



# Comparative ovarian function and reproductive monitoring of endangered mammals

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## ABSTRACT

The ability to track gonadal function is facilitated by the use of endocrine and ultrasound techniques, both of which are important tools for optimizing reproduction and ensuring sustainability of fragile populations. With so many species now endangered, captive breeding is increasingly viewed as a means to sustain important insurance populations. As reproduction is key to species survival, understanding how to control and monitor ovarian function is vital. Through decades of study, we now have a greater understanding of the diversity, and plasticity, of reproductive mechanisms across taxa. Even within related species, there are marked differences in seasonal, environmental and social influences on ovarian cycle dynamics, ovulatory mechanisms, and responses to assisted reproductive/ovulation induction protocols. For most wildlife species, endocrine function is assessed noninvasively through analyses of hormones or their metabolites excreted in urine or feces. Perhaps it should not be surprising then, that major differences in metabolism and routes of excretion exist, not only between species, but also among hormone types within a species. This means that a species by species, and sometimes hormone by hormone, approach is essential for developing effective reproductive monitoring and control strategies. Over the past 30 years, our laboratory has developed and validated a number of reproductive assay techniques, which has led to our amassing a database of ovarian cycle dynamics on over 100 species. This paper presents an overview of ovarian physiology, and summarizes comparative ovarian function research on some of our most well-studied species: felids, elephants, rhinos, tapirs and the giant panda, and how that information has been used to aid *ex situ* management. Each of these species represents a range of reproductive strategies, from the highly seasonal, monestrus giant panda to the aseasonal, polyestrus elephant. Some species exhibit spontaneous ovulations, while others are induced ovulators or both, with variations in ovarian cycle lengths that range from a few days to several months. These differences reinforce the need for studies of species basic biology to optimize breeding strategies.

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## 1. Introduction

Most wild mammals under human care are managed intensively, so the ability to monitor reproductive status can greatly facilitate breeding success for many species. More specifically, objective and reliable methods for monitoring key reproductive events, such as ovulation and pregnancy, not only find widespread application in the management of natural breeding, but also provide the basis for efforts to aid reproduction by assisted means. Control of ovarian function involves the interplay of hormones

within the hypothalamo-pituitary-gonadal (HPG) axis. Although there are many commonalities among mammalian species with regard to the endocrinology of reproductive function, marked differences in patterns and levels of hormones secreted and/or excreted make extrapolation of results from one species to another difficult and potentially misleading. Ultrasonography is another valuable tool for evaluating ovarian activity and function, especially when combined with endocrine assessments. Through research studies, collaborations and our diagnostic service laboratory, we have the largest reproductive database for wildlife in existence, with ovarian hormonal data on more than 100 mammalian species. This review summarizes information on several of our most well studied taxa: felids, rhinos, tapirs, elephants and giant panda, and highlights the diversity in reproductive mechanisms among and within those species, from the highly seasonal, monestrus giant

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panda to the aseasonal, polyestrus elephant. Also presented are examples of the interplay between adrenal (i.e., stress) and ovarian function, and how hormone monitoring can facilitate management decisions to improve overall well-being, including reproduction.

## 2. Overview of the hypothalamo-pituitary-ovarian axis

The control of ovarian function in mammals involves the interplay of hormones produced in the hypothalamus, pituitary, and ovary [see reviews, [1,2]. The arcuate nucleus in the mediobasal hypothalamus is an aggregation of neurons involved in synthesis and release of the polypeptide gonadotropin-releasing hormone (GnRH) that is transported to the anterior pituitary through a highly-specialized capillary network - the hypothalamic-hypophyseal portal system. GnRH binds receptors on gonadotroph cells of the anterior pituitary, which produce and release the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that are transported through the bloodstream to the ovaries, where they initiate a series of morphological changes that lead to ovulation and pregnancy if fertilization occurs. A general schematic of the ovary and its structures is presented in Fig. 1. Fig. 2 indicates typical hormonal relationships that result in follicle

recruitment, selection and ovulation. The fate of each follicle is controlled by endocrine as well as paracrine factors, and they develop through primordial, primary, and secondary stages. Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles. At the antral stage, most follicles undergo atresia, whereas one or a few of them, respond to cyclic gonadotropin stimulation, become dominant and ovulate. Rising FSH concentrations induce the emergence of follicle waves, sometimes multiple (Fig. 3), with the production of estrogens and inhibin from antral follicles under FSH control that are then responsible for a subsequent suppression in FSH. Follicle cohort attrition occurs during declining FSH concentrations, resulting in dominant follicle selection. The dominant follicle acquires relative FSH-independence to continue growth and differentiation during further declining FSH concentrations. In addition, FSH and insulin-like-growth factor synergize to stimulate follicle cell function. A transition from FSH- to LH-dependence is proposed as a mechanism for the continued development of the selected dominant follicle.

Anti-müllerian hormone (AMH) is a glycoprotein produced by granulosa cells primarily during the recruitment stage of folliculogenesis in preantral and small antral follicles that also plays a

