Chapter 2

Estrus Cycle Monitoring in Wild Mammals: Challenges and Perspectives

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69444

Abstract

The knowledge of reproductive physiology is of paramount importance to guide reproductive management and to make possible future application of assisted reproduction techniques (ARTs) aiming ex situ conservation of wild mammals. Nevertheless, information on the basic reproductive aspects of wild mammals remain scarce, and appropriate management practices have not yet been developed for all the species. This chapter discusses the methods most currently used for reproductive monitoring in wild females. Additionally, the difficulties regarding their use in different species and the possibilities of these procedures in captivity or in free-living mammals are addressed.

Keywords: wild animals, female reproductive physiology, hormonal profile, noninvasive monitoring, captive management

1. Introduction

Considering that reproduction is an essential process for species survival, the use of assisted reproduction techniques (ARTs) in wild mammals' conservation allows the storage and exchange of genetic material between populations. Nevertheless, conservation initiatives depend on a profound knowledge of the species' reproductive physiology, since it is not always possible, for some endangered species, to extrapolate from domestic species or even from other wild species counterparts [1].

Thus, ARTs will only be successfully applied for conservation after mastering the aspects related to anatomy and physiology, namely, the characteristics of the reproductive cycle,

seasonality, behavior, and other general mechanisms that regulate reproduction [2]. An important factor that hinders reproductive monitoring is the lack of knowledge about the reproductive biology of various wild mammals, which makes the knowledge on their reproductive behavior scarce [3]. Even though the observation of external estrus signs can be used for heat detection, it must be associated with other techniques, for example, vaginal cytology, hormone measurement, ultrasonography, or thermography, in order to determine the most appropriate time for mating or artificial insemination.

Thus, this chapter presents the methods most currently used for reproductive assessment in wild females. In addition, the difficulties regarding its use in different species and the possibilities for using these procedures in captivity or in free-living animals are addressed.

2. Reproductive behavior analysis

Behavioral expression is a major aspect of animal communication and easily reflects the reproductive status to other members of the species. Mammals display considerable variation in the display of behaviors during different physiological states. The study of wild animal behavior is essential for implementing captive breeding programs. The lack of knowledge of the species behavior in its natural environment limits our ability to meet their needs in captivity. In this sense, information about changes in their reproductive behavior can be used to aid monitor the cyclicity of wild females [4, 5].

The behavioral patterns can vary accordingly to the different phases of the estrus cycle. Among the female-specific behaviors, restlessness, characteristic vocalization, standing heat, vaginal mucus discharge, reduced milk secretion, and reduced food intake can be more frequent or intense during estrus [6]. In some wild ungulates, females generate signs of sexual receptivity as visually salient sexual swellings, olfactory cues, or copulation calls [7]. In the captive goral (*Naemorhedus griseus*), the most prevalent behavior is tail-up, which generally persists for 2–3 days associated with 35% of estrogen surges, followed by ovulation (based on elevation of progestogens). Captive goral females also performed head butts and whistles [8].

A study linked the behavioral and physiological reproductive patterns during the periovulatory period and beginning of pregnancy in collared peccaries (*Pecari tajacu*). In that study, Silva et al. [9] referred that behavioral monitoring is a useful procedure for recognition of this period, as long as associated to the other morphophysiological parameters and it should be useful for good practices of collared peccaries handling in captivity and for the improvement of ARTs.

Nonetheless, females in other species may have a silent estrus, in which the ovarian activity is not identified by external signs. External estrus signs are quite inconspicuous in elephants (*Elephas maximus*), and it is difficult to assess their estrus cyclicity using physical cues [10]. Even though elephants have a long estrus cycle of 14–16 weeks, the receptive period is relatively short, lasting for 2–10 days. In general, females display their receptive period through discreet chemical, auditory, and behavioral expressions to attract males [11]. Moreover, in

elephants, estrus behavior includes getting away from the herd in an arc-shaped trail, presenting its head tilted to the side to attract males or inform its state ("estrus walk"). They vocalize deep roaring sounds, flick their tail against the vulva, lift, and hold it in the air. When chased, female may first run away but eventually will return toward the bull and accept his mounting [12].

In addition, in many species in captivity, the estrus signs are not frequent or easily observed, mainly due to changes of social and natural habits or small enclosures, in addition to the stress caused by visitors, handling, and management [13]. The estrus cycle length in white rhinoceros (*Ceratotherium simum*) lasts from 4 to 10 weeks, but the reason for this variation remains unknown. Under captivity, this species undergoes long anovulatory periods without luteal activity, which are considered a major reason for their low reproductive rate [14].

Regarding wild felids, major estrus behavioral activities described in the domestic cat, as vocalization, rolling, and urine spray or marking, are also observed in Asiatic lion (*Panthera leo persica*). According to Umapathy et al. [15], vocalization was generally followed by rolling. Females immediately after a bout of vocalization rolled 3–4 times on their dorsal side, and the duration ranged from 10 to 30 s. The frequency of behavioral display is increased on the third day and decreased on the 6th day of estrus. Rubbing of the body against objects and lordosis were also observed during estrus in this species, alike in other small felid species (ocelots, tigrinas, and margays). Moreover, females may show restlessness, an increased frequency of urination (in small quantities), vocalization, and sexual receptivity reactions in the presence of the male, as well as courting acceptance [15].

