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Non-invasive measurement of adrenocortical activity in zoo and wildlife animals

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A multitude of endocrine mechanisms is involved in coping with challenges. Glucocorticoids, secreted by the adrenals, are front-line hormones to overcome stressful situations. They are usually determined in plasma samples as parameters of adrenal activity and thus of disturbance. Blood sample collection itself disturbs an animal and is dangerous or even impossible in some zoo and wildlife species. Non-invasive methods for the determination of glucocorticoids or their metabolites are therefore a prerequisite for assessing stress in these animals. Above all, fecal samples offer the advantage that they can be collected easily without any need to handle the animal. However, using this technique to reliably assess an animal's adrenocortical activity is not that simple and straightforward to apply. Because clear differences regarding the metabolism and excretion of glucocorticoid metabolites exist, a careful validation for each species and sex investigated is obligatory. Analytical issues regarding sample storage, extraction procedures, and immunoassays will be addressed and various examples of a successful application given.

Applied properly, non-invasive techniques to monitor stress hormone metabolites in fecal samples of various species are a useful tool in different research fields, such as ethology, field endocrinology, ecology, animal conservation and animal welfare, and can open new perspectives in biomedical and behavioral sciences.

Fecal steroid monitoring in free-ranging aardwolf (*Proteles cristatus*) – the importance of assessing the fraction of inorganic food intake

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The aardwolf *Proteles cristatus* is a small hyena which feeds almost exclusively on harvester termites. To evaluate the physiological consequences of such diet specialization, e.g., the physiological response to seasonal starvation, an enzyme immunoassay (EIA) was validated to measure glucocorticoid metabolites (GCM) in aardwolf feces. Bi-weekly fecal samples (n = 69) were collected from four previously radio collared aardwolves (two males and two females) at Benfontein Game Farm, South Africa between December 2009 and February 2010, and an ACTH challenge was performed on one of the males and one of the females. GCM immunoreactivity was tested in a cortisol EIA and an EIA that detects GCMs with a 3α-hydroxy-11-oxo structure.

Approximately 0.2 g of lyophilized fecal powder extracted in 3 ml 80% ethanol in water was sufficient to enable appropriate extract concentrations to conduct the assays. However, due to a highly variable amount of mineral content in the collected samples (49.5-92.5% of total dry weight), the organic content of the samples was determined by burning extracted samples in a muffle furnace and expressed the measured GCM levels as mass GCM/mass organic content in extracted feces.

Only the cortisol EIA detected the predicted increase in fecal GCM levels in response to intramuscular ACTH administration. GCM levels reached a peak 19- to 27-fold above baseline at about 6-20 h following treatment. Overall, the data indicate no significant difference in baseline GCM concentrations among animals (P = 0.66). However, some putative stressful events recorded seem to be associated with an elevation in GCM levels.

In conclusion, this assay system provides a reliable method for assessing adrenocortical function in Aardwolves based on fecal GCM analysis. However, the results indicate that hormone concentrations should be expressed per mass organic dry weight rather than percent mass dry weight of extracted fecal material, due to the unpredictable amount of physiologically inert sand within free-ranging Aardwolf feces.

What metabolites of testosterone do testosterone hormone assays target in the feces of carnivores?

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Several assays have been used to measure testosterone (T) metabolites in the feces of different carnivore species. These assays are normally based on an antibody directed against T metabolites, but these antibodies may not be specific and may have a significant cross-reactivity with non-target metabolites. For example, it has been shown that radio-labelled cortisol (C) is metabolized into androgens, which suggests that a group-specific antibody for T metabolite measurement may also measure androgenic C metabolites, leading to misleading results.

