used in this study. Zoos are listed in the order in which they joined the study; every institution holding *H. m. euryspilus* in North America participated.

Stbk ID	Zoo	Age (y)	Endocrine		Cytology		Behav video	Behav (0–3)	Swell (0–3)
			P	TE	M	C			
656[a]	WPZ	7	712	848	641	404	36	1048	93
671	WPZ	1.9	808	862	679	524	36	1060	98
672	SDZ	11	267	371	147	53	81	95	152
665[b]	SDZ	7	172	218	135	95	109	30	100
653[a]	AZG	11	164	177					
670[c]	AZG	6	102	113					
649[a]	MZG	13	226	372	304	223	182	279	266
647[a]	CMP	18	110	159	76	73	61		
655[a]	CMP	9	153	143	58	55	53		
651[a]	LPZ	12	157	175	110	110		60	31
673[c]	GPZ	19	109	107			33		
652[ac]	GPZ	15	128	113			39		
654[a]	SLZ	12	165	210				140	

Stbk ID: studbook identification number; Endocrine: fecal concentrations of progesterone (P) and total estrogens (TE); Cytology: cell morphology (M) and cell color (C); Behav: behavior from video (Behav video) and keeper-scored behaviors (Behav 0–3); Swell: vulva swell (scale, 0–3)
[a] Loaned from the Malaysian government in 1996, all others loaned in 2000.
[b] Female 665 became pregnant.
[c] Females 670, 673, 652 were not housed with a male at any point during the study.

2.2. Procedure

Several zoo staff at each facility were trained to use standardized protocols to collect data (described below). Site visits to all institutions collecting cytological data were conducted by the first author to assist with training and set-up. Subsets of the females also were studied for: 1) cytological evaluations; 2) behavioral monitoring (using frequency scored videotapes, keeper daily assessment ordinal scales, or both); and 3) ordinal scale scoring of vulva swell and color (Table 1).

2.3. Fecal sampling and hormone assays

Fecal samples were collected once daily an average of 3 d each week. A total of 3868 fecal samples were collected from the 13 females (range, 113 to 862 samples per female). When bears were not housed singly, they were fed either corn or birdseed 4 to16 h prior to sample collection to identify their feces. All fecal samples were frozen (−20 °C), shipped to the Woodland Park Zoo for processing, and extracted and assayed at the Center for Conservation Biology (University of Washington, Seattle, WA, USA). A standard methanol vortex method was used for hormone metabolite extraction. Briefly, a 0.2 g subsample of freeze-dried, sifted, well-mixed feces was combined with 2.0 mL of 90% methanol, vortexed for 30 min, and centrifuged at 1650 g-force for 20 min, producing a "1:2" methanol extract containing steroid hormone metabolites [35].

Female fecal extracts were diluted to 1:320 in assay buffer for a total-estrogen (TE) assay and to 1:30 for a progestin (P) assay. These dilutions were chosen to be close to 50% on the standard curve of each assay, the area of greatest assay precision. The TE assay was a commercially available 125-I double-antibody radioimmunoassay kit (catalog #140202, MP Biomedicals, Solon, OH USA), using a "total estrogen" antibody that binds to several estrogens. The P assay was an in-house 3-H radioimmunoassay using an antibody raised against progesterone (Munro progesterone antibody CL#425). Inter- and intra-assay variations were <10% for each assay. All samples and standards were done in duplicate, whereas non-specific binding tubes and blanks were done in quadruplicate. All assays included pooled controls. Samples were re-assayed if they had high %CV between duplicates, were outside the range of the standard curve for that assay, or if the assay controls were outside the normal range. In addition, female estrogen samples that were two SD above that individual's mean TE were repeated to authenticate high estrogen values (i.e. possible estrus). Each assay demonstrated good parallelism (P: $-y = 94.17–44.134\log(x)$ $R^2 = 0.99009$; TE: $y = 93.453–40.117\log(x)$ $R^2 = 0.98926$) and accuracy for pooled fecal extracts of Malayan sun bears. Recovery of added P and TE revealed an absence of assay interference (P: $y = 0.92832x + -.37389$ $R^2 = 0.99583$; TE: $y = 1.1068x + 3.1363$ $R^2 = 0.99898$). Reports regarding

the original assays [36–38] and modifications [39] should be consulted for further details and antibody cross-reactivities.

2.4. Reproductive cycle state designations

Hormonal data were used to create categories for analyses aimed at characterizing the reproductive cycle. The relative presence (+) or absence (–) of progesterone primarily defined each reproductive state and confirmed that estrus had occurred. To offset the individual variation in hormone metabolite concentrations, each female's P and TE data sets were transformed into a series of within subject z-scores ([raw score–mean]/SD). The (+) or (–) values of these scores were then used as standardized criteria for cycle designation into one of five states: high progesterone (at or above the mean when mean was high); low progesterone (at or above the mean when mean was low); baseline progesterone (below the mean), estrus, and high estrogen.

High progesterone (HP) was defined as a prolonged period (>1 mo) of a distinct rise in P (~500 to 2000+ ng/g) that transformed into + z-scores and ended with the first in a series (>2 wk) of negative z-scores. The HP state represented the luteal phase. Low progesterone (LP) was defined as periodic, low peaks in P (generally < 500 ng/g) that caused + z-scores when P profiles were low overall. The LP state was potentially associated with acyclic females. Baseline progesterone (BP), indicating very low relative P values (generally < 300 ng/g), was assigned to any value with a negative z-score for progesterone, except during HP (which may have intermittently low values) or estrus. The BP state was expected to correspond to the follicular phase of the cycle. Estrus (E) was designated as a standard 9-d interval consisting of the day of the peak estrogen value, plus the 4 d on either side, that preceded the onset of HP. It was assumed that the elevated progesterone of HP indicated ovulation had taken place [16]. High estrogen (HE) was also examined to explore potential (anovulatory) estrus when HP was not present. The HE designation was assigned to the top 10% of an individual's estrogen metabolite values that occurred outside of the HP and E states.

2.5. Vaginal cytology and vulva scoring

Operant conditioning was used to train the bears to present for, and accept, the brief insertion of a sterile, PBS moistened swab rotated 360° inside the vaginal tract to collect epithelial cells. Based on the methods of Schutte [17] and Durrant et al [20], the swab was then rolled on a slide and immediately sprayed with a commercially prepared alcohol based fixative (Wright Stain Fixative, Fisher Scientific catalog #04-330-4, Pittsburg, PA) and subsequently PAP stained. Slides were then evaluated for both cell morphology and color by an experienced cytotechnologist.

Changes in cell morphology were characterized by recording the relative proportions (counts) of three cell types present in the vaginal tract: parabasal, intermediate, and superficial epithelial cells. The trichrome PAP staining method also allowed for differential counts of cell color changes of basophilic (blue), acidophilic (pink), and keratinized (yellow) epithelial cells. Four of the five facilities collecting vaginal swabs also collected data on vulva changes. Vulva changes were scored using an ordinal ranking scale of 0–3 for vulva color (0 = none; 1 = light; 2 = moderate; and 3 = deep red) and vulva swell 0–3 (0 = little or no swelling with vaginal orifice barely visible; 1 = pronounced turgidity of the labia [not always seen]; 2 = partial vaginal opening enlargement; and 3 = a full and obvious "open" appearance). This scoring system was based on an established technique for giant panda [26] and adapted here for sun bear. All zoos used the same reference photos for scoring [40].

2.6. Behavior: video and ordinal

Videotaping of focal females included at least two, 30-min sessions per week. Frequencies of behaviors were converted to rate/h. Tapes were scored using either a Palm III Emulator program running Event 3.0 software created by J. Ha (University of Washington, Seattle, WA USA) or the Observational Coding System V 3.5 by Triangle Research Collaborative (Greensboro, NC; www.trctech.com). Both programs used keys corresponding to behaviors in all categories defined in an ethogram developed for use in this study (Table 2). All tapes were scored by one of two trained observers with > 90% inter-observer reliability.