Scoring of genital appearance, particularly if using digital cameras, is a noninvasive method that provides valuable information and does not require additional training time, laboratory work, or extra expense. Studies were carried out in sun bears (*Helarctos malayanus*) using video-recorded females to evaluate estrus behavior related to other parameters. The vulvar swelling and color were correlated; nevertheless, vulvar swelling appeared to be a more discriminating indicator of estrus. During the 4 days of interval before the estrogen peak, female bears in this study had more agonistic behavior, displayed noticeable declines in appetite, showed more vulvar opening, and increased the number of superficial and keratinized cells in vaginal cytology. At the estrogen peak (day 0 of estrus), a high number of superficial cells were observed, coincident with open vulva, a decrease in agonistic behavior, an increase in affiliate behaviors, and low appetite. In addition, sexual behavior occurred until 4 days after the estrogen peak, along with vaginal keratinized cells and presumably overlapped with ovulation [16]. The study not only confirmed the utility of behavioral measures but also showed that a simple keeper check sheet can be a valuable auxiliary tool for reproductive assessment, offering an alternative to data laboriously derived from video-scored recordings.

Matschie's tree kangaroo (*Dendrolagus matschiei*) is the predominant species of tree kangaroo held in North American zoos [17]. Importation of individuals from the wild is restricted, and, therefore, the captive population must be sustainable through oriented reproduction. Males and females are generally held separately in captivity and paired for mating during estrus, which is identified through observation of proceptive behaviors, for example, licking of the

forearms and affiliation with males. Additional information on tree kangaroo's reproductive biology is needed to advance captive propagation of this endangered species. In this sense, noninvasive techniques that eliminate blood collection associated stress are very welcome to study its reproduction [17].

Taking into account the importance of the knowledge of the reproductive behavior of wild animals as a method of estrus cycle monitoring, the main difficulties are especially the lack of knowledge on the physiology and behavior of various wild species in captivity. The perspectives of using this method associated with other noninvasive techniques are good, since it is increasingly necessary to minimize the stress associated with the management of captive animals and to affect as little as possible its reproductive function.

3. External features and vaginal cytology

The focus of an effective estrus detection is to determine the optimal time for mating and the ideal time for artificial insemination. Among the many methods available to identify the estrus cycle, the observation of external estrus signs and vaginal cytology is highlighted. In vaginal cytology (**Figure 1**), the epithelial cell morphology reflects the effect of the interaction of various hormones, particularly estrogen and progesterone, on the reproductive tract. Since the vaginal epithelium reflects the changes in hormone milieu, it follows that any abnormality in the sexual cycle due to either a direct hormonal involvement or disease condition would be reflected in changes in the cell types of vaginal epithelium. Additionally, this technique is simple, practical, economically viable, and in some wild mammal species can be used for characterizing the estrus cycle [18].

In elephants, the use of vaginal cytology has been described since the 1970s by Jainudeen et al. [19] and Watson and D'Souza [20], who described the smear from the vaginal vestibule or vagina in this species. In fact, gathering a vaginal vestibule smear from an elephant is relatively easy if the zoo conducts "free contact" animal training on a regular basis, which facilitates the monitoring of the estrus cycle [21]. A subsequent study conducted in elephants used a spectrum analysis, the Yule-Walker method, to verify the frequency of exfoliative cells. It was found that the markedly appearance of nucleated and enucleated superficial cells characterized the periods from proestrus to estrus, while an increase of intermediate and parabasal cells characterized the period from metestrus to diestrus [21]. In addition, other estrus signs include mucus droppings and the reddening and exposition of the clitoris and the emission of infrasonic sounds and olfactory chemicals, which can be transmitted over greater distances as verified both for Asian [22] and African individuals [23].

In wild carnivores, as the maned wolf (*Chrysocyon brachyurus*), the vaginal cytology is an effective procedure to determine the estrus cycle phases, but, unlike the domestic dogs, blood cells were scarce in all phases of the estrus cycle, including proestrus [24]. Furthermore, these findings may be associated with visible signs of estrus, which are characterized as swelling of the vulva and rosy or bloody vaginal secretions at the beginning of estrus. Already at the end of estrus, the vaginal secretion changes to a thick and yellowish appearance [25].