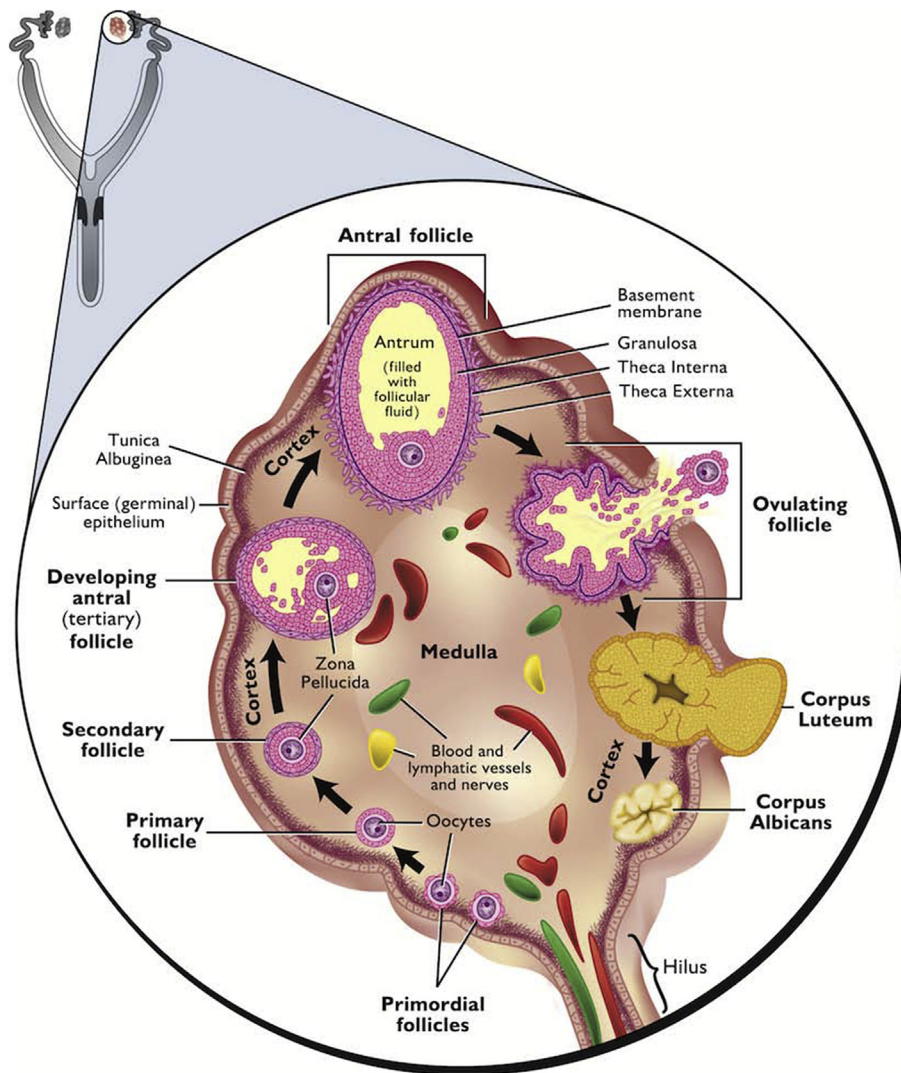


Fig. 1. Schematic of ovarian structures throughout the estrous cycle in mammals. Reprinted with permission, Pathways to Pregnancy & Parturition (Senger, 2012).

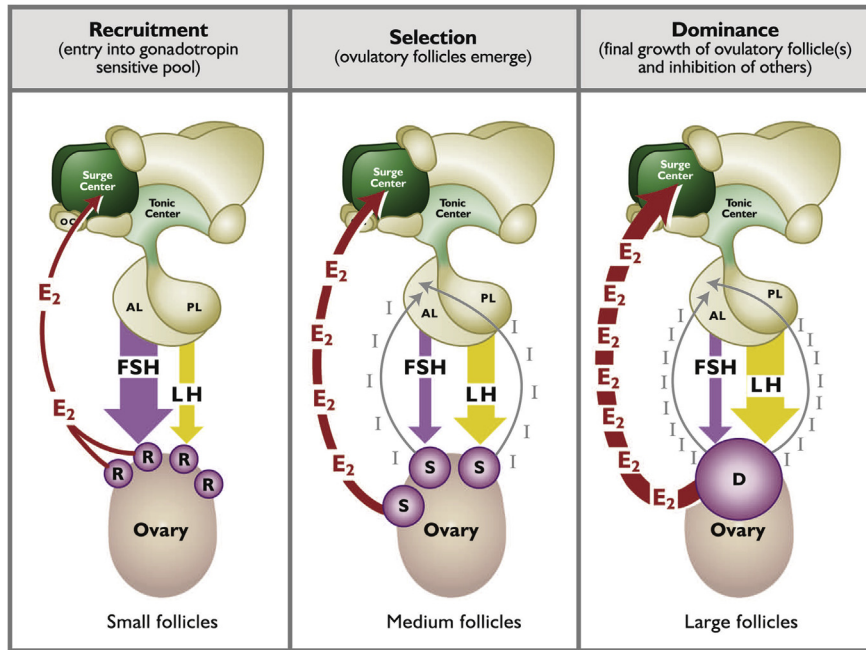


Fig. 2. Schematic of the hypothalamo-pituitary-ovarian axis during follicle recruitment (left panel, R), selection (center panel, S) and dominance (right panel, D) stages of the mammalian estrous cycle, showing patterns of estradiol-17β (E<sub>2</sub>), LH and FSH production. AL = anterior pituitary; PL = posterior pituitary; I = inhibitory pathway. Line width represents relative hormone concentration. Reprinted with permission, Pathways to Pregnancy & Parturition (Senger, 2012).

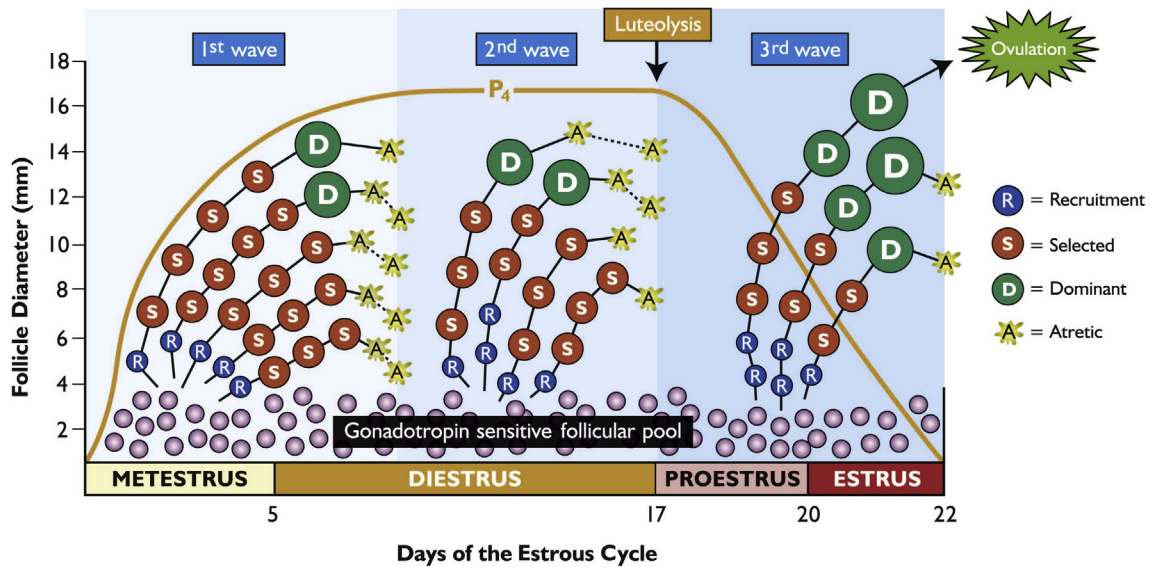
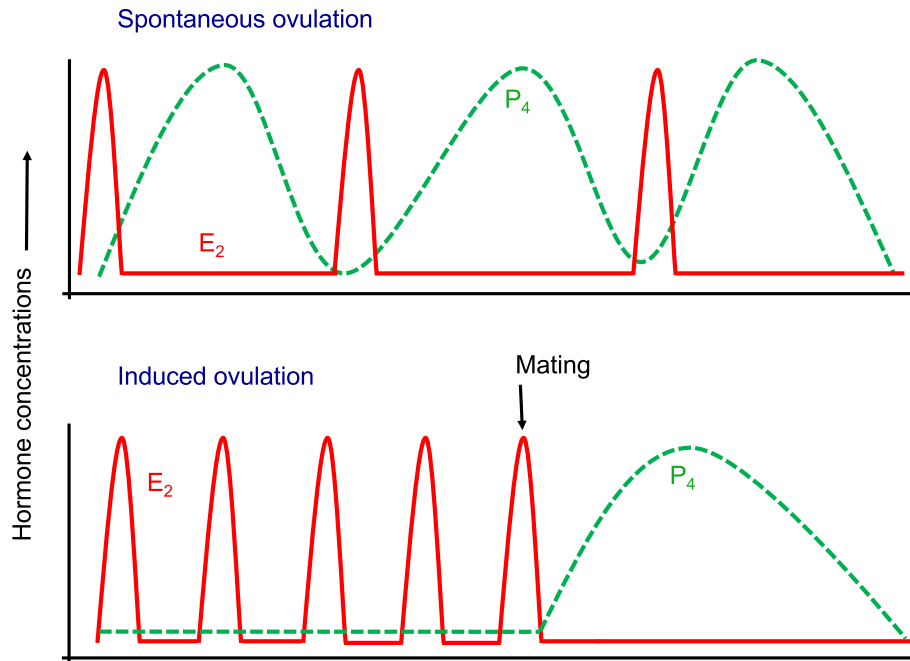