Using a system adapted from the San Diego Zoo's giant panda program, zookeepers recorded daily assessments of the bears' behavior based on a minimum of five observations made throughout the day. These ordinal measures (0–3) were of behaviors falling only under the categories stereotypic, sexual, affiliative, and agonistic (Table 2). Scores of 1, 2, 3 corresponded to low, medium, and high in terms of frequency/intensity, with 0 = baseline for that animal (a subjective assessment of what was considered typical for that individual). For example, if the bear had normal (for her) levels of stereotypic behavior, a score of 0 would be entered for that day, but if she was observed pacing

Table 2
Sun bear ethogram with category designations, behaviors and definitions.

Category	Behaviors	Definitions
Solitary	Sleep	Lies or sits with little or no head or body movement, passive, eyes closed.
	Self play	Spontaneous activity, w/out apparent purpose, often repetitive but not rigid.
	Self groom	Using tongue/paws, cleans/scratches body except nipple/genital area.
	Self genital groom	Using tongue/paws, cleaning focused on own nipple/genital area.
	Other	Solitary behavior not otherwise listed, typically travel and forage.
Stereotypic	Pace	Travels in a rigid, fixed pattern/route for >3 repetitionsany.
	Stereo other	Other rigid, repetitive behavior w/out apparent function, typically oral.
Sexual	(Self) Masturbate	Focused, repetitive genital manipulation >1 min, often w/vocalization.
	Genital groom other	Using tongue/paws to clean/manipulate genitals of other bear.
	Genital inspect other	Olfactory investigation of genitals/genital area of other bear.
	Mount	Male positions self behind and on top of female, may bite nape, thrust hips.
	Copulate	Female stays while male in mounted position, full intromission occurs.
Social	Approach	Clear, direct, intentional seeming movement to effect social contact.
	Social proximity	W/in 1 m of another bear, but not engaged in other social behavior listed.
Affiliative	Social play	Active, mutual, tackling/wrestling or non-aggressive chase, no vocals.
	Solicit	Initiates interaction, usually w/playface, sometimes while standing bipedal.
	Follow	Travels in social contact behind other for >5 m, no other apparent goal.
	Groom other	Using tongue/paws to clean/manipulate body of other bear except genitals.
	Muzzle-muzzle	Mutual contact of muzzle area, sniffing, licking, typically while standing.
	Social positive	Includes brief, positive contacts (touch, lean-in), gestures (playface).
Agonistic	Displace	Takes up a location or object relinquished by other when approached.
	Aggressive voxal	Aggressive vocal lengthy, deep, varied, rumbling growl, or short, staccato, abrupt bark.
	Aggress	Lunging, chasing, fighting (pinning and biting), always w/vocals.
	Socialnegative	Includes brief negative contacts (swipe), gestures (head down eyes up).
Appetite		Designated by the extent usual types and amounts of foods are: ignored, ingested or insufficient.

slightly more frequently or intensely than was considered usual, a score of 1 was recorded. The option to score below 0 existed but did not occur during this study. The single behavior appetite was assessed separately using a −3 to +3 scale (0 = normal appetite for that animal, +3 = extreme interest in food including aggression or distress behaviors, −3 = apathy toward and no ingestion of food; intermediate numbers indicate correspondingly low and moderate increases or decreases in appetitive behavior).

2.7. Statistical analyses

The MANOVAS were done between designated states of the reproductive cycle and cytology. Each state's daily counts for the six cell types were first averaged by individual to control for unequal data set sizes (results were entirely consistent with MANOVAs on raw data). Fisher's Protected Least Significant Difference (PLSD) was used as a post hoc test. Since power (< 0.3) was insufficient to test behavior means (video) in MANOVA, raw behavior frequencies were analyzed using a restricted number of unpaired t-tests for reproductive state and behavior category. An extremely conservative degrees of freedom estimate of n - number of females in that state was used to compensate for the unequal sample sizes contributing to the t-statistic. Spearman rank correlations were used to examine behavior (ordinal) categories. All statistical tests used an alpha of 0.05. All descriptive means presented in results are based on averages from individuals to control for unequal data set sizes (e.g. the interestrous interval was first averaged within females and then across females).

3. Results

3.1. Patterns of reproductive cycling

Results suggested a gradation in the frequency of reproductive cycling; 38.5% females spent 0% of their time in HP and were therefore considered "non-cyclers" (Figs. 1 and 2). In contrast, 30.8% females were

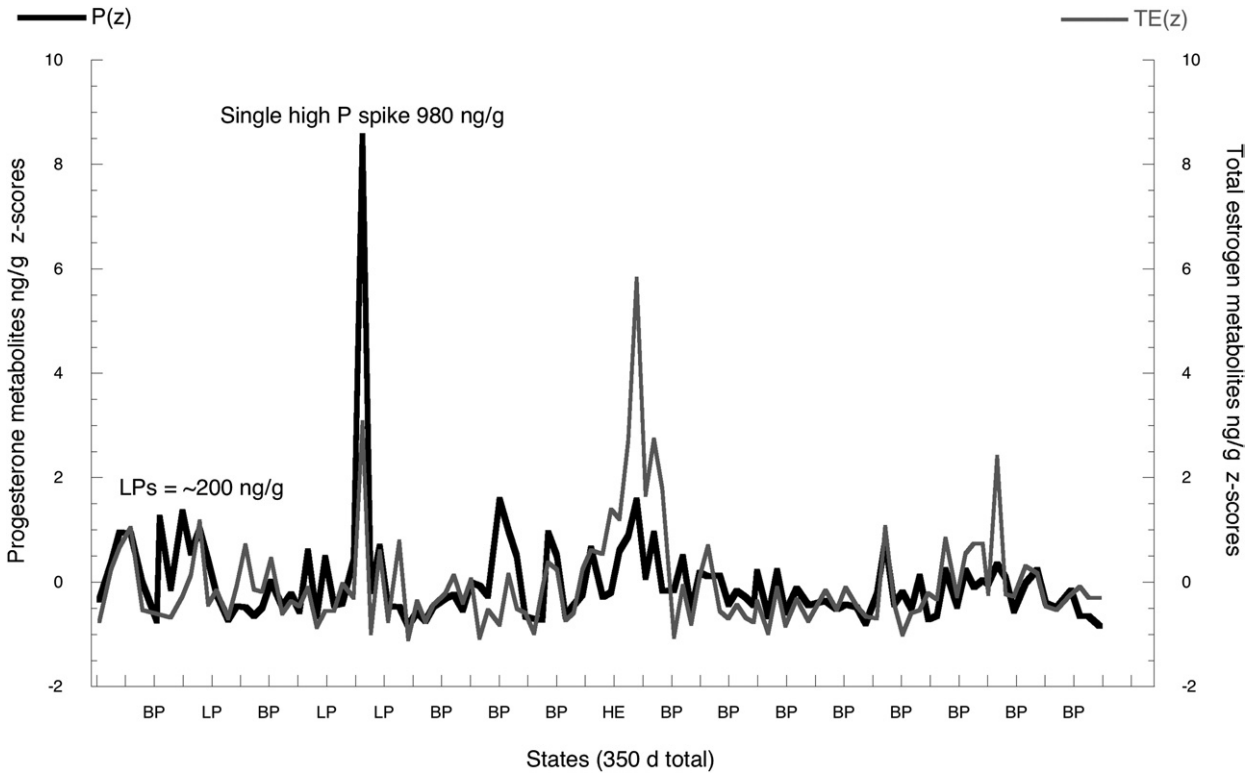

Fig. 1. Reproductive cycle states in female sun bears and z-score transformed hormone metabolite data in a non-cycler (No. 673). Note that P (progesterone) values below 0 are basal progesterone, whereas those above 0 are low progesterone.

in HP ~ 40% of the time, making them "repeat cyclers" (two or three HP states a year; Fig. 3), and 30.8% spent up to 25% of their time in HP and greater than 70% of their time in BP, indicating they were "single cyclers" (one HP a year; Fig. 4). A female having the maximum of three HP states a year spent an average of 29.4 ± 5 d in BP between HP and E (range = 17–49). Mean inter-estrous intervals (excluding outliers i.e. skipped cycles) ranged from 101 to 131 d, mean = 115.7 ± 6.3. The first documented estrous cycle for female 671 occurred at 2 y of age.