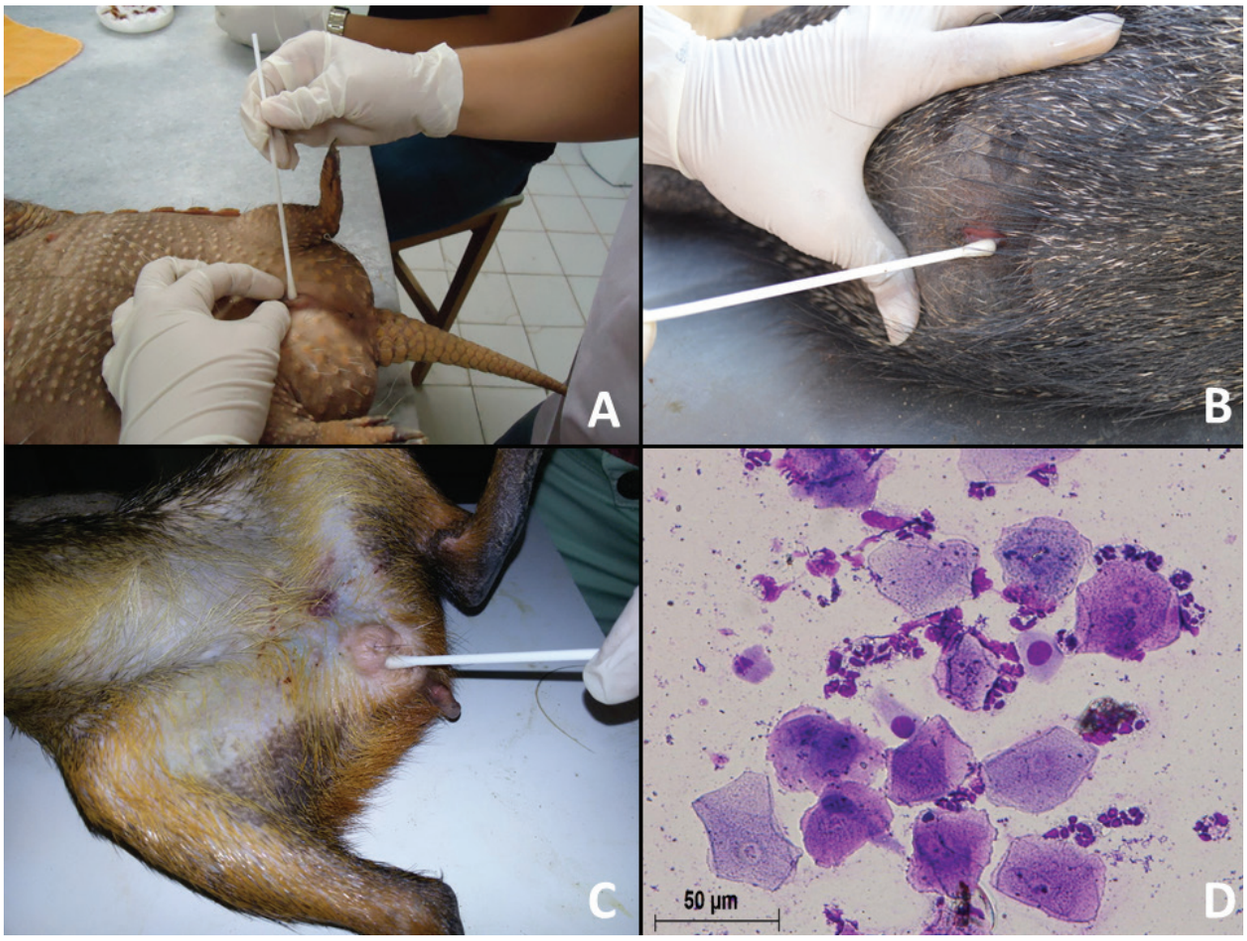

Figure 1. Collection of vaginal smears using swabs from female armadillo, *Euphractus sexcinctus* (A); collared peccary, *Pecari tajacu* (B); and agouti, *Dasyprocta leporina* (C). Cytological specimen presenting predominance of cornified cells indicating estrus in *E. sexcinctus* (D).

The reproduction in captive wild felids, even in relatively naturalistic enclosures, remains poor, especially in small species, which seem to be more susceptible to stress. Puma (*Puma concolor*) females vocalize characteristically during estrus, while ocelots show more estrus signs than other small felid species. In general, females rarely exhibit regular overt signs of sexual receptivity as a higher frequency of rubbing, vocalizing, rolling, urine spraying, and sniffing. These characteristics have been described in Siberian tigers (*Panthera tigris altaica*) [26], clouded leopard (*Neofelis nebulosa*) [27], and *Leopardus* genus [28]. For this reason, the detection of estrus by vaginal cytology is a resource in their reproductive evaluation but requires physical and/or chemical contention. In addition, this method has been described for lions (*Panthera leo*) [29], cheetahs (*Acinonyx jubatus*) [30], pumas [31], and ocelots (*Leopardus pardalis*) [32] in which the estrus was characterized by the presence of a high percentage of keratinized superficial cells.

In sun bears (*H. malayanus*), the vaginal cytology, vulvar changes, and behavior were essential for the characterization of the estrus cycle. Sexual behavior characteristics of estrus include self-masturbation; the interaction among partners, including mutual genital grooming, genital inspect, mount and copulate, affiliative (social play, solicit, follow, groom, and muzzle-muzzle

contact), and stereotyped (pacing and other repetitive movements) behaviors, which are displayed along with changes in genital appearance (as vulva color and swell); and the presence of superficial and keratinized cells in vaginal cytology. These characteristics are effective and inexpensive supplements or alternatives to fecal hormone assays and are highly recommended for the continued reproductive management of this and other captive bear populations [33].

Observations of changes in the external genitalia, as the presence of vaginal mucus, hyperemic vaginal mucosa, and separation of the vulvar lips, are also important for estrus identification in collared peccaries [34]. Regarding the use of vaginal cytology for estrus monitoring in this species, Guimarães et al. [35] suggested that it is possible to differentiate estrus cycle stages using this technique. Even though superficial and intermediate cells are present in higher numbers throughout the estrus cycle, the superficial ones significantly increase during the estrus. Nevertheless, authors highlighted that for the correct identification of estrus phases, it is necessary to consider other aspects, as the presence or absence of leukocytes and the relation between the number of intermediate and superficial cells, besides the signs of external genitalia. Conversely, Maia et al. [34] suggested that no significant differences between proportions of vaginal epithelial cells were identified when comparing follicular and luteal phases in collared peccaries. Therefore, an association is suggested among vaginal cytology, behavior and external genitalia observation, and ultrasound and hormonal analysis for correct estrus detection in this species.