Fig. 3. Representative schematic indicating three successive follicular waves throughout the mammalian estrous cycle culminating in ovulation. P<sub>4</sub> = progesterone. Reprinted with permission, Pathways to Pregnancy & Parturition (Senger, 2012).

role in selection of the dominant follicle [3]. AMH regulates folliculogenesis by inhibiting recruitment of follicles from the resting pool, and its levels decline as the size of the follicle increases. AMH is used as an indicator of follicle reserves in many species, including for menopause in women, and as a marker of ovarian dysfunction, such as polycystic ovarian disease. Estradiol is another important regulatory hormone in the control of ovarian function. At low concentrations, estrogens inhibit gonadotropin secretion, but as the follicle grows, theca cells acquire more LH receptors, producing androgens that are metabolized to estrogens by the granulosa cells in response to FSH. This positive feedback loop results in an

estradiol-triggered LH surge that causes ovulation. LH action then is responsible for the formation of the corpus luteum (CL), which produces progesterone that prevents cyclicity by acting on the anterior pituitary in a negative feedback fashion, thereby decreasing the release of FSH and LH. It also prepares the uterus for reception of a fertilized ovum and subsequent pregnancy. However, if pregnancy does not ensue, prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) is secreted by the endometrium of the uterus and causes the demise of the CL, and results in withdrawal of progesterone negative feedback so that cycles can resume. As indicated in Fig. 4, cyclic patterns of estrogens and progestagens vary depending on whether ovulation is induced



**Fig. 4.** Schematic demonstrating spontaneous (top panel) and induced (bottom panel) ovulation patterns of estradiol (E<sub>2</sub>, red line) and progesterone (P<sub>4</sub>, dashed green line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(requires mating stimulus to induce LH surges) or spontaneous. Species also vary in seasonality of hormone patterns, ranging from nonseasonal polyestrus to seasonal monestrus (Fig. 5).

### 3. Monitoring ovarian activity in wildlife

Hormones can be measured in a variety of biological matrices, including blood, saliva, urine, and feces, usually involving immunological methods, like radioimmunoassay (RIA) or enzyme immunoassay (EIA) techniques that rely on specific- or broad-spectrum antibodies [see review, [4]. The choice of what to use depends on a range of factors, including the species to be studied, type of information required, the assay techniques involved, mechanisms of steroid metabolism and routes of excretion, and the practicality of sample collection, particularly when longitudinal monitoring is desired. Measurement of hormones in blood remains the most informative and widely used approach for monitoring physiological function in laboratory and domestic animals, and there are a number of advantages: easier sample preparation, measurement of native hormone, no need for indexing concentrations, and real-time reflection of hormonal status (little or no time lag). We have considerable serum data on pituitary and ovarian hormones in elephants, rhinos and tapirs, three species that can easily be trained for routine blood collection to assess HPG activity.

In most wild animals, however, difficulties associated with capture or restraint necessary for blood collection makes this procedure impractical for routine (i.e. repeated and/or regular) assessments. Thus, noninvasive approaches have been developed, starting with urinary assays in the 1980's and fecal metabolite assays in the 1990's. From radiometabolism studies, we know that major differences exist, not only between species, but also among hormones within the same species in terms of where hormones are excreted [4]. For example, among primates, squirrel monkeys excrete most (70%) of the estrogen and progesterone metabolites in feces [5], whereas in another New World species, the cotton-top tamarin, excretion of estrogens is almost exclusively (~92%) in

feces, and progestagens (~95%) are eliminated via feces [6]. Similarly, both the Sumatran rhinoceros [7] and African elephant [8] excrete estrogens primarily via urine, but progestagens are found in both urine and feces. Finally, most excreted steroids (>90%) in felids are found in feces [9,10]. Thus, an understanding of the relative importance of urinary vs. fecal pathways in hormone excretion is vital to assist in the development of noninvasive techniques for diverse species. Identifying appropriate assays is also key because many steroids are metabolized, sometimes heavily, before excretion, thus requiring the use of antibodies that have broader crossreactivity to related steroids [4].

Another powerful tool for studying ovarian function is ultrasound imaging [11]. Ultrasonography was used as early as the 1950s to characterize soft tissues in humans, and today is used routinely in human and veterinary medicine. This technique offers opportunities to study structural changes within the ovary in relation to the sexual cycle and ovulation. In contrast to other imaging procedures (e.g. radiography, magnetic resonance, endoscopy), ultrasonography has advantages because it is non-invasive, provides real-time information, generates a high-resolution characterization of reproductive organs, and is portable and thus compatible with zoo and field studies. When combined with endocrine assessments, this technique offers opportunities to study reproductive biology in both captive and wild populations, and develop new, or modify established, reproductive technologies. In particular, the ability to assess ovarian responses to ovulation induction and artificial insemination (AI) or embryo transfer protocols is key to breeding success. Gonadotropins like equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), FSH and LH alone or in varying combinations are typically used to stimulate follicular development and induce ovulation, respectively. However, ovarian responses can be highly variable across species and even within individuals. The ability to assess follicular structural and estrogenic activity via ultrasound and endocrine monitoring is key to developing regimens that adequately stimulate follicular development and induce ovulation without overstimulating the ovary, a problem observed in many species.



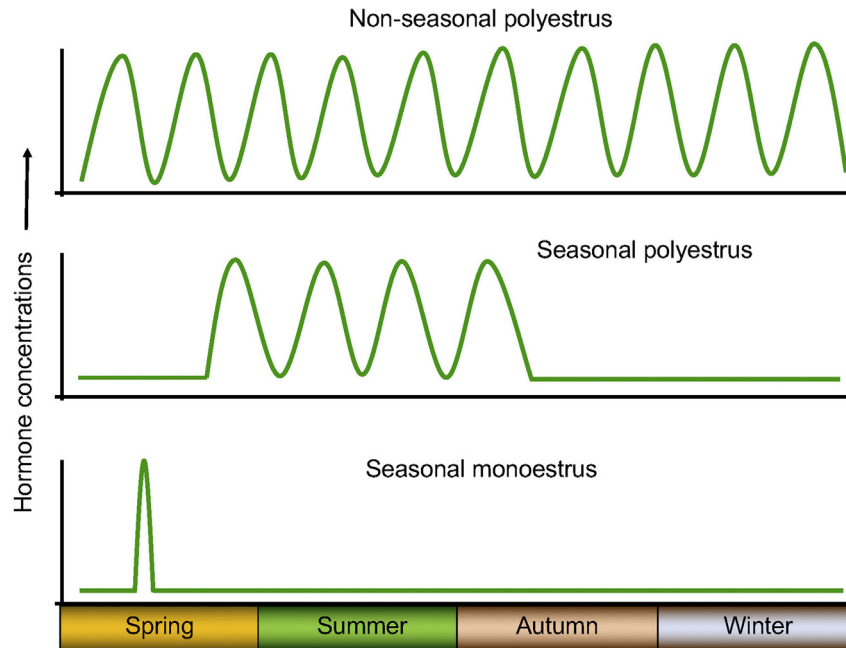


Fig. 5. Schematic demonstrating seasonality of hormone production representing non-seasonal polyestrus (top panel), seasonal polyestrus (middle panel) and seasonal monoestrus (bottom panel) secretion. Hormone concentrations can reflect patterns of estrogens or progestagens depending on the species.