3.2. Cytology and reproductive states

Vaginal epithelial cells varied between reproductive states; Wilks' Lambda = 0.053, $F_{24, 43} = 2.376$, P = 0.006). The five states with their distributions of cell types are shown (Fig. 5). The E state had higher frequencies of superficial cells than all other states except HE ($F_{4, 17} = 5.697$, P = 0.004). Keratinized cells did not vary significantly among states but, like superficial cells, attained their highest average during E. Intermediate cells were present in high numbers across all states, but did vary among them ($F_{4, 17} = 5.697$, P = 0.004); they were least frequent in E and greatest during HP. Acidophilic cells were high across all states except HP when they decreased ($F_{4, 17} = 5.690$, P = 0.004). The HP state instead had increased basophilic ($F_{4, 17} = 10.608$, P = 0.0002) and parabasal ($F_{4,17} = 3.370$, P = 0.03) cells.

The E and HP states were clearly different from one another, in contrast to LP and BP, which were overall extremely similar (Fig. 5). Both LP and BP were significantly lower in parabasal, intermediate and basophilic cells than HP, and significantly lower in superficial and keratinized cells than E. The HE state was not significantly different from E for any cell type (PLSD), but neither was it significantly different from LP or BP (Fig. 5).

3.3. Behavior (video) and reproductive states

Estrus (E) had higher rates of sexual behavior than BP did ($t_3 = 3.314$, P = 0.045), and E also had higher affiliative than LP and HP ($t_3 = 3.565$, P = 0.038; t_3 =

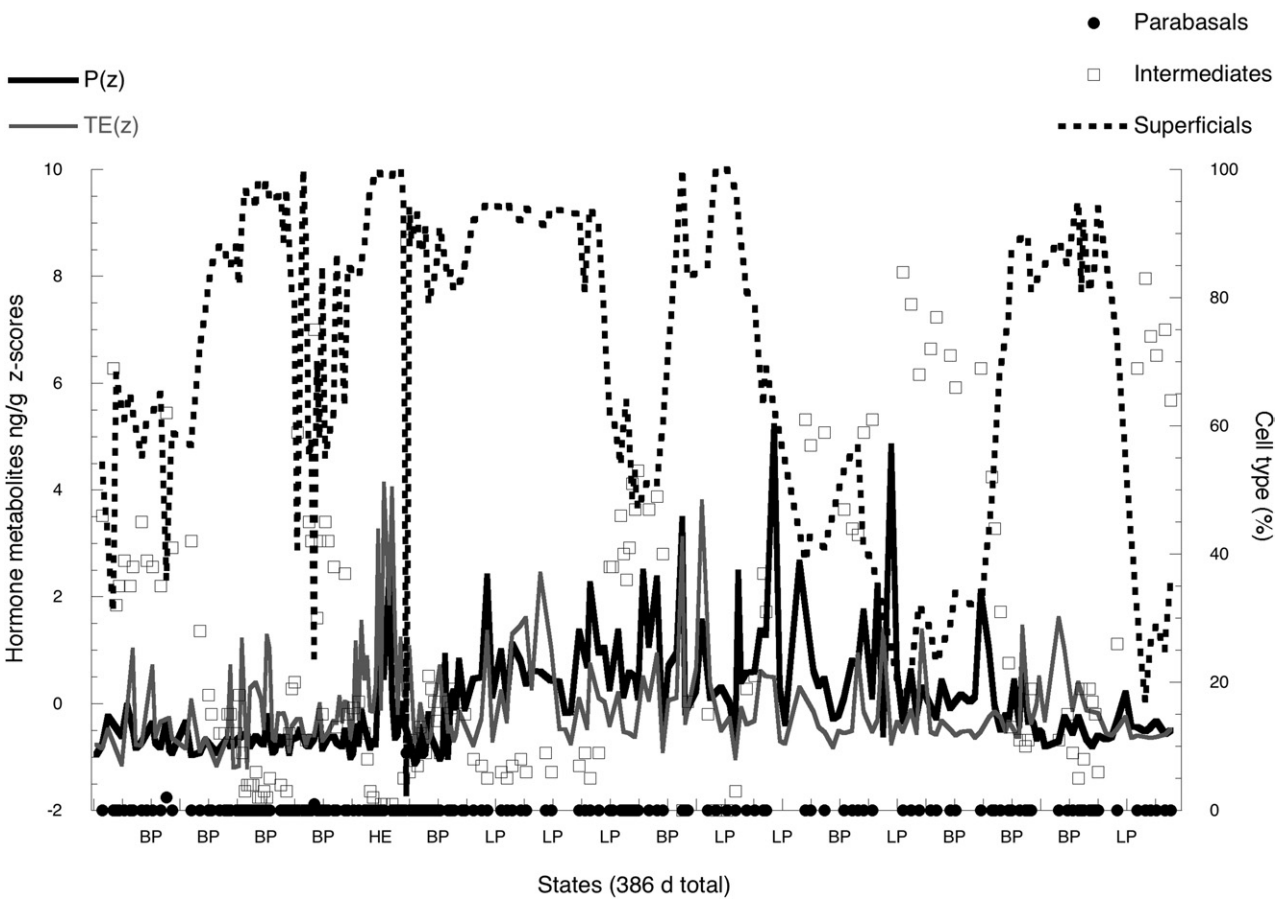

Fig. 2. Reproductive cycle states and z-score transformed hormone metabolite data in a non-cycling sun bear (No. 672). Note the corresponding lack of cyclicity in superficial cells.

4.993, P = 0.016); and greater stereotypic than LP did (t_3 = 04.688, P = 0.018). Affiliative was lower during HP than it was during E (see above), LP and HE (t_3 = 4.720, P = 0.018; t_3 = 4.490, P = 0.021). Social was lower during HP than it was during E, LP, BP, and HE (t_3 = 4.082, P = 0.027; t_3 = 5.204, P = 0.014; t_3 = 3.384, P = 0.043; and t_3 = 5.701, P = 0.011). Stereotypic was greater during HP than during LP and HE (t_3 = 6.236, P = 0.008; t_3 = 4.387, P = 0.022).

The two states E and HP had the most clearly contrasted behavioral profiles, whereas HE was not significantly different from E, BP, or LP (Fig. 6). Notably, BP was more similar to HP than LP was; for example, BP had higher stereotypic and lower affiliative and social than LP did (t_7 = 3.560, P = 0.009; t_7 = 2.475, P = 0.043; t_7 = 5.204, P = 0.001). Solitary and agonistic behaviors were not significantly different among states (Fig. 6).

3.4. Behavior (ordinal), cytology and genital changes

Correlations were determined for each of the four categories of behavior separately, with positive associations between behaviors recorded from video (frequency) data and their corresponding daily assessments (ordinal scores from keeper observations): stereotypic (r_{219} = .206, P = 0.002); sexual (r_{210} = .234, P = 0.0007); affiliative (r_{225} = .189, P = 0.005); and agonistic (r_{224} = .211, P = 0.002). Behaviors (ordinal) were then correlated with the measures described below. Spearman rank correlations were significant (i.e. were not due to chance) but were weak throughout, accounting for relatively little of the variability.