Despite the relative success of vaginal cytology described above, it is not always possible to distinguish among the phases of estrus cycle. In Xenarthras, as the maned sloths (*Bradypus torquatus*), this technique was used only to identify estrus, being characterized by the predominance of nucleated and enucleated superficial cells [36]. Moreover, in six-banded armadillos (*Euphractus sexcinctus*), the use of vaginal cytology is difficult because it requires the use of an anesthetic protocol due to their small vulvar commissure that hinders the swab introduction. Nevertheless, this technique does not allow a detailed identification of all phases of estrus cycle, being only possible to distinguish between the follicular and the luteal phase [37]. In fact, alterations in external genitalia seem to be very effective for estrus monitoring in Xenarthras. Both in *Tamandua* (*Tamandua tetradactyla*) [38] and in six-banded armadillos [37, 39], the presence of a vulvar bleeding was used as the main parameter to identify the beginning of the estrus cycle. Moreover, in armadillos, the presence of vulvar bleeding occurred approximately 3–7 days after estrogen rise, concomitant to the presence of vulvar edema and mucus [37]. In this species, the occurrence of clitoral hyperemia, varying between red and purple, and a pronounced clitoral erection was also described [39].

Some difficulties in the use of vaginal cytology for a detailed identification of the stages of estrus cycle have also been described for various wild rodents, as coypus (*Myocastor coypus*) [40], chinchillas (*Chinchilla lanigera*) [41], pacas (*Agouti paca*) [42], and agoutis (*Dasyprocta agouti*) [43]. The main reason for such difficulty is the existence of a vaginal occlusion membrane that tends to obstruct the external vaginal ostium, which remains until the estrus or parturition. The observation of vaginal opening, in parallel with the exfoliative cytology [44], allows the correct identification of estrus in *D. agouti* [43], *Dasyprocta prymnolopha* [45], *Cavia porcellus* [46], *Myoprocta pratti* [47], and chinchillas [48]. As an exception, the use of vaginal

cytology in the Spix's yellow-toothed cavy (*Galea spixii*) is reported to be very effective to distinguish the phases of the estrus cycle. In these rodents, a predominance of large intermediate cells is observed in proestrus, while superficial cells predominate in estrus, and the intermediate and parabasal cells prevail in diestrus [49].

The use of vaginal cytology has also been reported for common wombat (*Vombatus ursinus*), but the cycle stages are not accurately identified due to the high variability in the proportion of epithelial cells obtained in the smear analysis [50, 51]. In addition, the anatomy of the urogenital sinus, whose length varies between individuals and within an individual at different cycle stages [50], hinders the collection of an adequate cytological specimen [52]. As the vaginal swab collection procedure requires anesthesia in this species, repeated capture of the female wombat for sequential analysis is likely to be highly stressful, leading to potential reproductive failure [51]. As a marsupial, the condition of the pouch, namely, its depth, opening size, wall thickness, degree of cleanliness, and teat length, could also be indicatives for the reproductive status of wombats (i.e., whether cycling or not) [52, 53]. Alternatively, the observation of the external genitalia changes (clitoris and pericloacal region) that can become swollen and tumescent in different stages of the cycle was proposed for assessing the wombats' reproductive status [53]. However, this technique is not reliable due to the difficulty in detecting any noticeable genitalia changes [52]. An interesting study, conducted by Hogan et al. [54], showed that estrus was not detectable in female southern hairy-nosed wombat (*Lasiorhinus latifrons*) even when the continuous observations of physical activity via movement-sensitive transmitters were used. No difference in physical activity was recorded during estrus and anestrus, or there was any correlation between physical activity and the occurrence of reproductive behavior. In fact, even though numerous studies have examined Vombatidae reproductive behavior, estrus has rarely been observed and appears to be exceptionally short, as 15 h in the common wombat [55] or 13 h in the southern hairy-nosed wombat [56]. The reason why estrus is so short in wombats has yet to be determined. Further studies into reliable methods of estrus detection are urgently required, as the lack of specific information might be the most significant impediment to successfully breeding this species in captivity [57].

In general, the association between the vaginal cytology techniques and the observation of external estrus signs are useful for estrus cycle monitoring in various wild females. Thus, the ability to assess in an easy and safe way the reproductive status through noninvasive means is vital to understand the reproductive physiology of animals. Therefore, such methods ought to contribute to assist captive breeding of threatened species, additionally, in order to ensure better reproductive performance in animal production and the development of techniques and tools for assisted reproduction.

4. Endocrine monitoring and its metabolites

Endocrine monitoring enables the knowledge of endocrine activity as a tool to evaluate the ovarian cycle and to be used in a captive management, especially for endangered species, aiming to increase the number of individuals [58]. In wild mammals, the endocrine monitoring of

the estrus cycle can either be performed by invasive methods, as using blood samples [59], or noninvasive methods, by sampling from feces [8], urine [60], saliva [61], and hair [62].

The choice of the hormonal monitoring method depends on the type of assessment method selected (invasive and noninvasive) and on the requested information, as well as on the differences among species, hormone metabolism, excretion pathway, and viability during collection and processing [63]. In either method, the main analysis procedures available include the immunoassay, as enzyme immunoassay (EIA) [8], radioimmunoassay (RIA), and chemiluminescence [64], with antibodies directed to the hormone of interest [11] and also high-performance liquid chromatography (HPLC) [65].