In the spotted hyena we have shown that a specific T antibody-based assay (directed against T-11-HS-BSA) detected the stress response induced by an ACTH challenge. A radio-metabolism study on feces from an adult male spotted hyena that received [^{3}H]testosterone, revealed (by HPLC immunogram) that our T antibody predominantly traced unpolar metabolites that did not overlap with true radio-labeled T metabolites, and that radio-labelled testosterone was largely metabolised into polar (probably conjugated) metabolites. We assessed the efficacy of four different antibodies directed against T-11-HS-, T-3-CMO- and T-6-CMO-BSA, and one against epi-androsterone, a putative fecal T metabolite. All antibodies detected large amounts of metabolites not supported by radio-labeling and only minor portions of radio-labeled polar metabolites.

The results of this radio-labeled metabolism experiment indicate that antibody assays designed to measure fecal T metabolites may measure non-T metabolites, thereby producing false results. We hypothesize that these non-T metabolites are probably cortisol metabolites of adrenal origin that are cross-reacting with our T antibody. They may also be metabolites of precursors of gonadal or adrenal testosterone synthesis such as androstendione. To eliminate measurement of non-T metabolites we propose a preparatory solid-phase extraction step to separate the polar fraction of T metabolites prior to analyses.

Application of radio-labeled T to male cheetah revealed that the major proportion of radio-labeled T metabolites were in the polar fraction. In contrast to the results from spotted hyena, immunoreactivity was focused on this polar fraction and only a minor proportion consists of non-T metabolites. Verification of the efficacy of each assay for a specific species is essential to ensure the correct interpretation of results.

Environmental degradation of black rhino fecal hormone metabolites in Addo Elephant National Park, South Africa

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It is well known that non-invasive endocrine monitoring is a useful tool for assessing gonadal and adrenal activity in wildlife populations. However, it is not always possible to observe free-ranging animals defecating and collect fresh fecal samples. We have employed the use of camera traps to facilitate the collection of feces from the elusive black rhinoceros (*Diceros bicornis bicornis*) in Addo Elephant National Park, South Africa. The cameras enable us to identify the individual and provide an approximate time of defecation. To determine how frequently cameras have to be monitored and fecal samples collected after defecation, we tested the degradation of fecal hormone metabolites over time when exposed to environmental conditions. Cameras were checked and fresh fecal samples (n=10) collected for gonadal and adrenal hormone analyses. An aliquot of feces, our control sample, was collected immediately and frozen until extracted using a field method. At 2 hr intervals from the first collection (up to 12 hr) and 24, 48 and 72 hr post defecation, fecal matter was sampled and extracted. A 1 ml aliquot of all extracts was allowed to evaporate and then was heat treated to 72˚C for 30 min and exported to Lincoln Park Zoo (Chicago, IL) for hormonal analyses using previously validated EIA methods. Changes in fecal progestin, androgen and glucocorticoid metabolite concentrations over time were analyzed for each rhinoceros. Additionally, differences among the control sample and subsequent extracts were compared as well. Our results indicated small declines in corticoid and androgen concentrations and a slight increase in progestins as the samples age; however none of these trends were significant. Due to a high degree of individual variation, we believe it is best to collect only fecal samples that are <12hr old to maintain as much consistency as possible among the samples. Although camera traps have proven to be a useful tool for gathering feces from an elusive species, the effectiveness is limited by how frequently the cameras can be monitored.

Development, validation and comparison of field steroid extraction and assay techniques with standard laboratory methods for analyzing fecal reproductive steroid profiles of wild cotton-top tamarins *in situ* in Colombia, South America.

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Cotton-top tamarins (*Saguinus oedipus*) are small (0.5 kg) arboreal New World monkeys of the family *Callitrichidae*. Critically endangered and found only in northwestern Colombia, South America, populations are highly fragmented and estimated to be less than 7,500 individuals. Continued reproductive monitoring provides critical information on the health of this population. Changes and challenges in international shipping regulations over the past decade produced a need for development of field-friendly techniques for sample extraction and enzyme immunoassay (EIA) of pregnanediol-3-glucuronide (PdG) and estrone conjugates (E_1C) *in situ* that were comparable with standard in-house results for continued reproductive monitoring of the study population. Here we report development, validation of results from two field protocols for fecal sample extraction in comparison with standard in-house methods. Sample tests included 1) methanolic fecal extraction and EIA *in situ* using modified field equipment, sample extraction, storage and EIA protocols for the field; 2) methanolic then solid phase extracted (SPE) *in situ* with SPEs shipped to the USA for sample recovery and standard EIA; and 3) standard lab protocol for methanolic fecal extraction and EIA in the USA (control). These tests were conducted as part of a longitudinal reproductive study of 6500 fecals samples from 30 wild female cotton-top tamarins in 10 family groups in our field site in Santa Catalina, Colombia between 1999 and 2009. Results showed that both field methods produced fecal PdG and E_1C profiles of cycles and pregnancies with concentrations comparable to standard in-house analysis and were successfully used to assess reproductive condition in free-ranging female cotton-top tamarins.