#### 4. Ovarian physiology of felids

##### 4.1. General reproduction

Our laboratory has studied the reproductive biology of felids for over 30 years. As a taxon, these were the first non-primate species to benefit from the development of noninvasive hormone monitoring techniques [9]. Much of what we know about ovarian function in felids is based on initial studies of domestic cats [see reviews, [10,11]]. In nonmated cats, regular fluctuations in estrogens support an estrous cycle length of ~2 weeks, with estrus lasting 3–7 days. On Day 3 of estrus, behavioral signs are strongest in association with peak estrogen secretion. Multiple intromissions, often over several days, are necessary to stimulate release of GnRH from the medial basal hypothalamus and induce subsequent surges of LH from the anterior pituitary gland to induce ovulation, which normally is observed 24–48 h post-coitus depending on the time of mating onset. During diestrus, one or more CL produce progesterone that stays elevated for about 60 days if pregnant, and 40 days if conception did not occur after ovulation. Because frequent matings generally are needed to stimulate sufficient LH to cause ovulation, domestic cats have been characterized as induced ovulators; however, we now know that spontaneous ovulations do occur, sometimes frequently, in this species.

##### 4.2. Monitoring reproduction in nondomestic felids

In nondomestic felids, we assess ovarian follicular and luteal characteristics primarily through fecal hormone monitoring, using polyclonal antibodies against estrone-3-glucuronide (R583 or R522-1; U.C. Davis, CA) or estradiol-17 $\beta$  (R0008 or R4972, UC Davis, CA) depending on the species, and a monoclonal anti-progesterone antibody (Quidel clone, CL425) that generally works for all cat species, respectively. Patterns vary considerably though [10,12,13], and although many females cycle about every 2–3 weeks similar to the domestic cat, some average as short as 1–1.5 weeks (e.g., cheetahs, black-footed cat), to as long as 4–5 weeks (e.g., clouded

leopard, tiger, snow leopard). Because frequent matings are typical of wild felids as well, it has been assumed they too are primarily induced ovulators. However, like the domestic cat, we now know that the type of ovulation ranges from being almost exclusively induced (e.g., tiger, puma, snow leopard, cheetah, tigrina, jaguar, sand cat, lynx, ocelot), to occasionally (lion, leopard, black-footed cat, Pallas' cat, bobcat, fishing cat) or regularly (margay and clouded leopard) spontaneous. These differences occur not only across species, but also between individuals within a species. In fact, for some species, individual females display varied combinations of both induced and spontaneous ovulations over time. Thus, within this taxon, ovulatory mechanisms vary, regulated to a greater or lesser degree by species and perhaps individual-specific responses to physical and/or psychosocial stimuli. Seasonality in felids also exists on a continuum. Of the 41 wild cat species, at least 13 exhibit seasonal breeding in the wild and/or captivity, whereas for 15 others, births have been observed in every month of the year [13]. Seasonality of reproduction is controlled in part by photoperiod, although a few species (leopard cat, sand cat, serval) exhibit 'variable' seasonal reproduction that is linked to environmental conditions or prey availability rather than being restricted to specific months of the year.

Outliers among the felids in terms of ovarian function are the lynx species. Iberian and Eurasian lynx are strictly seasonal breeders with a single ovulatory cycle, and thus are the only monovulatory cat species [14,15]. Estrogen and progesterone immunoassays are ineffective in characterizing ovarian activity in part because estrogen concentrations increase throughout the breeding season, regardless of whether mating or pregnancy ensues, and due to persistent CL and that elevated progesterone concentrations are maintained for at least 2 years after ovulation, regardless of origin (pregnant or pseudopregnant) [16]. Though not as extensively studied, the Canadian lynx shares many of these traits, including persistent CL across cycles [17]. The bobcat is the only cat in the lynx genus that is seasonally polyestrous, but they too exhibit CL that persist in subsequent cycles [16].

Endocrine monitoring has been key to developing appropriate

hormonal therapies used with assisted reproductive procedures. The gonadotropins eCG and hCG are typically used to stimulate follicular development and induce ovulation in felids, respectively, but results often are inconsistent. In particular, standard chorionic gonadotropin regimens often induce ovarian hyperstimulation, resulting in estrogen concentrations that are several-fold higher than those observed during natural estrus [see review, 10]. Some excessive estrogen production after gonadotropin treatment is due to ancillary ovarian follicle development with subsequent hyperestrogenism causing an abnormal endocrine environment that is detrimental to fertilization, embryogenesis and implantation [18]. Approaches to temporarily shut-down ovarian activity before ovulation induction hopefully will result in more normal responses without hyperstimulation [18].

At least some hormonal data now exist for 24 of the 41 nondomestic cat species [13]), although for many, complete ovarian cycle characterizations are still lacking. Even within this limited dataset, with just over half of the species studied, it is clear that a wide range in ovarian control mechanisms exists among felids, which serves to reinforce the need to study each new species with the assumption that they may not fit into known parameters for this taxon.

## 5. Ovarian physiology of Perissodactyla

### 5.1. Rhinoceros

Roth et al. [19] provides a thorough review of rhinoceros reproduction and ovarian function in this issue, which will not be covered in detail here. However, this taxon is a good example of how our laboratory has been involved in studies to understand causes of poor reproductive performance in zoo populations, including rhinos. In the late 1990's, the Rhino Taxon Advisory Group (TAG) requested a multi-institutional study to assess reproductive problems in black and white rhinoceros females in U.S. zoos, including a thorough examination of ovarian cycle function. Thus, a total of 16 black and 13 white rhino females were assessed over 14- to 24-month periods [20], and immunoassays were validated to quantify steroid metabolites in feces using total estrogens (ICN Biomedicals) and broad spectrum progesterone (CL425) antibodies. Although analysis of urinary estrone (black rhinoceros) or estradiol-17 $\beta$  (white rhinoceros) had been shown early on to coincide with behavioral estrus [21], we found that fecal estrogens were uninformative in assessing follicular activity. By contrast, fecal progestagens provided good data on luteal function and ovarian cycle status. In our study, females cycled year-round, although erratic cycles were noted in both species. In the black rhino, 39% exhibited abnormal cycles that were shorter (<20 days; 18% of cycles) or longer (>32 days; 21%) than normal (27 days), as in other studies [20,22,23]. About 60% had periods of anestrus lasting several months, but none were completely acyclic. In a related study of ~70% of the black rhinos in North America, Carlstead et al. [24], identified three predictors of reproductive success: 1) females being behaviorally dominant to the male; 2) large enclosure size (>1000 m<sup>2</sup>); and 3) a minimal amount of high concrete wall exhibit construction. Edwards et al. [23] also reported that pregnancy rates in U.K. zoos were lower in black rhinos with estrous cycle lengths of <19 or >40 d. Altogether, although many females exhibit periods of acyclicity lasting several months that may be contributing to challenges with breeding, we concluded that population sustainability seems to be more affected by inappropriate management and to some extent high morbidity and mortality rates.