In general, endocrine monitoring in wild mammals has been carried out in blood samples for species that do not suffer so much stress during collection, as Elephantidae [58]. Already feces, urine, saliva, and hair were used in Cervidae [66], Rhinocerotidae [67], Felidae [28], and Ursidae [68].

4.1. Blood samples

Among the type of samples, the blood is the one that promotes a faster response to the endocrine cycle, also making possible to extrapolate the evaluation of steroids, for proteins, luteinizing hormone (LH), follicle-stimulating hormone (FSH), inhibin, prolactin, and relaxin [69]. The invasive method by blood sampling has the advantage of providing more immediate and accurate information regarding the peripheral hormone levels [58]. After collection, the blood is centrifuged to obtain serum or plasma that can be stored at −20°C until analysis [11].

This method has been used in armadillos, collared peccaries, elephants, and agoutis. In armadillos, a clear identification of a 23.5 days of estrus cycle was made, consisting of 8.8 days for follicular and 15.6 days for luteal phase [37]. In collared peccaries, the estrus cycle lasts 21 days, with a follicular phase of 6 days and 15 days for the luteal phase [34]. In Asian elephants, the estrus cycle has an overall duration of 12–19 weeks, the luteal phase extending between 4 and 15 weeks, and the follicular phase lengthening for 2–12 weeks [11]. In red-rumped agoutis, the estrus cycle lasts for 31 days, the follicular phase ranging from 6 to 9 days, and the luteal phase from 19 to 23 days [59].

Nevertheless, this method has the inconvenience of causing a high level of stress in several wild mammals, associated with blood sampling, whose collection needs a more laborious procedure, as physical and chemical contention of the animal [37]. Moreover, the generated stress can result in a change in hormonal levels [64]. Additionally, the blood collection requires the training of the operator that will collect the sample, besides the adaptation of the animal to this type of management [58]. Thus, although the blood samples are quite sensitive to hormonal changes and allow the evaluation of a greater number of hormones, in wild mammals it is preferable to use noninvasive methods, so to avoid contact with the animal and reduce stress into a minimum.

In addition to animal stress, difficulties and risks associated with blood collection and sometimes training requirements supported the development of alternative methods for hormonal assessment. In this sense, noninvasive methods have the advantage of an easy collection of

the sample, without causing stress to the animal. These methods assume that hormones that circulate in the bloodstream are secreted into the saliva, deposited in the hair, and excreted via feces or urine [63]. Nevertheless, it has the disadvantages that the immunogenic form of hormones in urine and feces is different in some species, because they are metabolized in the liver and kidney, mainly in a biologically inactive form [58].

4.2. Fecal samples

In general, fecal steroid metabolites are the most common noninvasive method to screen the endocrine function in wild mammals, allowing the knowledge of the reproductive biology of several species. The metabolic pathway involves the inactivation or excretion of hormones and metabolites of steroids that have different routes according to species and the type of steroid in the same species [63]. The main estrogen metabolites present in fecal samples are estrone, estradiol-17α, and estradiol-17β [70]. Already progesterone metabolites present in fecal samples are allopregnanolone (5α-P-3OH) [10], 17a-hydroxyprogesterone [15], and pregnanediol-3-glucuronide (PdG) [71]. The fecal samples have a pattern of steroid concentration similar to the one found in plasma, with a delay in relation to blood due to metabolism and excretion (lag time), which can vary from hours to days depending on the species [63].

The collection of fecal samples is simple; nevertheless, the preparation of this sample requires a longer time. This type of sample should be stored at −20°C because of the presence of gastrointestinal bacteria that can degrade the hormones and cause changes in concentrations [27]. Subsequently, fecal samples need to be homogenized prior to the steroid uniformity, the extraction in the presence of methanol or ethanol, and the evaluation of hormones in the supernatant after centrifugation [72]. Steroid and prostaglandin metabolites are lipophilic and are usually conjugated in the liver to soluble portions for excretion into feces [73].

The enzyme immunoassay for monitoring fecal metabolites has been successfully used in wild felid species, as ocelots, tigrinas (*Leopardus tigrinus*), and margays (*Leopardus wiedii*), allowing to determine the mean length of the estrus cycle as 18.4, 16.7, and 17.6 days, respectively [28]. Results derived from hormonal assessment in feces from several other wild mammals are reported in **Table 1**.

4.3. Urine samples

In most cases, fecal analysis can measure estradiol-17β, estrone conjugates (E1C), progesterone, and PdG, whereas urine analysis (**Table 1**) is generally used to measure E1C and PdG [71]. Moreover, peptide hormones can be filtered through the renal glomerulus and excreted in urine [64]. Analysis of urinary hormones or their metabolites in many cetacean species has been successful in detecting estrus, developing the ability to define patterns of endocrine excretion [60].