There was a positive correlation between agonistic behavior and appetite (r_{2469} = .072, P = 0.0003). Sexual behavior was positively correlated with superficial and keratinized cells (r_{663} = .103, P = 0.008;

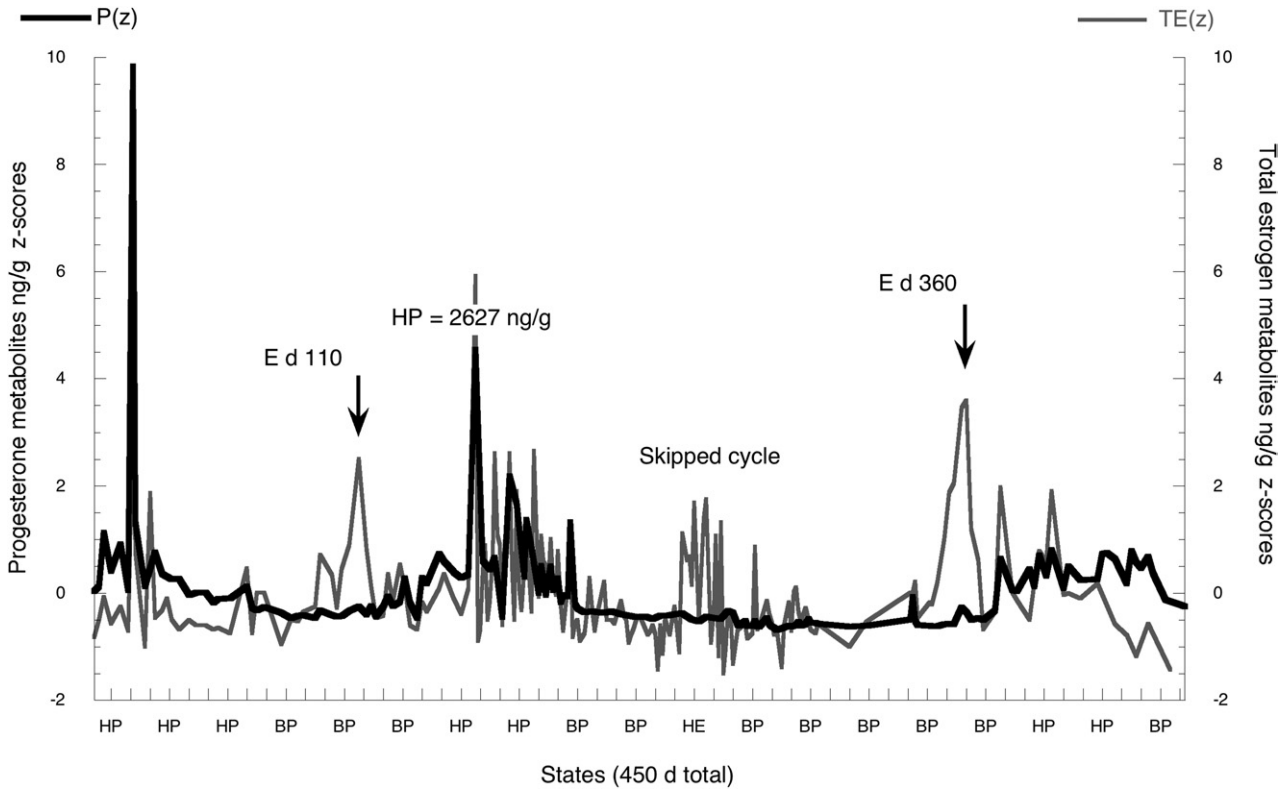

Fig. 3. Reproductive cycle states and z-score transformed hormone metabolite data in a repeat cycler sun bear (No. 654). Note that P (progesterone) values below 0 are basal progesterone, whereas those above 0 are high progesterone.

r_{568}= .172, P = 0.0001) and negatively correlated with parabasal and basophilic cells (r_{663} = −.099, P = 0.011; r_{568} = −.098, P = 0.018). Sexual and affiliative behaviors were positively associated with vulva swell and color. Vulva swell and color were correlated with one another (r_{560} = .374, P < .0001). However, only vulva swell had a positive correlation with superficial cells (r_{564} = .222, P <. 0001), and a negative correlation with parabasal and basophilic cells (r_{564} = −.198, P < .0001; r_{394} = −.375, P = 0.0001). There were no significant correlations between stereotypic behavior and vulva swell and color or between agonistic behavior and swell.

When agonistic behavior and vulva color were considered in cycling and non-cycling females (those that never had HP) separately, only non-cycling females had a positive relationship (r_{320} = .417, P < .0001). A key distinction between cycling and non-cycling females was that, whereas both types attained rank 3 of vulva color, only cycling females ever attained rank 3 of vulva swell. Vulva swell had a positive correlation with keratinized cells in cycling females only (r_{167} = .326, P < .0001); in non-cyclers this correlation was negative (r_{227} = −.250, P = 0.0002). In cycling females, appetite had a negative association with keratinized and superficial cells (r_{568} = −.152, P = 0.009; r_{298} = −.115, P < .047) and a positive association with basophilic cells (r_{290} = .146, P = 0.013). In non-cycling females, the opposite patterns were seen; appetite correlated positively with superficial cells (r_{344} = .114, P = 0.035) and negatively with basophilic cells (r_{278} = −.130, P = 0.031).

3.5. The estrus profile

A summary of measures potentially associated with E is shown (Table 3); it was derived from females that both showed HP and were studied for multiple parameters. The average of the individual means of cycling females for each measure was taken for three periods within designated E: −4 d before peak estrogen, Day 0 of peak estrogen, and +4 d after peak estrogen. Also considered were the 4 d prior to (−8) and the 4 d after (+8) designated E. All measures attained their most

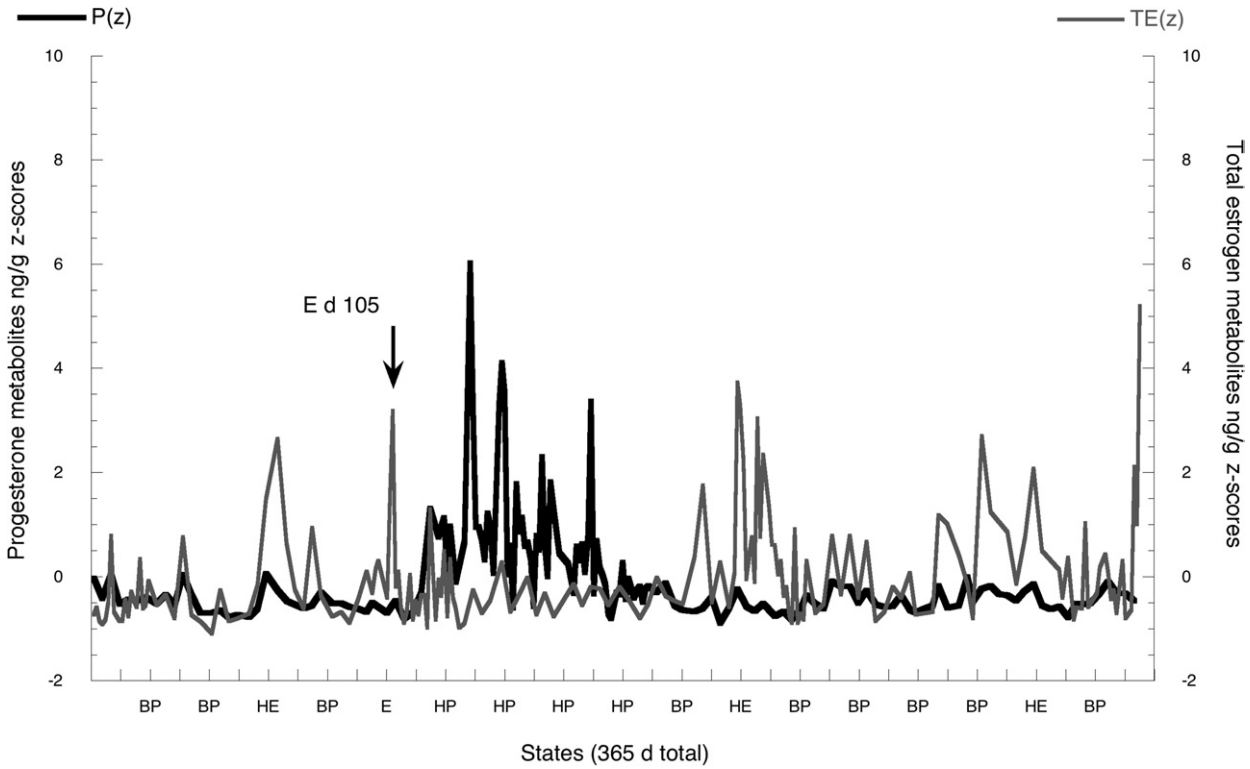

Fig. 4. Reproductive cycle states and z-score transformed hormone metabolite data in a single cycler sun bear (No. 651). Note that P (progesterone) values below 0 are basal progesterone, whereas those above 0 are high progesterone.

