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REVIEW ARTICLE

MONITORING OVARIAN FUNCTION IN CAPTIVE AND FREE-RANGING WILDLIFE BY MEANS OF URINARY AND FECAL STEROIDS

Bill L. Lasley, Ph.D., and Jay F. Kirkpatrick, Ph.D.

Abstract: Methods for monitoring and assessing reproductive status have been developed to allow both captive and free-ranging wildlife to be evaluated while avoiding chemical immobilization or physical restraint. The broad application of these methods to a wide range of species is related to the conservation of the steroid hormone molecule across taxa and the stability of steroid hormone metabolites in the excreta. In general, conjugated steroid hormone metabolites of the sex steroids are measured in the urine, whereas the unconjugated metabolites are measured in feces; both methods are used to monitor gonadal function or pregnancy. Most methods are appropriate for monitoring females and measure metabolites of estradiol or progesterone. The measurement of metabolites of testosterone to monitor testicular activity in males has been used, but to a lesser degree. When correctly applied, these approaches allow the assessment of reproductive function for prolonged time periods, which permits the objective description of cyclic physiologic processes or the detection of infrequent events. A relatively large number of zoo species have been studied, and valid methods and baseline data exist for many nondomestic primates and ungulates. Although a smaller number of free-ranging wildlife species have been characterized at this time, the current trend indicates increased application of these methods in this research area.

Key words: Sex steroids, urine, feces, reproductive status.

INTRODUCTION AND OVERVIEW

Assessing reproductive status is important to the effective management of captive and free-ranging wildlife species. Immediate and long-range plans for a group are largely dictated by the current reproductive potential of available individuals. Evaluations of sexual maturity, fertility, and reproductive status are needed to provide specific guidelines for culling, mating, or separation and are also useful for forecasting the success or failure of a breeding group. Beyond reproductive potential, the general health and stability of a population is a reflection of its capacity to reproduce because this non-life-threatening aspect of adaptive physiology is usually the first and only physiologic loss resulting from severe stress.

Fecundability and population dynamics are not useful indices for measuring reproductive capacity when an immediate analysis is required. In such cases, reproductive status must be assessed in individuals. This assessment can be accomplished by measuring endocrine characteristics because all aspects of reproduction are mediated through hormonal signals produced only in response to adequate environmental and/or maturational cues. Neural peptide, pituitary glycoprotein, and steroid hormone interactions occur in a species-specific format to support gonadal function, germ cell maturation, behavioral patterns of courtship and mating, the growth and development of the conceptus, and the nurturing of the young. Patterns of hormone secretion in female mammals can be used to detect or characterize physiologic events and to diagnose the specific reproductive dysfunctions. This approach is less well suited for monitoring males because of the less tightly coupled relatibnship among male germ cell production, behavior, and hormonal secretion.

From the Institute for Toxicology and Health Research, University of California, Davis, California 95616, USA (Lasley); and the Department of Biological Sciences, Eastern Montana College, Billings, Montana 59101, USA (Kirkpatrick).

Practical assays to monitor and assess reproductive function have been developed for humans and domestic and laboratory animals. The species-specific nature of both the structure of the reproductive protein and peptide hormones and the patterns in which they are secreted are limiting factors for applying existing assays to all mammalian species. More importantly, the requirement for blood collection, which is not always possible with nondomestic and nonlaboratory species, has prevented the application of such techniques to most species. Of the more than 4,000 mammalian species, complete reproductive hormone profiles have been characterized for <50 species.

Two characteristics of comparative reproductive physiology are currently being exploited to develop general evaluative strategies applicable to virtually all mammalian species. The first universal trait is that gonadal steroid hormones have the same molecular structure throughout the animal kingdom. For example, estradiol and its major metabolite estrone, along with progesterone, are the key hormones secreted by the healthy mammalian ovary. Similarly, testosterone is the primary androgen secreted by the mammalian testis. The second universal trait is that all steroid hormones are secreted into the vascular space and then cleared within minutes after undergoing only modest physical change. The resulting metabolites are concentrated in either the urine and/or feces and are relatively stable for a prolonged time. The excreta containing the metabolites can be collected and preserved indefinitely by simple freezing. Thus, steroid hormone metabolites can be measured in the excreta of virtually any mammalian species by utilizing a relatively small number of similar hormone assays. Sufficient data are now available to suggest that, in general, the excretion profiles of gonadal steroid metabolites reflect gonadal activity and provide an accurate assessment of overall endocrine status.