Environmental fecal glucocorticoid degradation: Impacts for assessing non-invasive adrenal activity in the Belizean jaguar (*Pantheraonca*)

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Non-invasive fecal hormone metabolite (e.g. glucocorticoids) analysis to monitor adrenal activity has been performed extensively in zoo-housed wild felids; however little work has been conducted on free-ranging populations, in part due to potential hormonal degradation in feces exposed to field environmental conditions. The aim of this study was to validate laboratory techniques and evaluate environmental effects on jaguar fecal glucocorticoid concentration (FGC) stability in a biologically relevant habitat, Belize, Central America. At the Belize Zoo, fresh scat from jaguars (6 males and 4 females) was collected and randomly exposed to two environmental conditions: shade and sun. Collected samples were homogenized prior to being divided into several subsamples for further tests. A control (first subsample) was immediately frozen. Thereafter, subsamples were collected and frozen daily over a 5 day period in both the dry and wet seasons. Weather parameters were measured with data loggers at ground level. In the lab, scat was freeze-dried, homogenized and extracted by boiling the sample in ethanol (90%v/v).The cortisol ELISA R-4846 (C. Munro) was validated for jaguar feces by showing parallelism between the cortisol standard and fecal extracts.

Biological validity of this assay was demonstrated by showing a significant difference in FGC metabolites among individuals in relation to the time spent in captivity. Problem jaguars recently captured from the wild and brought to captivity excreted 5-fold more FGCs than captive-born or long-term captive jaguars. Repeated measures analysis over time indicated that FGCs were stable for 5 days during the dry season but less than 1 day during the wet season. Exposure of jaguar scats to sun or shade had no effect on FGC degradation, despite significant differences found in weather parameters. In conclusion, non-invasive FGC measurements in free-ranging Belizean jaguars can be conducted employing a practical collection regimen by surveying the same areas every 5 days in the dry season. It is not suggested to conduct field surveys for FGC in the wet season to ensure physiologically relevant FGC concentrations. Furthermore, warmer and dryer weather seem to be ideal to minimize variation in results due to FGC degradation. Assessing adrenal activity in jaguars ranging in areas of varying human disturbance is a timely application of this methodology in Belize, a country that has experienced increasing levels of human-jaguar conflict.

Using fecal samples to diagnose pregnancy in wild giraffe

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Noninvasive measurements of hormone levels allow researchers to gather information about an animal's internal state with minimal stress and disruption. While these measurements were developed using captive animals, they have also been used to determine internal states of wild animals during field studies. Reproductive state[1] and estrous cycle phase[2] of captive giraffe have been assessed by ether extraction and radioimmunoassay (RIA) of fecal progesterone metabolites (pregnanes). With 4-5 fecal samples per week, pregnancy could be diagnosed about 6 weeks after conception. The current study aimed to diagnose pregnancy in wild giraffe using single samples.