extreme values during E. Agonistic data (video and ordinal) were greater up to Day 0; both then decreased to their lowest values. In contrast, affiliative data (video and ordinal) were both highest on Day 0, but stayed relatively high for the next +4 d after peak. Superficial cells, vulva swell, and color, all also had their highest

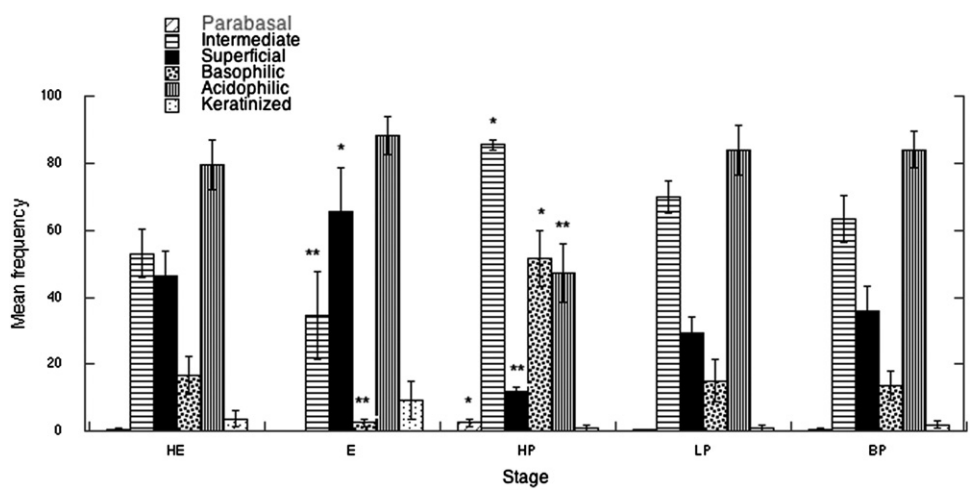

Fig. 5. Grand mean frequency and SEM of cell types (morphology and color) across designated reproductive cycle states in female sun bears. HE: high estrogen; E: estrus; HP: high progesterone; LP: low progesterone; BP: baseline progesterone.

* Highest mean differed ($P < 0.05$) from one or more other states.

** Lowest mean differed ($P < 0.05$) from one or more other states.

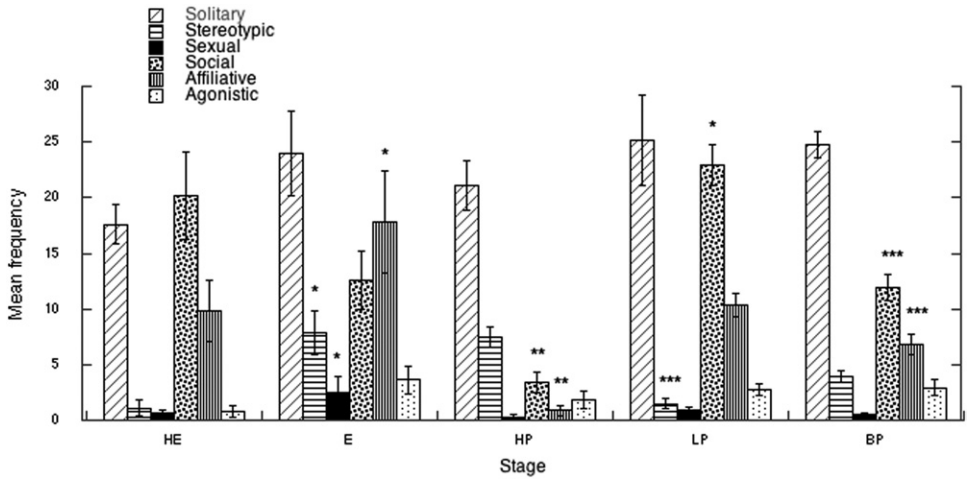

Fig. 6. Grand mean frequency and SEM of videotaped behaviors combined into behavioral categories across designated reproductive cycle states in female sun bears.

HE: high estrogen; E: estrus; HP: high progesterone; LP: low progesterone; BP: baseline progesterone.

* Highest mean differed ($P < 0.05$) from one or more other states.

** Lowest mean differed ($P < 0.05$) from one or more other states.

*** Difference ($P < 0.05$) between the LP and BP states.

values on Day 0. The range for superficial cells during E (all females, not averaged) was 18–100%. Keratinized cells and sexual measures (both video and ordinal) had their greatest values +4 d peak estrogen. The range for keratinized cells during E was 0–48%. A decrease in appetite occurred prior to E −8 d, and was greatest −4 d peak.

Relationships among hormonal, cytological, vulva swell and sexual (ordinal) measures across three cycles in a single year in WPZ Female 671 (note that no male was present before or during this period) are shown (Fig. 7). Overall, agreement among measures during the estrous cycle was strong and conformed to the patterns previously described in this paper, although

Table 3
Measures during designated estrous (E) of peak estrogen and for the two 4 d intervals flanking it (Day 0 +/− 4 d) in female sun bears.

	4 d before E		Estrous stage E peak (0) +/− 4 d						4 d after E	
Measure	−8		−4		0		+ 4		+8	
Superficial cells[b]	42.5	18.9	65.9	17.9	85.8	11.3	65	20.9	57.2	22
Keratinized cells	7.3	5.2	9.4	9.6	12	17	13.8	13.4	12.9	8.6
Swell ordinal	2.2	0.5	2	0.9	2.6	0.2	2.6	0.4	2	0.9
Color ordinal	1.9	0.3	1.6	0.3	2.2	0.4	1.8	0.5	1.4	0.6
Appetite ordinal[c]	−.08	0.2	−0.3	0.5	−0.2	0.3	−0.1	0.3	0	0
Sex video	1.7	2.5	4.2	3.5	2	0	6.2	7.2	1.9	3.4
Sex ordinal[d]	0.2	0.2	0.2	0.2	0.5	0.3	1.2	0.3	0.3	0.4
Agonistic video[e]	2.6	3.5	3	3.2	4.6	0	2.2	3.3	1.6	3.2
Agonistic ordinal	0.5	0.1	0.8	0.9	0.3	1.2	0.3	0.8	0.3	0.3
Affiliative video	12.3	7.9	13.6	10.1	23.3	21.3	15.1	8.6	13.7	9.9
Affiliative ordinal	0.8	0.3	0.9	0.1	1.5	0.7	1	0.2	0.5	0.2

Grand means are in the left columns and SD in the right columns. Means were based on data from 2–4 females, depending on the measure and interval.[a]

[a] One peak estrogen timing was questionable, so those data were excluded from all relevant analyses.

[b] Cell averages were based on daily proportions (%).

[c] Ordinal values of 0 = baseline; appetite daily averages were based on a −3 to +3 scale.

[d] All other daily ordinal averages were based on a 0 to +3 scale.

[e] All video daily averages were based on frequencies (rate/h).