This strategy was first applied to humans and domestic species decades ago when most analytical methods still were insufficiently sensitive to detect steroid hormones circulating in the vascular bed. As assay methods became more sensitive (primarily through the advent of the immunoassay), measurement of the active substance in circulation became more appropriate, and excretion profile monitoring generally was abandoned. Recently, the recognition of the decided advantages to collecting and analyzing excreted steroid metabolites has focused new attention on this approach.

The primary benefits of urine or fecal monitoring are that a collection protocol can be imposed for prolonged time periods without manipulating or stressing the animal, which is essential when events to be studied are infrequent, take place over long periods, or the amount of blood available for collection is limited. The most obvious candidates for this type of monitoring are studies of cyclic events in small fragile species. A secondary advantage is that metabolite concentrations frequently are two to four orders of magnitude higher than that of the parent steroid in blood. These higher concentrations allow a wider range of assays to be employed and even permit automation and the development of noninstrumented kits. The tertiary (and perhaps the most practical) advantage of monitoring urine or feces is that the samples can be collected even under the most adverse conditions and from the most pugnacious of subjects without great risk to either the subject or the investigator.

Epidemiologists who study humans have subjects collect their own samples and temporarily store them in their own freezers.[51] In contrast, zoo keepers adapt their daily routines to accommodate urine or fecal collection needs[28–30] or train their subjects to provide a sample on command.[49] The biologist working under field conditions, in contrast, must follow close behind the subject to opportunistically identify and collect samples.[21,40] Usually, samples can be stored frozen without preservatives until assayed. In the near future, assays will be field usable.

totally eliminating the need for sample storage.

The assays currently used to measure steroidal metabolites are relatively simple, efficient, and easily adapted from one species to another. This does not imply that new assays do not require validation or that all assays will provide comparable data among closely related species. In most cases, however, validations can be performed quickly with the use of differential extractions, and high performance liquid cochromatography (using known radiolabeled standards) will prevent inappropriate assay use.

Interpretation of results is difficult without validation of a given assay in a given species. Assay validation should include serial sampling and comparison of the urine/fecal data to an independent and traditional method of evaluating the reproductive process. Indexing of hormone results often is required but sometimes not essential, particularly when crude estimates are all that is needed to diagnose or understand a specific reproductive event. Whereas steroids and their metabolites are relatively ubiquitous across mammalian taxa, enough differences have been documented among species to warrant caution at all steps.

The classical validation approach has been to demonstrate that the assay does not detect other known or unknown compounds that might interfere through cross-reactivity. This kind of proof has little value when steroid metabolites of various species are concerned because there is no possibility of predicting which metabolites might be present and in what proportions. It is impossible to test (or even to obtain) all the potential reactors. Furthermore, the lack of absolute specificity and the detection of more than one metabolite are not necessarily weaknesses of an assay. Certain advantages in sensitivity are realized when the cross-reacting compounds are all metabolites of one parent circulating bioactive steroid molecule. Better approaches to validating urinary steroid metabolite assays include, as a basic step, the cochromatographic evaluation of the sample using known radiolabeled markers and using the assay as the end-point. By evaluating a range of samples, the number of cross-reacting substances and their individual relationships to the physiologic event can be determined. Finally, whenever possible, steroid concentrations in the vascular bed should be compared with matched urine concentrations of the appropriate metabolites, thereby establishing a positive correlation between hormone concentrations in the two compartments.[38]

MONITORING REPRODUCTION IN ZOO ANIMALS

Hormone metabolite monitoring was originally developed in zoos. During the past 10 yr, >100 reports have been published in which the reproductive biology of zoo animals is evaluated through excretion profiles of gonadal steroid metabolites. The initial need was to obtain a biological sample without imposing a capture and restraint stress. Initial work was directed at developing methods for predicting sex of monomorphic birds, detecting pregnancy and impending parturition, and performing general assessments of breeding potential. More recently, method refinements have allowed characterization of reproductive cycles and the monitoring of treatment therapies and have provided insight into the mechanisms associated with hormones, behavior, and seasonality.