More problems with ovarian cycle problems were observed in white rhinoceros females [20], where 46% showed no evidence of ovarian activity, and only 15% cycled continuously. An unusual

feature of white rhinoceros reproduction is that females exhibit two distinct cycle lengths: ~30 days or ~70 days [19] rather than a range as observed in black rhinoceros. In our study, the majority of females (89%) displayed long cycles, although some females exhibited both over time, similar to other reports [25–27]. These results indicate a physiological basis for poor reproduction in white rhinos in captivity. Patton et al. [25] suggested the extended luteal phases in white rhinos could be the result of pyometra, endometritis or embryonic loss, all of which have been documented in females exhibiting prolonged progestagen excretion [25,27]. By contrast, Schwarzenberger et al. [26] proposed that some short luteal phases associated with low progestagen production are caused by cystic or hemorrhagic follicles and anovulation. Conceptions have been observed in white rhinos displaying both short and long cycles [26], although pregnancy are reported to be higher in association with the shorter cycles [25,27]. Thus, there are conflicting theories as to what cycle type is actually normal for this species. Finally, the development of reproductive tract pathologies in older captive white rhinos is a concern [28], and given the age structure of the U.S. zoo population, it suggests problems with erratic or totally quiescent ovarian activity likely will continue or worsen.

Whatever the etiology of abnormal cyclicity in rhinoceroses, it is clear these reproductive problems need to be addressed. A high priority must be to use noninvasive hormone monitoring in conjunction with frequent ovarian examinations via ultrasound to document the physiology (or pathology) associated with each cycle type, and assess how management and husbandry practices affects fitness, both on an individual and population level.

### 5.2. Tapir

Our studies of tapirs began in the late 1980's because of a need to understand their reproductive biology to sustain dwindling captive populations. The first species we studied was the Baird's tapir [29], but we now have ovarian steroid data for the other three species: lowland, mountain, and Malayan tapir. All four species currently are maintained in captivity either in zoos or breeding centers throughout the world, with one priority being to develop assisted reproductive techniques to improve genetic management of often isolated populations [see review, 30].

#### 5.2.1. General reproduction

Female tapirs reach puberty at 14–30 months of age, with nutrition and the compatibility of breeding pairs appearing to influence the onset of sexual maturity [30,31]. Females maintain fertility well into their 20's, and have been known to reproduce past 30 years of age [31]. Tapirs are nonseasonal, polyestrous, spontaneous ovulators, although females display few overt behavioral signs of estrus [32]. In the Malayan tapir, vulvar swelling and/or discharge has been observed during estrus when progesterone is low, and can be used as a visual detection aide [33]. As reviewed by Pukazhenthil et al. [30], the paired ovaries are similar in structure to that of the horse and located on the ventral surface of the os ilium near the coxal tuber. The average length and width of the ovary in an adult tapir is 2.8 cm and 1.3 cm, respectively.

Female tapirs typically give birth to a single offspring every 1.5–2 years. The interbirth interval is ~18 months for consecutive pregnancies, but can be as short as 14 months because the first postpartum estrus is fertile [29,31]. Although tapirs in the wild breed primarily during the rainy season, no seasonality has been observed in animals managed in captivity [29,31,33].

#### 5.2.2. Reproductive endocrinology

An advantage to studying tapirs is that many females are

tractable and will permit serial blood collection in the absence of anesthesia or extensive physical restraint. Blood can be drawn safely from a cephalic vein in the forelimb or a medial saphenous vein in the hind limb, or the jugular vein, and a weekly sampling protocol is adequate to assess cycles. As such, at least some longitudinal characterizations of blood serum estradiol and/or progesterone during the estrous cycle and/or pregnancy are available for all four tapir species [30]. Hormone monitoring is a particularly valuable breeding management tool as females often copulate during early gestation, and males can be aggressive to offspring [31], so it is important to know if a female is pregnant. For females not trained for blood collection, urinary steroid metabolite monitoring has been used for diagnosing and monitoring pregnancy [34,35], but more work is needed to develop other non-invasive approaches for assessing estrous cyclicity and fertility status in these species.

Our initial studies with Baird's tapirs utilized progesterone and estradiol RIAs developed for human serum (ICN Biomedical, Costa Mesa, CA) [29], but other commercial assays also work well, as does the monoclonal progesterone antibody, CL425, for assessing luteal activity. Estrous cycle duration is  $30.8 \pm 2.6$  days (range, 25–38 days), with a luteal phase length of  $18.1 \pm 0.4$  days (range, 15–20 days) and an interluteal period of  $12.6 \pm 0.5$  days (range, 11–14 days). Distinct surges of estradiol precede luteal phase progesterone increases.

There is less information on estrous cycle hormone patterns in lowland and mountain tapirs, but we have determined that the cycle length of these two species is similar to Baird's, at about monthly intervals [30]. An evaluation of milk progesterone in one lowland tapir indicated an estrous cycle of 31 days [36]. In one case report, a single lowland tapir experienced recurrent vaginal prolapses every 38 days over 10 cycles, a condition that was alleviated by bilateral ovariectomy [37]. Data on mountain tapir are limited to a single evaluation of a female we evaluated that was blood sampled weekly for 1 year, which revealed a clear, monthly cycle pattern in serum progesterone, similar to that described for the Baird's and lowland tapirs [30].

The Malayan tapir differs from the other three species by exhibiting variable cycle lengths: short (~30 days) and long (~70 days), with both cycle types often occurring in the same individual [30], not unlike the white rhinoceros. In general, the two categories of cycles occur with nearly equal distribution in Malayan tapir. It is not known if the two cycle lengths represent normal vs. abnormal conditions, so it is an area that deserves further research. Endocrine patterns in this species have been associated with changes in vulvar appearance and mucous discharge, although not always consistently [33]. Thus, the only accurate method of tracking ovarian activity remains some form of hormone monitoring.