In general, urine collection requires proper training of the animal, to avoid contamination of the samples [61]. In case of untrained animals, this material is collected on the ground, and it is necessary to isolate the animal, which causes stress besides requiring a time for the isolation and urine recovery [58]. These uses of urine samples also require a previous step, that is, creatinine

Samples	Species	Metabolites	Hormonal assay	Follicular phase	Luteal phase	Ovarian cycle	References
Fecal	*Myrmecophaga tridactyla*	E2/P4	EIA/HPCL	34.5 d	16.9 d	51.4 d	Patzl et al. [77]
Fecal	*Naemorhedus griseus*	E2/P4	EIA	3 d	18 d	21 d	Khonmee et al. [8]
Fecal	*Gorilla beringei beringei*	E2/P4	EIA/LC-MS/ HPLC	21 d	8 d	29 d	Habumuremyi et al. [65]
Fecal	*Rhinoceros unicornis*	E2/P4	EIA/HPCL	15.9 d	19.1 d	43.4 d	Schwarzenberger et al. [67]
Fecal	*Ceratotherium simum*	20-oxo-P	EIA/HPCL	12.4 d	55.9 d	68.3 d	Schwarzenberger et al. [78]
Fecal	*Tragulus javanicus*	E2/P4	EIA/HPCL	11.5 d	3 d	14.5 d	Kusuda et al. [72]
Fecal	*Perodicticus potto*	E2/P4	EIA	9.1 d	19.89 d	36.06 d	MacKinnon et al. [79]
Urine/fecal	*Rhinopithecus roxellana*	E2G/PdG/E2/PdG	EIA	14.7 d	10.4 d	25.1 d	Muren et al. [71]
Urine	*Delphinapterus leucas*	E2/P4	EIA/HPCL	25 d	25 d	50 d	Steinman et al. [60]
Urine	*Tursiops truncatus*	EC/LH/UP	EIA/UP/HPLC	8 d	19 d	27 d	Robeck et al. [80]
Urine	*Lagenorhynchus obliquidens*	EC/LH/UP	EIA/UP/HPLC	10 d	21 d	31 d	Robeck et al. [81]
Saliva	*Loxodonta africana*	P4	EIA	4.6 wk	18.6 wk	23.2 wk	Illera et al. [61]
Saliva	*Trichechus inunguis*	E2/P4	EIA	19 d	27.33 d	47.67 d	Amaral et al. [75]

Saliva and fecal samples were stored at −20°C and urine samples at −70°C.

Abbreviations: enzyme immunoassay (EIA), liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC), estradiol-17β (E2), progesterone (P4), total immunoreactive 20-oxo-pregnanes (20-oxo-P), estradiol-3-glucuronide (E2G), pregnanediol-3-glucuronide (PdG), estrogen conjugates (EC), luteinizing hormone (LH), urinary progesterone (UP) metabolites, day (d), week (wk).

Table 1. Monitoring the ovarian cycle of some wild mammals using noninvasive methods.

analysis to evaluate if the sample is much diluted for subsequent hormonal evaluation [74]. It also includes centrifugation for separation of particles that can cause contamination.

Urine samples can be stored for 24 h at room temperature; if there is an interest in measuring proteo- or peptide hormones, it is advisable to freeze the sample since these particles are easily degraded. For the gonadotrophins analysis, it is usual to add glycerol in the sample, to avoid dissociation in subunits. On the other hand, sex steroid hormones are secreted as conjugates soluble in water [63]; estrone (E1) and PdG represent the urinary metabolites of estradiol and progesterone, respectively, in most primate species [71].

4.4. Saliva samples

The sex steroid hormones found in saliva retain the same form as in blood because circulating steroid hormones pass through the epithelium of exocrine glands by passive diffusion [75]. Thus, the saliva becomes the suitable sample for endocrine monitoring, since it has unaltered steroid and whole peptide hormones [64]. In relation to the hormonal proportions of the blood in the saliva, it is possible to detect a smaller amount of steroid and peptide/proteo-hormones [63]. The saliva reflects the hormonal changes in the blood, allowing for its immediate analysis [61]. The hormonal levels in saliva have a difficult interpretation since this is easily changed in a short period [75]. Moreover, as the hormones detected in saliva are quite similar to the blood, these suffer less the specific species effect, allowing the use of commercial kits [63].

Salivary samples are obtained with the aid of swab and stored at −20°C. In addition, the samples can be previously lyophilized or simply centrifuged and suspended in buffer for subsequent EIA [61]. Nevertheless, the method is still seldom used because of the difficulty in collection that requires a closer contact to the animal to obtain the sample [66], being performed in few species (**Table 1**).

4.5. Hair samples

The hair can also be used as a source for measuring hormone levels, since through the bloodstream, hormones are deposited in the hair follicle [62]. The hair is considered as a form of long-term monitoring because it will detect endocrine activity for months or weeks and will not represent hormone levels for hours or days; nevertheless, the hormones are structurally similar to the forms found in blood [76].

In Canada lynx (*Lynx canadensis*), the hormone measurements from hair samples are foreseen as a promising method for reproductive surveillance; nevertheless, it still requires more studies and validation to be reliable and widely applied [62]. In general, the hair is pre-washed with methanol, collected with commercial clippers, and stored at room temperature in aluminum foil until analysis. In primates, the use of hair to measure the hormonal exposure of fetuses was possible through mass spectrometry (MS) and high-performance liquid chromatography (HPLC), demonstrating that this method has the ability to predict hormone levels [76]. Although the method of endocrine monitoring via hair is very interesting for the knowledge of the estrus cycle, further studies are necessary, because this method is more directed to the measurement of cortisol levels [64].

In summary, the availability of different methods of endocrine monitoring in wild animals makes it possible to choose the most appropriate method for the species of interest, considering the hormonal metabolism and the metabolite evaluated. Although some species allow blood collection, for most wild mammals, noninvasive methods are preferable to minimize stress during collection. This knowledge of the endocrine mechanism concurs to the conservation of wild mammals, fostering the study of species of unknown physiology and the assessment of endocrine profiles in reproductive biotechnology. Therefore, the endocrine monitoring is an important tool to study hormonal ovarian activity of wild mammals.