Sex determination in monomorphic birds, such as the Hispaniolan parrot (*Amazona ventralis*), was accomplished by measuring free fecal steroids and examining the estrogen:androgen ratios.[3] Most of the early mammalian research was accomplished by measuring urinary concentrations of just two groups of metabolites, estrone conjugates (E_1C) and pregnanediol-3-glucuronide (PdG). Estrone conjugates include estrone sulfate and estrone glucuronide, which collectively reflect plasma estrogens, whereas urinary PdG reflects a poorly defined group of progesterone metabolites. Urinary E_1C

and/or PdG was measured to characterize the estrous cycles, reproductive seasonality, and breeding potential of a variety of captive exotic species, including the okapi (*Okapia johnstoni*),[28] giraffe (*Giraffa camelopardalis*),[30] Indian rhinoceros (*Rhinoceros unicornis*),[17,18] Asian elephant (*Elephas maximus*),[41] lion-tailed macaque (*Macaca silenus*),[47] Goeldi's monkey (*Callimico goeldii*),[6] gorilla (*Gorilla gorilla*),[8,35,36] and ruffed lemur (*Lemur variegatus*).[43] In these examples, urinary E_1C concentrations were useful for identifying preovulatory estrogen surges, whereas urinary PdG concentrations provided proof of ovulation and the establishment of luteal phases.

The detection of pregnancy is also important for managing zoo animals, and monitoring certain urinary hormone metabolites can provide a useful index of fetal health or impending fetal demise.[13] In many species, the fetoplacental unit produces large quantities of estrogen precursors, and urinary E_1C concentrations rise markedly at some point in pregnancy. Accurate pregnancy detection has been accomplished by measuring urinary E_1C in species as diverse as the gorilla and orangutan (*Pongo pygmaeus*),[8] the ruffed lemur,[43] Eld's deer (*Cervus eldi thamin*),[39] tapirs (*Tapirus terrestris* and *T. indicus*),[16] Przewalski's horse (*Equus przewalskii*), and Hartmann's zebra (*E. zebra*).[9] In other species, including the baboon (*Papio anubis*)[14] and Indian rhinoceros,[17] pregnancy can be predicted by the rise in urinary PdG concentrations above luteal phase concentrations. Even the subtle differences between a true pregnancy and a pseudopregnancy, as in the case of the giant panda (*Ailuropoda melanoleuca*), can be detected by monitoring urinary PdG and E_1C concentrations.[5]

The correlation of reproductive behavior with underlying endocrine mechanisms has also been examined through urinary steroids or their metabolites. Estrogen–behavior correlates have been found in the ruffed lemur,[46] and positive correlations between musth activity and urinary androstenedione and luteinizing hormone have been found in African elephants (*Loxodonta africana*).[4]

Although much valuable reproductive information has been derived from zoo animals by monitoring urinary E_1C and PdG, not all species metabolize estrogens and progesterone similarly; thus, both assays may not be useful in all species. Among the Perissodactyla[29] and Old World monkeys,[27] specific urinary PdG measurements were inadequate for accurately reflecting luteal function. This problem was partially resolved by varying the PdG assay and using an antiserum with significant cross-reactivity with 20-hydroxyprogesterone.[47] This nonspecific assay was used successfully to measure luteal function in the macaque,[47] the killer whale (*Orcinus orca*),[49] and, more recently, the domestic horse.[22] The failure of a single urinary progesterone metabolite assay to accurately reflect the luteal phase in all species results from species-specific differences in progesterone metabolism. The same is undoubtedly true for estrogen metabolites, but these differences have not yet been systematically addressed.

Fecal steroid metabolites have also been measured in zoo animals. Pregnancy was diagnosed in the red buffalo (*Syncerus caffer nanus*), yak (*Bos mutus*), Grevy's zebra (*Equus grevyi*), Nubian ibex (*Capra ibex nubiana*), and hippopotamus (*Hippopotamus amphibius*) by measuring total fecal estrogens.[42] Cyclic changes of estradiol-17β and progesterone also were detectable in feces collected during the menstrual cycle of the pigtailed macaque (*M. nemestrina*) and baboon (*P. cynocephalus*).[50]