Fifteen giraffe fecal samples were extracted twice: once using the published lab technique and once using a field technique adapted from rhinoceros[3] with extracts heated above 72ºC for 30 minutes to meet USDA import requirements. The published RIA with a monoclonal antibody produced against 4-pregnen-11-ol-3,20-dione hemisuccinate:BSA was characterized and used to assay both sets of extracts. The two extractions produced comparable results after adjusting for efficiency and water content. The field technique and RIA were then used to find pregnane concentrations in 111 fecal samples from 71 females in Etosha National Park, Namibia, which were converted to lab equivalents and compared to the published threshold for pregnancy[1]. About half of the samples yielded concentrations above the threshold and in most cases this diagnosis agreed with observations suggesting reproductive state. Field extraction techniques appear to be acceptable for giraffe samples and pregnant females can be identified using RIA of a single fecal sample. However, many samples classified as non-pregnant may have come from females in early pregnancy.

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Noninvasive pregnancy diagnosis in carnivores based on fecal prostaglandin F2α metabolites

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In mammals, uterine and placental prostaglandin (PG)F2α are involved in the regulation of reproductive related processes like embryonic development, initiation of parturition and resumption of ovarian activity. PGF2α is rapidly metabolized to its plasma metabolite PGFM (13,14-dihydro-15-keto-PGF2α) that has also been detected in urine and feces. We recently developed an efficient, quick and inexpensive EIA for PGFM estimation in feces of felid species for pregnancy monitoring and for differentiation between pregnancy and pseudopregnancy. A significant PGFM increase in pregnant lynxes was detectable at the beginning of the last trimester of pregnancy whereas in pseudo-pregnant females PGFM levels remained at baseline. Similar pregnancy related profiles were demonstrated in the sand cat (*Felis margarita*), Oncilla *(Leopardus tigrinus*), Geoffroy's cat (*Felis geoffroyi*), the cheetah (*Acinonyx jubatus*) and the Sumatran tiger (*Panthera tigris sumatrae*).

In lynx high-performance liquid chromatography (HPLC) immunograms and liquid chromatography–mass spectrometry (LCMS) were performed to identify PGFM within fecal samples. Besides PGFM two additional immunoreactivities were detected. As our PGFM antibody does not show any cross-reactivity toward other prostaglandins we assume that both immunoreactivities might belong to a PGFM isomer.

However, our cautious optimism that fecal PGFM might serve as specific pregnancy indicator and its differentiator from pseudopregnancy in other carnivores and probably non-carnivorous mammals was impaired. In fecal samples from the European mink *(Mustela lutreola*), the red wolf (*Canis lupus rufus*) and the giant panda (*Ailuropoda melanoleuca*) a continuous increase indicating pregnancy was not obtained. In these species fecal PGFM peaked immediately one day prior parturition indicating the known luteolytic action of PGF2□. These findings were also supported by data obtained in one pregnant domestic dog. An exception was the fecal PGFM course in one pregnant spectacled bear (*Tremarctos ornatus*) increasing three weeks prior parturition.

In some pregnant non-carnivorous mammals such as the white rhino (*Ceratotherium simum*), the red-fronted lemur (*Eulemur rufifrons*) and the hare (*Lepus europaeus*) no PGFM was detectable in fecal samples. We assume that in these species PGFM is either not excreted with the feces or metabolized in a way that it is unrecognizable for the antibody. Further research is needed to validate whether PGFM can be applied as pregnancy marker in other felid species and to investigate the biological function of PGF2a that hides behind the PGFM course in felids.

Development of an enzyme immunoassay for the non-invasive determination of corticosterone metabolites in a variety of wildlife species

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The current study describes the development an enzyme immunoassay to non-invasively measure adrenal activity in the feces of okapi, Asian elephant and black rhino. The antiserum was raised in rabbits to a corticosterone-3CMO-BSA immunogen. The resultant polyclonal antibody (CJM006) cross-reacted with corticosterone 100%, desoxycorticosterone 14.2%, progesterone 2.7%, tetrahydrocorticosterone 0.9%, testosterone 0.64% and less than 0.2% with all other steroids tested. To establish an accurate and repeatable method, a number of factors were manipulated to produce an enzyme immunoassay with low inter- and intra-assay variation: plate type (Nunc maxisorp II versus Immulon II plates), type (artificial versus natural) and presence (light versus dark) of light during incubation, plate loading temperature (4^{o}C versus room temperature), substrate reagent temperature (4^{o}C versus room temperature) and the addition of a non-specific IgG. The optimum assay conditions include Nunc maxisorp plates, room temperature substrate reagents and dark incubation. Once established, the enzyme immunoassay was validated for each species using parallel displacement curves, interference assessment and biological responses to a challenging event. This enzyme immunoassay provides a consistent enzyme immunoassay method which can potentially be utilized for a range of species.