5. Ultrasonography

Ultrasonography is a classical and reliable method for monitoring ovarian dynamic in mammals (**Figure 2**). In wild females, ultrasound is an integral part of ART procedures allowing the monitoring of sexual cycles. Moreover, ultrasound aids to confirm the efficiency of estrus synchronization and superovulation protocols and to identify the presence of follicles and corpora lutea and the follow up of follicular dynamics [82]. In addition, ultrasound can assist in the study of corpus luteum regression mechanisms, thus allowing to confirm the response to hormonal treatments for estrus control. Nevertheless, the effective application of ultrasound varies among different species, being dependent of several characteristics, as ovary size [83].

Follicles within the ovaries appear as anechoic spherical structures, while the corpus luteum appears with distinctive margins and non-smooth surfaces that are hypoechoic or anechoic in the center, presenting homogeneous fluid dark spaces. This description, observed in the majority of mammalian species, can be extrapolated for wild animals [84].

Among nonhuman primates, the initial studies in the common marmoset (*Callithrix jacchus*) showed that ultrasound provides a reliable and noninvasive method for ovarian cycle evaluation. The cycles were monitored by plasma progesterone, and ultrasound reliability was validated by comparing the findings with direct observation of the ovaries (number and position of structures) through laparotomy. In those animals, 92% of the follicles and 78% of corpus luteum were correctly determined by ultrasound [85]. In capuchin monkeys (*Sapajus paella*), the dominant follicle was recognized at 6 days prior to ovulation with the use of 2D ultrasound, the diameter and mean volume of preovulatory follicle being estimated as 9.6 mm and 0.54 mL, respectively [86]. By ultrasound, the occurrence of ovulation was observed when the mean diameter of the ovulatory follicle was 9 mm, the follicle size being an important parameter to estimate the ovulation day in this species [87].

For ungulates as cervids, the transrectal ultrasonography has been described for evaluating the ovarian response in wapitis (*Cervus elaphus*) subjected to estrus synchronization protocol (CIDR-B, 1.9 g of progesterone and 200 IU of eCG) used for fixed-time artificial insemination (FTAI). In this occasion, corpus luteum and ovulatory follicles (≥8 mm) were easily detected [88]. In Jilin sika deer (*Cervus nippon hortulorum*), the transrectal ultrasonography enabled the consistent visualization of both ovaries and allowed the detailed characterization of follicular dynamics during the estrus cycle. In this species, it has been shown that the follicular wave started with a follicle with ≥4 mm diameter, and it ended in the day when the number of

Figure 2. Placement of the ultrasound transducer for ovary monitoring in armadillo (A), agouti (B), and collared peccary (C).

follicles <4 mm increased and the number follicles ≥4 mm decreased in the same proportion. Additionally, the dominant follicle was defined as a follicle that attained a diameter ≥8 mm, and these findings provide rationale for the hypothesis that the increase in follicular size was associated with an increase in estradiol concentration. After ovulation, the corpus luteum was observed at the same location within the next 3 days [84].

For wood bison (*Bison bison athabascae*), the ultrasound was used for transvaginal ultrasound-guided follicular aspiration after an effective superovulatory protocol (association of PGF, eCG, and FSH). Numerous follicles ≥5 mm were easily detected on day 14 after treatment, featuring the technique as effective [89].

Transrectal ultrasound (4–7 MHz) exams were performed to follow the appearance of ovarian follicles after different synchronization protocols in the Przewalski's horse (*Equus ferus przewalskii*). The characterization of ovarian structures, that is, numbers of follicle, follicle size, and the presence of a corpus luteum, was easily performed [90].

The use of ultrasound in African elephants has been well characterized. It has proven to be a valuable tool for use with ARTs and has enormous potential for evaluating the efficiency of hormonal therapies used to treat reproductive dysfunction. Transrectal ultrasound showed

that this species presents a peculiar pattern of follicular development in the ovary, associated with two LH surges: the first with formation of multiple small follicles and the second with a single large ovulatory follicle [91].

In order to determine the ideal day for artificial insemination in white rhinoceros, the ovarian follicle sizes were visualized by ultrasound. After measurement of preovulatory follicle (mean 2.7 cm), ovulation was induced with GnRH analog administration. The artificial insemination procedure resulted in two pregnancies. In addition, ultrasound documented the postpartum involution of the uterus, complete reabsorption of accumulated intrauterine fluid, and the development of a preovulatory follicle 30 days postpartum [14].

Many studies using ultrasonography have been described for estrus monitoring [34] and synchronization [92] in collared peccaries. Ovarian follicles measuring 0.2 ± 0.1 cm were visualized during the estrogen peak; corpora lutea, presented as hyperechoic regions measuring 0.4 ± 0.2 cm, were identified during luteal phase [34].

Regarding carnivores, the ultrasound was useful to characterize the ovaries of maned wolf (*C. brachyurus*) in captivity. In this species, the description of the ovaries (mean 1.02 cm length and 0.67 cm width) and follicles (mean 1.12 cm length and 0.32 cm width) is similar to that reported for domestic bitches [93].

The lynx (*Lynx* sp.), a most critically endangered felid, presents unique reproductive strategy with a monoestrus cycle persisting corpora lutea over the years. Painer et al. [94] evaluated whether artificial luteolysis could be achieved with common luteolytic drugs and if luteolysis would induce a subsequent natural estrus. In this case, the ultrasound was used as a primordial method for the identification of nonstructural regression of corpora lutea and subsequent spontaneous estrus induction after treatment with PGF2α analog (cloprostenol, 2.5 mg/kg).