MONITORING REPRODUCTION IN FREE-RANGING WILDLIFE

Successful management of wildlife requires understanding the species' reproductive biology. Noninvasive urinary and fecal monitoring technology, designed for captive animals, can be extended to free-ranging counterparts. The live capture of wild and feral animals to assess reproductive status varies in cost, difficulty, and risk, depending

on species. Small mammals and birds can be live-trapped and released without irreversible harm, but this approach can confound a reproductive physiology study by introducing a stress component. In studies of population densities and reproductive physiology in rodents, the subject populations were captured and handled to obtain blood samples.[1] The biases produced by trapping, the inadvertent spread of pheromones by investigators, and the negative physiologic consequences of extended trap confinement on reproduction, recruitment, dispersal, and general demography have been well documented.[32] Many of the same problems, when applied to primate studies, are exacerbated by the behavioral alterations common to captive populations.[43] Behavioral observations have been made and the endocrine status of the animals inferred.[11] Large free-ranging species pose even greater problems. Reproductive studies of deer, moose (*Alces alces*), caribou (*Rangifer tarandus*), feral horses, or bison (*Bison bison*) traditionally require immobilization and/or capture, events associated with enormous animal stress and sometimes significant mortality,[44,48] disruption of reproductive events,[2,26] and/or confounded endocrine status.[20] The cost of safe immobilization often exceeds $50/animal and, depending upon the species, capture can also be hazardous to the investigator.

The earliest reported use of urinary steroid monitoring in a free-ranging species was an examination of urinary androgens in male African elephants.[40] Endocrine correlates of musth were investigated by aspirating fresh urine pooled on the ground and measuring urinary androgen content. Free testosterone and dihydrotestosterone were measured in the unextracted urine samples and positively correlated to the increased sexual activity of the males.

Pregnancy in feral mares (*Equus caballus*) in Montana was diagnosed by measuring E_1C in urine extracted from the soil.[21] The urine was collected by placing recently soaked soil in gauze squares that were then placed in plastic bags and hand-centrifuged. The urine collected in the bottom of the plastic bag was poured into storage vials. This same method has been used successfully to monitor pregnancy rates among contracepted feral horses,[23] thereby evaluating the success or failure of fertility control experiments up to 8 mo before the foaling season. Noncapture pregnancy diagnosis by examination of E_1C and nonspecific progesterone metabolites (iPdG) has approached 100% accuracy in feral horses.[22] Under certain conditions, urine-soaked snow has been collected and melted, and E_1C has been measured to successfully diagnose pregnancy in feral horses.[25] Free-ranging breeding zebra stallions (*E. burchelli* and *E. grevyi*) in one study had significantly higher urinary androgen concentrations than bachelor males.[7] In a study in progress, urinary steroid metabolites are being used to study fertility in free-ranging mountain gorillas (*G. g. beringei*) in Rawanda (N. Czekala, pers. comm.).

Although there are few published reports concerning the reproductive physiology of truly free-ranging wildlife, several studies have been conducted or are in progress with animals living semifree in reserves. These same approaches to pregnancy testing (via urinary metabolites) have been applied to North American bison maintained in a 200-ha reserve[19] and to Przewalski's horses and Hartmann's zebras living in the San Diego Wild Animal Park.[9]

These urinary metabolite assays are accurate, but procedures for extracting urine from the soil are sometimes time consuming, and certain soils may interfere with hormone or creatinine assays. Pregnancy testing in free-ranging animals may be simplified by measuring free steroids or their metabolites in feces. Pregnancy diagnosis has been accomplished in feral horses by assessing total fecal estrogens[25] and fecal steroid conjugates[24] with the same high degree of accuracy as achieved by urinary E_1C and PdG. This method has also been used to characterize fetal loss among feral horses

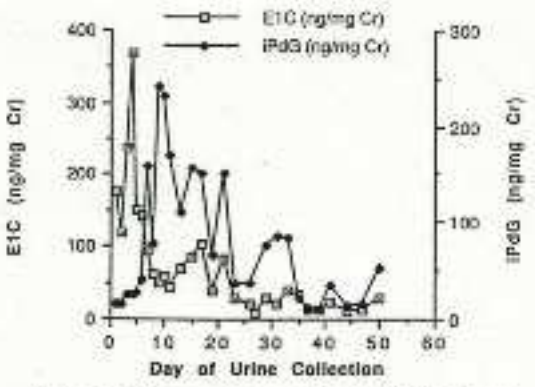

Figure 1. Urinary estrone conjugate (E_1C) and pregnanediol-3-glucuronide (PdG) concentrations in a feral mare from samples collected over a 51-day period during the breeding season. The hormone metabolite profile reveals a nonconceptive ovulatory cycle, characterized by a preovulatory E_1C peak occurring coincident with a PdG nadir, followed by a luteal phase PdG profile.