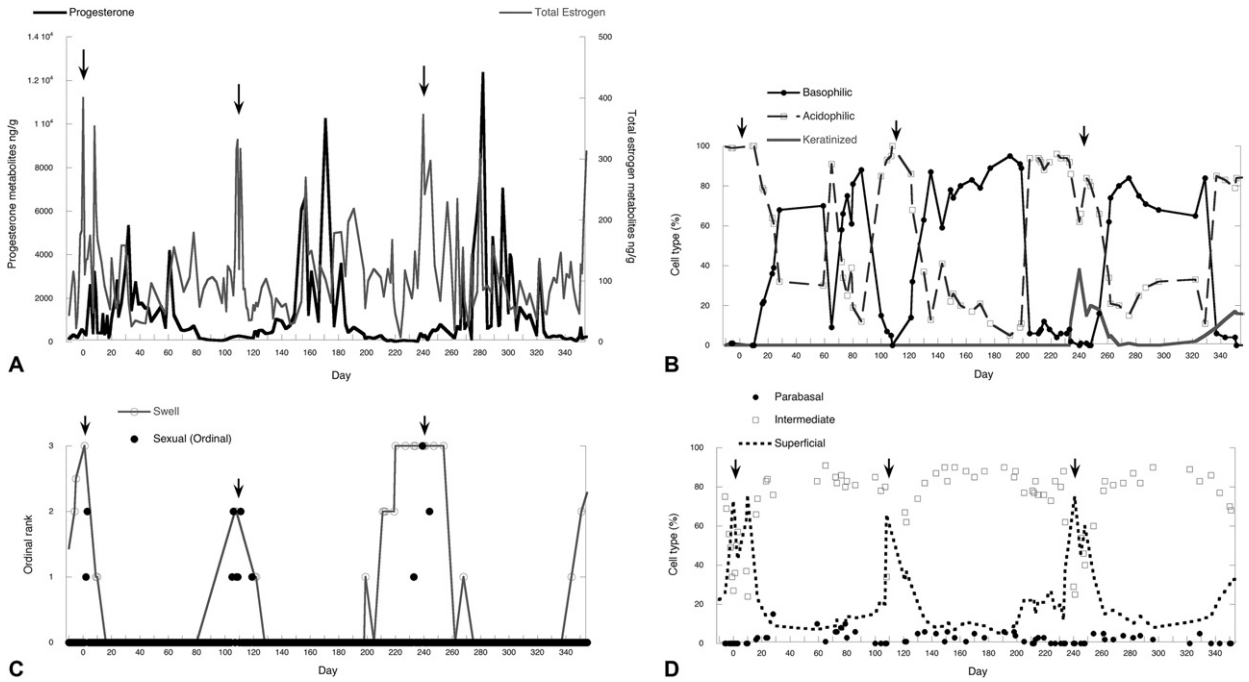

Fig. 7. One year of reproductive cycles, Days -10 to 355 (Day 0 was peak estrogen [denoted by arrows] for the first of the three cycles) in repeat cycler female sun bear (No. 671), showing patterns and agreement between reproductive measures.

A) hormone metabolites (untransformed); B) vaginal cytology (trichromic color); C) ordinal scores for vulva swell and sexual behavior; D) vaginal cytology (morphology).

these cycles also had variability within measures (e.g. the timing of HP onset).

4. Discussion

4.1. Evaluating methods for reproductive cycle assessment

In the present study, hormone metabolites, vaginal epithelial cells, changes in genital appearance, and behaviors were evaluated as indicators of the sun bear's reproductive cycle. Contrary to the findings of Schwarzenberger et al [8], estrogen and progesterone radioimmunoassays were effective at reliably detailing the estrous cycle of the sun bear. The other indices examined also supported this conclusion. Hormones and cytology in the sun bear had expected relationships as reported in a myriad of species studied to date [13,41,42].

Estrogens appeared to induce the maturation and proliferation of superficial and keratinized cells associated with estrus, consistent with the maturational index [22]. When high estrogen concentrations were overshadowed (masked) by progesterone, they were instead associated with primarily intermediate cells [28], some parabasal cells, and increased basophilic cells, reflecting a more alkaline vaginal pH [43,44]. These patterns were also present in females when males were absent and pregnancy was not a potential confounding factor (e.g. female 671). Cytology therefore acted like a filter for estrogen "noise" in cycling females by usually reflecting biologically meaningful hormone changes rather than naturally occurring hormone fluctuations that were nonetheless unrelated to estrus. Cytology was comparably representative and in some ways preferable to endocrine measures for evaluating the cycle. In that regard, it provided more immediate and less expensive data regarding cycle status than hormone metabolites from fecal samples. However, some training must take place for voluntary sample collection to occur, cytological sampling needs to be done regularly [17] and, as with any measure, cycle peaks are only known retrospectively.

Scoring of genital appearance (particularly if using digital photos) is non-invasive, provides valuable information, and does not require additional training time, laboratory work or expense. Vulva swell and color were correlated with one another, but swell appeared to be a more discriminating indicator of estrus. Only vulva swell was associated with superficial and keratinized

cells. The highest swelling score of rank 3 indicating a fully open appearance was only seen in cycling females. Vulva color assessment may have been more subjective than physical changes and therefore more variable. Also, flushing of the tissue may occur under other arousal states, as suggested by its association with agonistic behavior in non-cycling females (see also [45]). Although vulva swell may be too variable to predict or give precise information on the timing of ovulation [26], it provided supporting evidence of a follicular phase (seen in scores 2 and 3).

Behavioral analysis offered another potentially useful method for characterizing reproductive states. We examined six broad categories of behavior for possible changes that could help to identify and better understand the estrous cycle. There was a continuum of sociality (solitary versus social versus affiliative) and receptivity (agonistic versus sexual) and finally stereotypic behavior as a potential metric for agitation or distress. Although neither solitary nor agonistic behaviors varied significantly among states in the females of this study, some behavioral differences were apparent. For example, social and affiliative behaviors were strongly dichotomized between the follicular and luteal phases. Further, we concluded that in cycling females there were three reproductive states with distinctive behavioral profiles: Estrus (E), High Progesterone (HP), and the Baseline Progesterone (BP) state described below. Our study not only confirmed the utility of behavioral measures, but also showed that a simple keeper check-sheet can be an effective tool for reproductive assessment, offering an alternative to data laboriously derived from scored videotapes. In addition, some highly diagnostic behaviors like appetite, which changes throughout the cycle (see also [34]), can only be monitored via a keeper's systematic input.

4.2. Differentiating reproductive states

Complications can arise when discussing the luteal phase, particularly in species with pseudopregnancy. Pregnancy and pseudopregnancy appeared endocrinologically indistinguishable in bears [8,9,48–50]. In non-pregnant bears, a profile of prolonged progesterone rise (HP in this study) was typically defined as pseudopregnancy [9,46,47]. In the mammalian reproductive literature, the terms "diestrus" and "anestrus" can vary in their meaning and usage. We suggest here that "diestrus" be used to refer to an active luteal phase (i.e. pseudopregnant), whereas "anestrus" be used to describe prolonged periods of reproductive inactivity in females that cycle at other times (i.e. a female that skips a cycle). We refer to females that never showed luteal activity as acyclic or non-cyclers. The terms "diestrus" and "anestrus" were not applied to these non-cycling females, because we lacked evidence that they ever demonstrated any aspect of the estrous cycle.

The BP state of prolonged minimal progesterone values primarily occurred: 1) along with LP in females that were acyclic; 2) during anestrus; and 3) in the period between the end of HP and the next E (i.e. the follicular phase). The LP state consisted of periodic low progesterone peaks that, nevertheless, were higher than average in those individual bears. We wanted to empirically test whether LP represented a reproductively neutral state (like BP) or simple individual variation in magnitude of hormone concentrations. It was anticipated that LP would be highly similar to BP. As expected, they were not significantly different from one another with respect to any cell type, and BP was actually more behaviorally similar to HP than was LP. These results confirmed that low peaks of progesterone lacked detectable physiological or behavioral impact in the females that showed them, consistent with the idea that they were acyclic.

We investigated high estrogen (HE) to determine the likelihood that estrus was occurring when not followed by HP. High Estrogen did mirror E in having a cytological profile that contrasted with HP, but was otherwise not greatly similar. Unlike E, HE was never significantly different from BP or LP in either cytology or behavior. We therefore failed to find any compelling evidence that females experienced estrus in the absence of ovulation.

Our study supported the assumption that the diestrus portion of the reproductive cycle of the sun bear is typically represented by a pseudopregnancy [8,9]. In that regard, HP was distinguished from other states by significantly higher proportions of intermediate and basophilic cells, also associated with increased appetite. Behaviorally, females showed less affiliative and social behavior, and higher stereotypic behavior during HP. Since pregnant bears socially isolate themselves [51], it was not surprising that affiliative and social behavior were lowest during the HP state. Stereotypic behavior, also prevalent during E, may be associated with a general hormonal increase. Stereotypy has been reported to increase during the breeding season in both black [52] and polar bears [53].