Gestation in the Baird's tapir is about 390 days (~13 weeks) [29], which is slightly longer than the 11–12 months reported for horses, but shorter than the 15- to 16-month gestation of black and white rhinoceros [20,25]. As reviewed by Pukazhenthil et al. [30], female Baird's tapirs exhibit a biphasic pattern of progesterone secretion during early gestation [29]. In the domestic horse, increased progesterone secretion after Day 30 is due to the development of secondary corpora lutea that form in response to the production of eCG by placental endometrial cups [38]. Several nondomestic Perissodactyla species, such as Przewalski's horse and zebra, also produce a CG that crossreacts in eCG immunoassays [35,39]. However, only low levels of eCG immunoactivity have been found in the urine of pregnant tapirs (all four species), and so are of limited use as a diagnostic pregnancy test [35]. A secondary dip in progesterone secretion occurs later in pregnancy, at 4–5 months [29], which in the horse is attributed to the transition from luteal to fetoplacental sources. Thereafter, serum progesterone

concentrations gradually increase, peaking at concentrations up to 40-fold higher than baseline in the weeks before parturition. Serum estradiol concentrations also increase above baseline by about the fifth week of gestation and continue to increase thereafter until a precipitous decline at parturition. Based on serum progesterone, females resume cycling  $16.2 \pm 2.5$  days after parturition, and can conceive during the first postpartum estrus [29]. The rapid return to fertility in Baird's tapirs is similar to 'foal heat' described for mares; the first generally occurs within 20 days postpartum, and is characterized by normal follicular development and ovulation. Similarly, in black rhinoceros, increases in fecal progestagens demonstrate resumption of estrous cyclicity within 1–3 months, before calves are weaned [20].

### 5.2.3. Feasibility of noninvasive hormone monitoring

Urinary estrogen metabolite analyses are effective for diagnosing and monitoring pregnancy noninvasively during the last two thirds in all four tapir species [30]; however, urinary progestagen analyses have been less reliable. Ramsay et al. (35) tested a small number of samples (3–24) in each species using an immunoassay for C-19/C-21 metabolites and found reasonable specificity (93%), but low sensitivity (73%) for diagnosing pregnancy. A urinary assay for pregnanediol-3-glucuronide also could not detect pregnancy in any of the four species [35]. Analysis of fecal estrogens has shown promise as a potential pregnancy test, increasing during late gestation in Malayan tapirs [40,41], and may be applicable to other tapir species.

Although fecal progestagen analyses can be used to assess luteal activity in many Perissodactyla [25,42–44], use of several different broad-spectrum progestagen antibodies, including CL425, has failed to yield biologically relevant data in tapirs [30]. It is possible that progesterone metabolites are not excreted in appreciable quantities in tapir feces, or that they are not recognized by currently available antibodies. Conducting a radiometabolism study (i.e., injecting  $^{14}\text{C}$ -labelled progesterone and collecting excreta) would determine if appreciable amounts of progestagens are excreted in feces, and what the major metabolites are. This would be an important step in developing appropriate noninvasive techniques for monitoring follicular and luteal function in tapirs.

## 6. Ovarian physiology of elephants

### 6.1. General reproduction

Elephants are the most well-studied species in our laboratory, and we have hormonal data on over 200 individuals, some evaluated for up to 30 years. A review of basic elephant ovarian physiology is presented by Thitaram and Brown [45] and also Brown [46,47], which describe much of our work conducted in western zoos and in Thailand. Elephants are relatively easy to study because they can be trained for regular blood collection and ultrasound examinations. Early on it was discovered that in contrast to other species, the main luteal product in the elephant is not progesterone, but several  $5\alpha$ -reduced pregnanes (progestagens). This has required the use of immunoassays that use more broad-spectrum progesterone antibodies [46,55]. Originally, we relied on a solid-phase progesterone RIA from Siemens Medical Solutions to assess luteal activity, but after its discontinuation, we began using our monoclonal progesterone CL425 antibody, which is effective for use with blood serum/plasma, saliva, urine, and also fecal extracts. Estradiol concentrations are low in circulation due to rapid conjugation and excretion primarily into urine [8,48], and so we are generally limited to characterizing follicular activity via urine analyses and ultrasound examinations.

## 6.2. Estrous cycle

In general, both species are polyestrous spontaneous ovulators, generally aseasonal, and have the longest mammalian ovarian cycle of 14–17 weeks in duration (8- to 10-week luteal phase, 4- to 7-week follicular phase). One of the most significant findings was the discovery of a 'double LH surge' utilizing an anti-bovine LH antisera (518-B7), where two distinct LH surges (LH1 and LH2) occur 18–22 days apart in association with two follicular waves. Using a combination of serial endocrine and transrectal ultrasound assessments, multiple follicles develop during the first wave, but none ovulate in response to LH1. Rather, some of the follicles luteinize, herein referred to as accessory CL (acCL), although they do not immediately produce progestagens [49,50]. During the second wave, additional follicles develop, but only one becomes dominant, ovulates in response to LH2, and forms the ovulatory CL (ovCL). Thus, although the elephant is monovular, multiple CLs (ovCL and acCL) are present in any given cycle, and many remain functional in subsequent luteal periods. By repeatedly forming two distinct types of CLs during every reproductive cycle, elephants may have developed a mechanism to ensure there is sufficient luteal capacity for maintaining a 22-month gestation should conception occur [49].

Simultaneous with luteinized follicle formation after LH1, inhibin concentrations increase [46,47,51–53] and through a negative feedback mechanism are inversely related to FSH. Inhibin is produced by estrogenic follicles in other species [54]; however, little is produced by large estrogenic follicles of the first follicular wave [52]. Rather, it appears the cells of luteinized follicles and acCLs leading up to LH2 are sources of inhibin in elephants [52]. Dominant follicle deviation begins about 5–6 days before ovulation when inhibin concentrations are high and FSH concentrations return to baseline [46,47,51,55]. Prolactin is folliculogenic in many species, and in African elephants is elevated during the follicular phase of the cycle [56,57]. There is a significant species difference in the secretion of prolactin because concentrations are unvaried throughout the cycle in Asian elephants [58], and ovarian cycle problems associated with abnormal prolactin secretion are observed primarily in African elephants [46].

## 6.3. Gestation

Elephants and horses share some gestational traits, such as the presence of multiple large CL in the maternal ovaries [see review, 59]. However, there are some notable differences. In the elephant, acCLs are produced throughout the follicular phase of preceding cycles [49,52], and there are no additional CLs produced during gestation [50]. By contrast, the mare produces one CL at ovulation, with additional CLs formed as the result of ovulation/luteinization of mature follicles after conception due to eCG [60,61]. In both Asian and African elephants, acCLs and the ovCL begin to regress about 5–6 weeks after conception (the normal luteal phase lifespan), but then rebound and grow significantly larger than those in a non-conceptive luteal phase [50], commensurate with the marked secondary rise in progestagen concentrations in the maternal circulation after the second gestational month [50,62]. By contrast, mature follicles never develop in pregnant elephants [50], nor is there evidence of gestational gonadotropin-like activity in serum or placental extracts [62,63]. In many species, particularly primates, inhibin produced by follicles, CLs and/or the placenta is involved in the establishment and maintenance of pregnancy. In the elephant, CLs are a major source of inhibin during the estrous cycle [51,53], but do not appear to produce much inhibin in the pregnant elephant [53]. Rather, both progestagens and inhibin are increased immediately post-conception and then decrease at 7–8

weeks. After that, progestagens rebound to even higher concentrations on average, whereas inhibin concentrations continue to decline. This pattern suggests a shift in luteal cell function during early gestation, and a deviation in the secretory ability of CLs between cycling and pregnant elephants.