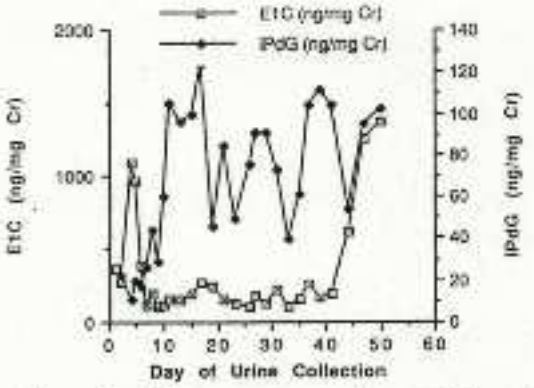

Figure 2. Urinary estrone conjugate (E_1C) and pregnanediol-3-glucuronide (PdG) concentrations in a feral mare from samples collected over a 51-day period during the breeding season. The hormone metabolite profile reveals a conceptive ovulatory cycle, characterized by the preovulatory E_1C peak, the luteal rise in PdG following ovulation, and a marked increase in E_1C at about the 35th day of pregnancy.

inhabiting Sable Island in Canada.[31] Pregnancy rates among captive and hunter-killed caribou in northern Canada were accurately assessed by measuring immunoreactive fecal progestins and total estrogens.[34]

Monitoring cyclic reproductive events through assay of urinary or fecal hormones represents a more advanced application of this technology. Estrous cycles have been tracked successfully in semicaptive muskox (*Ovibus moschatus*) by measuring fecal progesterone and total fecal estrogens,[10] in bison living in a reserve by measuring urinary PdG concentrations,[19] and in semi-free-ranging Przewalski's horses by measuring urinary E_1C.[37] Nonconceptive (Fig. 1) and a conceptive (Fig. 2) ovulatory cycles were monitored in feral mares using urinary E_1C and PdG (Kirkpatrick, unpubl. data).

The applications of noninvasive endocrine assessments to free-ranging wildlife are numerous. The field behaviorist can determine the precise reproductive status of animals without unduly altering the subjects' behaviors. Early pregnancy rates among ungulates can be compared with subsequent birth rates to assess fetal mortality rates. Pregnancy rates determined late in gestation can be compared with the number of newborns to more accurately evaluate neonatal mortality. Ovulation can be detected and compared with pregnancy rate, providing fecundability data. Together, these types of information can provide useful insights into the mechanisms of environmental pressures associated with population density, disease, nutrition, genetics, environmental contamination, or even climate. For example, in an innovative approach to the study of oil pollution-induced toxicity among seabirds, droppings were collected from Cassin's auklets (*Ptychoramphus aleuticus*), and testosterone, estradiol, and progesterone were measured during the breeding season.[12]

Increased emphasis on understanding the biology of free-ranging endangered species also provides a useful application for this noncapture technology. In Yellowstone National Park, research-related mortality rates in the endangered grizzly bear (*Ursus arctos*) is about 4% (J. Varley, pers. comm.); sex determination, juvenile/adult status, and pregnancy determination (based on fecal steroids) could provide important demographic data without the stresses and dangers of capture. Recent characterization of

the reproductive cycle of the steppe polecat (*Mustela evermanni*) by urinary progesterone tracking[35] and of the endangered black-footed ferret (*Mustela nigripes*) by tracking fecal steroids[23] provides an excellent database for future noncapture monitoring of black-footed ferrets after their eventual reintroduction into historical native habitat.

SUMMARY

Modern fundamental studies of zoo animals and wildlife biology will not eliminate the need for the occasional capture or immobilization of animals. There is, however, much valuable information that can be collected without capture, through urinary and/or fecal hormone metabolite monitoring. Wherever possible, the first choice for studying reproduction or diagnosing reproductive/endocrine status in captive or free-ranging wildlife should be these noninvasive techniques. These approaches benefit the animals studied and improve the overall quality of the science. Additional technical advancements in the near future will expand the capabilities, but the application of this technology for conservation biology will depend on the willingness of researchers to routinely use these new methods.

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