4.3. Detecting estrus

We focused on the E state to characterize the cytological and behavioral changes associated with poten-

tial female receptivity. Estrus in this study occurred approximately every 3.5 to 4 mo, comparable to what has been previously reported [47,54]. We selected a 9 d interval to examine potential behavioral and biological changes corresponding with estrus. These measures (described below) changed most substantially during designated estrus, relative to the periods flanking it, thereby supporting it as the appropriate timeframe. Superficial cells were significantly higher during E than any other state. Keratinized cells were also highest during E, as were sexual, affiliative, and stereotypic behaviors. It is worth noting that averaging within and between females for keratinized cells resulted in means well below what a given individual may show. Behavioral estrus in sun bears has been reported to last 1–2 d, and up to 5–7 d [55,56] and even 11 d [8]. In the present study, in the one cycle in which breeding took place it occurred over the 4 d interval after peak estrogen.

In the 4 d interval before peak estrogen, the bears in this study had more agonistic behaviors, noticeable declines in appetite, more open vulvas, and increases in superficial and keratinized cells. At peak estrogen (Day 0 of estrus), superficial cells, an open vulva appearance, decrease in agonistic behaviors, increase in affiliative behaviors, and low appetite were all manifest in the female sun bear. Sexual behaviors may well have started, but these, along with keratinized cells were most frequent in the 4 d after the estrogen peak and presumably overlapped with ovulation. At 8 d after the estrogen peak, these females had clear signs that estrus had ended; a decline in superficial cells and vulva swell was seen along with a return to normal appetite. Variability between and even within females was considerable, not only in timing of onset, but also in magnitude (e.g. peaks in hormone metabolites concentrations and cell frequencies). Maximum values for cell counts for keratinized and superficial cells varied widely within and among cycles. Schwarzenberger et al [8] also observed high inter- and intra-female variability in sun bear estrous cycle expression.

4.4. Summarizing the captive H. m. euryspilus population

The age range for females in this study was from ~ 2 to 19 y. Little is known about the lifespan or reproductive span for wild sun bears. Breeding may occur as early as 3 y of age [55]. Females attain approximate adult body weight at 1.5–2 y, and in captivity live well into their 30s, and reproduce between 4 and 27 y of age (reviewed in [40,57]). The youngest female in this study had an estrous cycle at 2 y of age.

Sun bears do not appear to show reproductive seasonality [40,47,57]. The study females had a gradation in the frequency of cycling. Some females had three reproductive cycles a year, others only one estrus cycle and were therefore considered single or low frequency cyclers, whereas still others were acyclic. Of the females studied, only two-thirds had at least one full reproductive cycle (estrus + diestrus) and therefore some level of reproductive potential. Schwarzenberger et al [8] studied 12 sun bear females and also found four to be "missing luteal activity". There was no simple or clear answer for why some females were single cyclers and others repeat cyclers. Perhaps age, health or stressful circumstances at the time of study contributed to this variation. A more detailed examination of the potential causes for cycling differences between females will be presented separately.

Cycling and non-cycling females were distinguished by several different measures. Under the sexual category of behavior, masturbatory and breeding behaviors were only seen in cycling females (though not all cycling females showed either; data not shown). Only cycling females had the fully open vulva and state-specific changes in appetite. Unfortunately, since all females had fluctuating proportions in cell types, it may be difficult to distinguish between cycling and non-cycling females based solely on cytology alone. However, cycling females usually had clearer transitional patterns, with distinct periods of higher superficial-keratinized cells occurring only at 3 to 4 mo intervals, with long periods of predominantly intermediate-basophilic cells in between.

Progesterone output clearly delineated cycling from non-cycling females, but alone did not give precise (post hoc) information on estrus timing, since the onset of the progesterone rise was variable. High estrogen was often detected outside of estrus, so estrogen was only diagnostic of estrus when it preceded a prolonged period of high progesterone. Therefore, estrus is best determined by examining both hormones in combination.

The use of vaginal cytology, vulva scoring, and behavior monitoring offered effective and inexpensive supplements or alternatives to fecal hormone metabolite assays and are highly recommended in the continued reproductive management of this and other captive sun bear populations. Approximately one third of the study females were acyclic and thus may have little or no reproductive potential if their profiles remain unchanged. Further study to successfully determine and implement mechanisms for stimulating reproductive

cycling in this species may prove critical to maintaining these small captive populations.

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References

[1] Fitzgerald CS, Krausman PR. *Helarctos malayanus*. Mam Spec 2002;696:1–5.

[2] Servheen C. Sun bear conservation action plan. In: Servheen C, Herrero S, Peyton B, editors. Bears. Status Survey and Conservation Action Plan. − IUCN/SSC Bear and Polar Bear Specialist Groups; 1999. p. 219–23.

[3] Payne J, Francis CM. A field guide of the mammals of Borneo. Malaysia: Sabah Society; 1998.

[4] Meijaard E. Craniometric differente among Malayan sun bears (*Ursus malayanus*). Raffles Bull Zool 2004;52:665–72.

[5] IUCN. IUCN Red List of Threatened Species. Available at: www.iucnredlist.org. Accessed 20 June 2008. International Union for the Conservation of Nature; 2007.

[6] Frederick C. North American Regional Studbook for the sun bear (*Helarctos malayanus*). Seattle, WA: Woodland Park Zoo; 1998.

[7] Schwarzenberger F, Schaller K, Chaduc Y, Pagan O, Kolter L. Faecal steroid analysis for monitoring ovarian function and the effect of PZP (porcine zona pellucida protein) in the sun bear (*Helarctos malayanus*). Proc European Assoc Zoo Wildl Vet 1998;2:387–95.

[8] Schwarzenberger F, Fredricksson GM, Schaller K, Kolter L. Faecal steroid analysis for monitoring reproduction in the sun bear (*Helarctos malayanus*). Theriogenology 2004;62:1677–92.

[9] Onuma M, Suzuki M, Ohtaishi N. Reproductive pattern of the sun bear (*Helarctos malayanus*) in Sarawak. J Vet Med Sci 2001;63:293–7.

[10] Onuma M, Suzuki M, Uchida E, Niiyama M, Ohtaishi N. Annual changes in fecal estradiol-17beta concentration of the sun bear (*Helarctos malayanus*) in Sarawak, Malaysia. J Vet Med Sci 2002;64:309–13.

[11] Monfort SL, Dahl KD, Czekala NM, Stevens L, Bush M, Wildt DE. Monitoring ovarian function and pregnancy in the giant panda (*Ailuropoda melanoleuca*) by evaluating urinary bioactive FSH and steroid metabolites. J Reprod Fertil 1989;85: 203–12.

[12] Wasser SK, Hunt KE, Brown JL, Cooper KC, Crockett CM, Bechert U, Millspaugh JJ, Larson S, Monfort SL. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen Comp Endocrinol 2000;102:260–75.

[13] Moreira N, Monteiro-Filho ELA, Moraes W, Swanson WF, Graham LH, Pasquali OL, Gomes MLF, Morais RN, Wildt DE, Brown JL. Reproductive steroid hormones and ovarian activity in felids of the *Leopardus* genus. Zoo Biol 2001;20:103–16.

[14] Larson S, Casson CJ, Wasser SK. Noninvasive reproductive steroid hormone estimates from fecal samples of captive female sea otters (*Enhydra lutris*). Gen Comp Endocrinol 2003;134: 18–25.

[15] Hesterman H, Wasser SK, Cockrem JF. Longitudinal monitoring of fecal testosterone in male Malayan sun bears (*U. malayanus*). Zoo Biol 2005;24:403–17.

[16] Nelson RJ. An Introduction to Behavioral Endocrinology. Sunderland, MA: Sinauer Associates Inc.; 1995.

[17] Schutte AP. Canine vaginal cytology I. Technique and cytological morphology. J Small Anim Pract 1967;8:301–6.

[18] Mills JN, Valli VE, Lumsden JH. Cyclical changes of vaginal cytology in the cat. Can Vet J 1979;20:95–101.