## 6.4. Reproductive monitoring for management

What has aided our understanding of basic elephant endocrinology is the establishment of our endocrine diagnostic laboratory in 1994, which works with the Elephant TAG to identify reproductively viable females and aid in breeding management. Through evaluations of weekly serum samples, we can precisely time breeding by identifying LH1 and scheduling natural mating or AI for 3 weeks later, and pregnancy can be diagnosed by the continual elevation in progestagens past 12 weeks, or an increase in serum prolactin concentrations after ~5 months [46]. The sex of a calf can be determined, at least in Asians, by measuring the concentrations of testosterone in the maternal circulation after a year of gestation [64], and birth can be predicted within ~5 days after the drop in progestagens to baseline [46]. In fact, in the U.S., a zoo cannot receive a breeding recommendation without at least a year of hormone monitoring to establish regular ovarian cycles, in conjunction with a normal reproductive tract ultrasound assessment.

## 6.5. Reproductive problems

Our diagnostic laboratory was instrumental in identifying significant reproductive problems of captive elephants in the U.S. zoo population. For Africans in particular, a high rate of ovarian acyclicity across the age categories thwarts population sustainability [46]. In North America, 52% of African elephant females exhibit abnormal cycles, with 38% not cycling at all [65]. Comparatively, the acyclicity rate in Asian elephants is lower (<20%) and affects mostly older females [65,66], although it is not believed that elephants experience a true 'menopause' like that in humans [67]. Thus, these abnormalities in ovarian function likely signal a form of maladaptation of elephants to the captive environment, which has led to 20 years of research trying to understand the causes.

Several conditions known to be associated with infertility in other species are not related to ovarian cycle problems in African elephants, such as hyperandrogenism, hyperestrogenism, thyroid derangements, pituitary dysfunction and elevated cortisol [46]. Lack of observed differences between cycling and acyclic elephants in anti-müllerian hormone (AMH) concentrations suggests the problem is not due to a lack of functional follicles [68]. One association with ovarian acyclicity in African elephants is hyperprolactinemia [56–58], which is the most common disorder of the hypothalamic-pituitary axis related to infertility in women [69]. Over half of noncycling elephants exhibit levels of prolactin that are 5- to 30-fold higher than those in normal cycling females. Thus, it may be significant that prolactin is related to follicular development in African elephants only, as are ovarian cycle problems associated with abnormal prolactin secretion [57]. As in other species, pituitary prolactin secretion in elephants is under inhibitory control by dopamine [46]. The dopamine agonist, cabergoline, effectively suppresses prolactin secretion in hyperprolactinemic elephants, but has yet to induce a resumption of ovarian cyclicity even after a year of treatment [70]. No underlying physiological cause of hyperprolactinemia has yet been found, but it does not appear to be related to abnormal production of cortisol, estradiol or thyroid hormones (TSH, T<sub>3</sub>, T<sub>4</sub>) [58].

Prolonged acyclicity does not appear to occur in wild African elephants when resources are available based on physiological



studies, including noninvasive fecal progesterone monitoring, that show females can cycle into their 50's, although capacity does decline with age [71,72]. It is unlikely that any one management factor is responsible, as 52% of zoos house both cycling and non-cycling females [73]. Some elephants can alternate between a cyclic and non-cyclic status [46], so there likely are multiple etiologies, and it will be key to ascertain if problems are of physical or behavioral origin, and what the best approach is to ameliorate them (e.g., exercise programs for overweight elephants, altered social groupings, creation of multi-generational herds, increased space, etc.). It is important to develop targeted treatments for these conditions, especially given the complexity of trying to control endocrine function by therapeutic means.

## 7. Ovarian physiology of the giant panda

### 7.1. General reproduction

The National Zoo has been a leader in giant panda reproduction research since 1972 when China gifted the U.S. with a breeding pair (Ling-Ling and Hsing-Hsing). Early on it was obvious that reproduction in the giant panda is particularly challenging, as outlined in a recent paper by Kersey et al. [74]. Female giant pandas are seasonally monestrous, typically experiencing a single estrus between February and May, during which time the female is only fertile for about 48 h. Outside of China, there are few pairs of pandas that mate naturally, due to lack of female interest or displays of lordosis, or inability of the male to properly position the female for intromission. When natural mating fails, transcervical AI can be used, and has been proven to be highly successful [75]. Key to breeding giant pandas, whether by natural means or use of AI, is the ability to assess ovarian activity noninvasively as a supplement to behavioral observations of sexual receptivity. Ovarian steroids are excreted in both urine and feces of giant pandas [76], but the ability to generate data within a few hours of collection has made urinary testing the most practical approach. In our laboratory, estrogens in urine or feces are monitored using polyclonal antibodies against estrone-3-glucuronide (R583 or R522-1) and the monoclonal progesterone antibody (CL425).

### 7.2. Estrous monitoring and timing breeding

The peri-estrous period in the giant panda is characterized by a gradual increase in estrogens over a 10- to 18-day period in association with a decrease in progesterone to sub baseline levels. Progesterone remains low while estrogens continue to increase, and then increase back to or just above baseline as estrogens begin to decline, a signal that peaks estrogens have been reached and ovulation has occurred. It is this confluence of a return of progesterone to pre-estrous levels and a decline in estrogens that is used to time AI, generally as two inseminations between 12- to 36-h post estradiol peak, 7- to 12-h apart.

### 7.3. Luteal function and delayed implantation

The giant panda exhibits delayed implantation, and the ensuing biphasic luteal phase post-ovulation is indistinguishable between pregnant and nonpregnant pandas, making pregnancy diagnosis by urinary or fecal progesterone analyses impossible. Although pregnancy can be confirmed via ultrasonography, one or more fetuses are not generally visible before 2 weeks of birth [77,78]. Ceruloplasmin evaluation, quantified from urine via oxidase activity, holds promise for early pregnancy detection, as concentrations

increase after ovulation and remain elevated until about 3 weeks before birth [79].

The nearly simultaneous decline in late gestational progesterone and ceruloplasmin levels, manifestation of maternal behaviors, and the emergence of a conceptus on ultrasound suggests these physiological events are linked [74]. The increase in progesterone during the secondary rise likely prepares the uterus for attachment [76]. Moreover, because ceruloplasmin, an acute phase protein, modulates inflammatory responses that may maintain or sustain embryonic diapause [79], a decline in ceruloplasmin would permit zona pellucida hatching, and expose the embryo to a uterus primed for embryonic attachment. Maternal behavioral and morphologic changes (e.g. mammary development, restlessness, nest building, depressed appetite, and vulva dilation, reddening, and licking) also do not initiate until progesterone declines. Finally, the visualization of the conceptus only when progesterone and ceruloplasmin begin to decline suggests these changes are needed to prepare for embryo attachment and growth, with the last 20–25 days of gestation reserved for rapid fetal growth. After parturition, females experience a period of acyclicity likely tied to lactation, only to resume reproductive cyclicity in the spring after offspring have been weaned [81]. Using a multi-disciplinary approach involving physiological measures and behavioral assessments has been key to successful reproduction of this species in both western zoos and China.