However, in the marsupial wombat (*L. latifrons*), because of the opacity of the ovarian bursa, the transabdominal ultrasonography was unsuccessful for confirming ovulation, detecting the number of follicles in stimulated ovaries or the presence of the preovulatory follicle [95].

Recently, the monitoring of reproductive physiology in a Xenarthra, the six-banded armadillo, was made possible by ultrasound screening of the ovary. Using a microconvex transducer (8.0 MHz), it was possible to detect the ovary in 88.3% of the attempts, with defined structures, rounded and slightly hypoechoic compared to adjacent tissue [37]. The same study showed that, in 52% of the monitored ovaries in the follicular phase, it was possible to identify the presence of growing ovarian follicles, measuring on average 0.2 ± 0.1 × 0.2 ± 0.2 cm. In addition, during the luteal phase, the corpus luteum was observed in 60% of the ovaries, ranging from 0.1 to 0.2 cm [37].

Regarding rodents, a study carried out in red-rumped agoutis used different techniques to monitor the estrus cycle, including the ultrasound. Although it failed to differentiate the ovarian morphology during the different phases of the estrus cycle, the ultrasound was efficient to identify and measure follicles during the follicular phase, with an average diameter of 1 ± 0.5 mm; conversely, only in 12.5% of luteal phase, corpora lutea measuring 1.4 ± 0.9 mm were identified. Authors related the difficulty in identifying the ovary to its reduced size, as well as to the presence of adjacent fat [43].

http://dx.doi.org/10.5772/intechopen.69444

6. Other possibilities

Thermography is a modern, noninvasive, and safe technique that measures the temperature in a surface based on its infrared radiation emission, given that the superficial heating of an animal is influenced by local circulation and tissue metabolism, which are generally constant [96]. Areas with higher metabolic rates show a higher temperature than areas with less tissue activity; therefore, surface temperature changes are caused by changes in local perfusion [97]. The increased local blood flow is linked to the rising of plasma estrogens, reflected by vulvar reddening and swelling that have been widely reported as typical estrus signs [98]. Altogether, infrared thermography has the potential to evaluate these physiological changes by monitoring the increase of temperature on the vulvar skin, with the objective of establishing a relationship between vulvar temperature fluctuation and ovulation [99].

Unfortunately, thermography has some limitations: good quality thermo-cameras can be very expensive, and also the maintenance of the camera can be expensive [97]; care must be taken when getting images in sunlight or in high humidity conditions, also with convective heat loss due to wind or when surfaces are dirty. Radiation measured by the camera does not only depend upon the temperature of the object but is also a function of its emissivity and conductivity [100]. Infrared thermography has proved to be highly sensitive to changes in the environmental conditions. Factors such as air flow, moisture, fluctuations in the environmental temperature, level of physical activity, and animal's stance before the measurement can induce a considerable variation in these readings, which may limit the applicability of this technology under field conditions, where these factors are difficult to control [99].

Currently, thermography is being used in some domestic species for estrus cycle monitoring as bovine [96, 99], swine [101], and equine [102]. In wild animals, this technique is still underused; however, it is noteworthy that, in addition to its other advantages, this is a noninvasive technique, which in certain conditions may be very useful, to avoid the immobilization of the animal [103]. Sykes et al. [104] defend that infrared thermography could be valuable for estrus detection in zoological species due to the possibility of observing and monitoring the animals in a natural environment with little human interference. However, variation among species could hinder the accurate estrus detection in all species. Difference in the length of estrus cycle and in the temperature gradients of the vulva also needs to be mapped out over continuous cycles to assess uniformity. In this context, continuous research is needed for both domestic and zoological species to validate thermography as a reliable tool for estrus detection.

In dealing with wildlife management, the preferential use of less or noninvasive techniques is required since this is necessary for maintaining the physiological behavior of the animals and reducing stressful situations. Therefore, several modern and practical methods having the potential to be adapted from domestic to wild animals have been developed, such as the use of pedometers, video cameras, and electronic odor detector, among others.

Pedometer is a real-time watch used for time interval measuring of the animal activity [100]. This activity measurer is commonly used at the neck or legs in cows, being connected to a computerized receiver for movement analysis. Some pedometer emits signals in a form of light when cows show increased activity. It is observed that cows in heat are more mobile

and walk two to four times more when compared to non-estrus animals. Data of cow activity recoded with the help of pedometer has good correlation with estrus, thus resulting in a heat detection efficiency from 90 to 96%. Its main limitations are the high cost for acquisition and replacement of lost equipment [105].

The principle of the electronic odor detector is based on the detection of sex pheromones related to heat. The pheromones are the natural olfactory signal for male that cow emits during estrus. It is up to 90% efficient. Even if the project is running for a successful future, further development steps are anticipated [106].

6.1. General considerations

The development of reliable and less-invasive techniques for monitoring the reproductive cycle of wild mammals is required to optimize the captive breeding management. These techniques are needed for the use of reproductive biotechnologies applied for either preservation or production. Understanding the changes in reproductive behavior of wild animals is therefore critical to better estrus monitoring—which allows the application of reproductive biotechnologies—as well as improving the management of these animals [11, 107]. Therefore, the use of noninvasive techniques to monitor the reproductive status is of paramount importance to avoid stress and its induced changes in physiology.

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