[19] Williams ES, Thorne ET, Kwiatkowski DR, Lutz K, Anderson SL. Comparative vaginal cytology of the estrous cycle of black-footed ferrets (*Mustela nigripes*), Siberian polecats (*M. eversmanni*) and domestic ferrets (*M. putorius furo*). J Vet Diagn Invest 1992;4:38–44.

[20] Durrant B, Czekala NM, Olson MA, Anderson A, Amodeo D, Campos-Morales R, Gual-Sill F, Ramos-Garza J. Papanicolaou staining of exfoliated vaginal epithelial cells facilitates the prediction of ovulation in the giant panda. Theriogenology 2002; 57:1855–65.

[21] Mayor P, Lopez-Bejar M, Jori F, Fenech M, Lopez-Gatius F. Reproductive functional anatomy and oestrous cycle pattern of the female brush-tailed porcupine (*Atherurus africanus*, Gray 1842) from Gabon. Anim Repro Science 2003;77:247–59.

[22] Schutte AP. Canine vaginal cytology II. Cyclic changes. J Small Anim Pract 1967;8:307–11.

[23] Wied GL, Bibbo M, Keebler CM. Evaluation of the endocrinologic condition of the female genital tract by exfoliative cytology. In: Wied GL, Bibbo M, Keebler CM, Koss LG, Patten SF, Rosenthal DL, editors. Compendium on Diagnostic Cytology, 8th edition, Tutorials of Cytology; 1997. p. 55–64.

[24] Desmond T, Laule G. Use of positive reinforcement training in the management of species. Zoo Biol 1994;13:471–7.

[25] Hinds LA, Reader M, Wernberg-Moller S, Saunders NR. Hormonal evidence for induced ovulation in *Monodelphis domestica*. J Reprod Fertil 1992;95:303–12.

[26] Durrant B, Olson MA, Amodeo D, Anderson A, Russ KD, Campos-Morales R, Gual-Sill F, Ramos-Garza J. Vaginal cytology and vulvar swelling as indicators of impending oestrus and ovulation in the giant panda (*Ailuropoda melanoleuca*). Zoo Biol 2003;22:313–21.

[27] Mayor P, Galvez H, Guimaraes DA, Lopez-Gatius F, Lopez-Bejar M. Serum oestradiol 17b, vaginal cytology and vulval appearance as predictors of oestrus cyclicity in the female col-

lared peccary (*Tayassu tajacu*) from the eastern Amazon region. Anim Reprod Sci 2007;97:165–74.
[28] McEndree B. Clinical application of the vaginal maturation index. Nurs Pract 1999;24:48–56.
[29] Hubscher CH, Brooks DL, Johnson JR. A quantitative method for assessing stages of the rat estrous cycle. Biotech Histochem 2005;80:79–87.
[30] Lindburg DG, Fitch-Snyder H. Use of behavior to evaluate reproductive problems in captive mammals. Zoo Biol 1994;13: 433–45.
[31] Bonney RC, Wood DJ, Kleiman DG. Endocrine correlates of behavioral oestrus in the female giant panda (*Ailuropoda melanoleuca*) and associated hormonal changes in the male. J Reprod Fertil 1982;64:209–15.
[32] Lindburg DG, Czekala NM, Swaisgood RR. Hormonal and behavioral relationships during estrus in the giant panda. Zoo Biol 2001;20:537–43.
[33] Swaisgood RR, Lindburg DG, Zhang H. Discrimination of oestrus status in giant pandas (*Ailuropoda melanoleuca*) via chemical cues in urine. J Zool London 2002;257:381–6.
[34] McGeehan L, Li X, Jackintell L, Huang S, Wang A, Czekala NM. Hormonal and behavioral correlates of estrus in captive giant pandas. Zoo Biol 2002;21:449–66.
[35] Hunt KE, Wasser SK. Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant. Physiol Biochem Zool 2003;76:918–28.
[36] Wasser SK, Monfort SL, Southers J, Wildt DE. Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces. J Rep Fert 1994;101: 213–20.
[37] Wasser SK. Reproductive control in wild baboons measured by fecal steroids. Biol Reprod 1996;55:393–9.
[38] Wasser SK, Papageorge S, Foley C, Brown JL. Excretory fate of estradiol and progesterone in the African elephant (Loxodonta africana) and patterns of fecal steroid concentrations throughout the estrous cycle. Gen Comp Endocrinol 1996;102:255–62.
[39] Rolland RM, Hunt KE, Kraus SD, Wasser SK. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. Gen Comp Endocrinol 2005;142:308–17.
[40] Frederick C. The Reproductive Biology and Behavior of the Sun Bear (*Ursus malayanus*). Seattle, WA: University of Washington; 2008.
[41] Bouchard G, Youngquist RS, Clark B, Concannon PW, Braun WF. Estrus induction in the bitch using a combination diethylstilbestrol and FSH-P. Theriogenology 1991;36:51–65.
[42] Finlayson GR, Shimmin GA, Taggart DA, Skinner JF, Gilmore A, Paris MCJ. Oestrus cycle of captive southern hairy-nosed wombats (*Lasiorhinus latifrons*) in South Australia. Anim Reprod Sci 2006;95:295–306.
[43] Nilsson K, Risberg B, Heimer G. The vaginal epithelium in the postmenopause - cytology, histology and pH as methods of assessment. Maturitas 1995;21:51–6.
[44] Brizzolara S, Killeen J, Severino R. Vaginal pH and parabasal cells in post menopausal women. Obstet Gynecol 1999;94:700–2.
[45] Higham JP, MacLarnon A, Ross C, Heistermann M, Semple S. Baboon sexual swellings: information content of size and color. Horm Behav 2008;53:452–62.
[46] Goritz F, Hildebrandt T, Jewgenow K, Wagner N, Hermes R, Straub MH. Transrectal ultrasonographic examination of the female urogenital tract in nonpregnant and pregnant captive bears (Ursidae). J Reprod Fert Supp 1997;51:303–12.
[47] Spadey TJ, Lindgurg DG, Durrant BS. Evolution of reproductive seasonality in bears. Mammal Rev 2007;37:21–53.
[48] Tsubota T, Takahashi Y, Kanagawa H. Changes in serum progesterone levels and growth of fetuses in Hokkaido brown bear. Int Conf Bear Res Management 1987;7:355–8.
[49] Sato M, Tsubota T, Komatsu T, Watanabe G, Taya K, Murase T, Kita I, Kudo T. Changes in sex steroids, gonadotropins, prolactin, and inhibin in pregnant and nonpregnant Japanese black bears (*Ursus thibetanus japonicus*). Biol Reprod 2001;65: 1006–13.
[50] Schulz LC, Nelson RA, Pyter LM, Bahr JM. Induction of pseudopregnancy in the American Black Bear (*Ursus americanus*). J Exp Zool A 2003;298:162–6.
[51] Stirling I. Bears - Majestic Creatures of the Wild. Emaus, PA: Rodale Press; 1993.
[52] Carlstead K, Seidensticker J. Seasonal variation in stereotypic pacing in an American black bear (*Ursus americanus*). Behav Proc 1991;25:155–61.
[53] Zlamal A, Wieczorek M. Stereotypies in polar bears kept at Warsaw Zoological Garden. Advances in Ethology, Supplements to Ethology, 4th International Symposium on Physiology and Behavior of Wild and Zoo Animals 2002;37.
[54] Smith S. Propagation techniques and hand-rearing problems with Malayan sun bears and Roeding Park Zoo. Sixth Annual American Association of Zoo Keepers National Conference Proceedings 1979.
[55] Domico T. Bears of the World. New York, NY: Facts on File, 1988.
[56] Johnston LA, Donoghue AM, Igo W, Simmons LG, Wildt DE, Rieffenberger J. Oocyte recovery and maturation in American black bears (*Ursus americanus*): a model for endangered ursids. J Exp Zool 1994;269:53–61.
[57] Frederick C, Shrake D, North American Regional Studbook for the sun bear (*Helarctos malayanus*). Seattle, WA: Woodland Park Zoo; 2002.