## 8. Management, stress and reproduction

Breeding of wildlife *ex situ* often is difficult; some species exhibit more problems in general, whereas acute or chronic bouts of infertility appear to be related more to individuals, perhaps in response to factors in the captive environment. We have several examples of how management or husbandry factors affect reproduction in a variety of species, many of these identified by means of routine hormonal monitoring. For instance, infertility related to a lack of normal ovarian activity has been observed in many zoo-managed felids (e.g., cheetah, ocelot, fishing cat) [10,12]. In some cats, suppression of estrous cyclicity occurs when females are housed together [cheetahs, [82]; Canadian lynx [17], whereas in cheetahs, separation of pairs results in normal cyclic ovarian activity [83]. In ocelots, ovarian inactivity has been associated with poor husbandry management, which was ameliorated by addition of enrichment items and hiding places [84]. A multi-institutional study of fishing cats identified a number of ways zoos could improve reproduction, including: 1) transferring males in the late spring or early summer to allow at least 3 months post-quarantine to allow glucocorticoid concentrations to return to baseline before breeding introductions; 2) reducing stress levels with positive animal keeper interactions through training; 3) providing indoor off-exhibit refuge areas; and 4) providing at least 1–2 nest boxes for the females [85]. Because many cat species are seasonal, management can affect reproduction. Clouded leopards exhibit anestrus in the late summer/early fall both in northern latitudes and in Asia, but if housed indoors on a 12-h light cycle, they will cycle year-round, which can affect breeding strategies [86]. Pallas' cats are highly seasonal, so a sudden transition to 'long days' during a November zoo lights festival stimulated premature follicular activation that had a negative impact on reproduction. Moving the cats away from the festival area the following year resulted in the successful production of kittens [87].

Based on fecal progesterone metabolite analyses, problems with abnormal ovarian cycles plague both white and black rhinoceros populations in zoos. Achieving appropriate social groupings to

stimulate normal reproductive behavior likely are essential. In the white rhinoceros, non-breeding females exposed to new males within the same facility or translocated to a new facility have become breeders [25]. The most compatible pairs of black rhinoceros are those in which the female is dominant to the male [24]. Longer cycles (>40 days) in black rhinoceros were more common in nulliparous females, and periods of acyclicity were observed more often in females that had not bred for at least 7 years. Even when endocrine data indicate clear ovarian activity, overt behavioral signs of estrus are not always apparent, particularly among nulliparous females. In black rhino, fecal glucocorticoid concentrations did not differ between parous and nulliparous females, but were related to temperament; more unpredictable temperaments were associated with higher glucocorticoids, and nulliparous females were rated as more unpredictable.

In elephants, several studies in the U.S. and Europe have applied epidemiological methods to identify factors in the zoo environment that are associated with reproductive success, and noted several differences between the Asian and African species [65,88]. For both, the chance a female will cycle is greater when they are housed in a mixed-sex herd, and decreases with age. For African elephants, social stability and compatibility, and enrichment are important for promoting normal pituitary-ovarian function, whereas social isolation has a negative effect. There also appears to be a social aspect to hyperprolactinemia in African elephants, which is more prevalent in females exposed to many social groups throughout the day, perhaps related to social instability [65]. The ability to longitudinally monitor hormone patterns, coupled with occasional ultrasound examinations, will be key to assessing how management changes impact ovarian function in elephants, and guide successful strategies in the future.

Some effects of management or husbandry are associated with glucocorticoid production (the major stress hormone). In white rhinos, higher fecal glucocorticoids were associated with ovarian acyclicity [89]. However, no relationship between ovarian cycle problems and circulating cortisol has been identified in African elephants, a species with significant fertility problems [90]. Rather, in that species, social stress perhaps mediated by prolactin rather than cortisol appears to be more of a problem [65]. In pygmy rabbits, higher glucocorticoid concentrations during the breeding season were associated with lower reproductive success. Females that failed to conceive had higher fecal glucocorticoid and lower progesterone baseline concentrations than females that did conceive [91]. Providing rabbits with soil for digging reduced fecal glucocorticoid concentrations. Soil also plays a vital role in pygmy rabbit reproduction because females give birth in underground natal burrows. Thus, given the fossorial nature of pygmy rabbits, soil appears to be an important component of their environment as it provides opportunities for natural behaviours such as digging, exploring and hiding [92].

Some populations reproduce too well, requiring the use of contraceptives, which can have unintended consequences. Because of poor reproduction after the lifting of an 8-year breeding moratorium, a biomedical survey of female lions in U.S. zoos was initiated in 2007 [93]. Analyses of fecal estrogens, progesterone, and glucocorticoids determined problems were not related to altered adrenal or ovarian steroid activity, but may have been a consequence of females having been contracepted during the moratorium. The return to cyclicity after treatment withdrawal, although variable, was ~4.0 yr, and much longer than the 1-yr expected efficacy, especially for those implanted with the GnRH analog, Suprelorin. Captive lion numbers now are increasing to the extent that contraceptives again are being used. For long-term management, newer drugs or safer, more reliable treatment protocols are needed to avoid such boom and bust cycles of breeding, not only for

lions, but other highly managed animal collections.

## 9. Conclusion

As comparative biologists, we never cease to be amazed by how varied reproductive mechanisms are across the species, even those that are closely related, and even sometimes between individuals within the same species. What this means is that a good understanding of the reproductive biology of one does not mean it will be the same for other related species. For each, we need to identify the type of ovulation (induced versus spontaneous), determine the effect of seasonality on reproduction, and assess ovarian responses to natural or assisted reproductive procedures to maximize fitness, especially for highly managed or captive populations. Ultimately, a thorough understanding of fundamental reproductive processes can facilitate breeding, management and overall conservation activities.

The ability to easily and safely assess reproductive status, especially through noninvasive means like urinary or fecal hormone monitoring, allows identifying reproductive problems and developing mediating solutions. A benefit of endocrine and ultrasound monitoring, in particular, is identifying causes of poor fertility in response to assisted reproductive procedures, allowing these tools to be optimized so they can more reliably contribute to species propagation. A high priority is developing ovulation induction protocols that result in consistent responses, without ovarian hyperstimulation, to provide an optimal maternal environment for fertilization and embryo development. Along with this effort is the need to control the reproductive cycle, including down-regulating endogenous ovarian activity, synchronizing estrus, and in some cases, contraception.

Ultimately, by utilizing a variety of physiological and behavioral assessments, wildlife managers can better assess how well environmental conditions meet individual needs. Particularly important is knowledge of species' natural history, which can be used to design proper exhibits and husbandry protocols that take into consideration: 1) natural social dynamics; 2) individual temperament and mate compatibility; 3) enclosure and enrichment diversity (e.g., branches, climbing structures, water feature, nest boxes); 4) nocturnal versus diurnal activity patterns; 5) dietary needs; and 6) appropriate choice options. All of these factors can, to varying degrees, affect reproductive function. In addition to reproductive monitoring, long-term evaluations of adrenal activity allow identifying captive environments compatible with welfare needs and maximal fitness.

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