The levels of active urinary ceruloplasmin as an indicator of pregnancy status in ursids

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Members of the Ursidae (bear) family undergo pseudopregnancy after ovulation, during which non-pregnant females exhibit physiological and hormonal changes similar to pregnancy. In many other species, pregnancy can be diagnosed by monitoring the steroid hormone progesterone or its metabolites, but this is inconclusive at determining the pregnancy status in many species that experience pseudopregnancy. Therefore, the objective of this study was to develop a non-invasive assay for pregnancy detection for ursids. For the giant panda (*Ailuropoda melanoleuca*), urine was collected 3-7 times weekly over the course of 18-25 weeks from 16 reproductive cycles. An assay for measuring the levels of urinary ceruloplasmin was then validated using oxidasic activity measurement, wherein the concentration of ceruloplasmin is determined by the rate of formation of a colored product produced from ceruloplasmin and the substrate, N,N-dimethyl-p-phenylendiamine. Creatinine was also measured in each urine sample to account for the concentration of water.

In term pregnancies, the activity of urinary ceruloplasmin was higher during the pregnant luteal phase compared to the pseudopregnant luteal phase ($P \leq 0.05$). In all term pregnancies examined, levels of active ceruloplasmin were elevated the first week of pregnancy and remained elevated until 20-24 days prior to parturition. The levels of active ceruloplasmin also increased during lost pregnancies; however, the pattern of elevated ceruloplasmin was different compared to the pattern observed in term pregnancies. In the polar bear (*Ursus maritimus)*, preliminary results indicate that measuring active ceruloplasmin in urine is also applicable for the determination of pregnancy status. After natural mating in the polar bear housed at the Memphis Zoo, urine was collected 1-4 times weekly over the course of 13 weeks during the luteal phase. Samples collected the same year prior to breeding and banked samples collected previous years were used as baseline control levels. Weekly luteal phase levels of active ceruloplasmin were significantly higher after breeding than baseline levels prior to breeding and in proceeding years ($P \leq 0.05$). The levels of active urinary ceruloplasmin obtained during the luteal phase after breeding were also elevated compared to samples collected from a control female on contraceptives.

Non-invasive monitoring of adrenal activity in the saiga antelope

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During the 1950s and '60s there were up to 1,250,000 antelopes (*Saiga tatarica tatarica*) in the North-West Pre-Caspian (NWPC) region. Intensive development of agriculture, construction of irrigation channels, and severe poaching, have led to the decline in saiga populations, the restriction of their range size, and several changes in saiga nomadic behaviour. Currently there are less than 20,000 individuals in the NWPC region. The Center for Wild Animals (CWA) of the Republic of Kalmykia was established in 2000 with the aim of protecting the genetic diversity of saiga antelope and establishing recommendations for restoring saiga populations. Non-invasive monitoring of adrenal activity could help to improve population restoration efforts. To develop and validate an enzyme immunoassay (EIA) for monitoring fecal glucocorticoid metabolites (GCM) in saiga, we used four captive adult males and four females housed at CWA.. Four different EIAs were tested. The best results was obtained using an enzyme immunoassay measuring 11,17-dioxoandrostanes (11,17-DOA). To provide physiological validation, we used two basic approaches: ACTH (Synacthen® Depo, Novartis) injections and dexamethasone application. Fecal samples were collected 24 hours before and 48 hours after injections. We extracted GC metabolites with 80% methanol. In males (n=4), a five to nine-fold increase in fecal GC metabolites was observed 17 hours after ACTH injection (25mg/kg); in females (n=4), an analogous peak was observed 12 hours after ACTH injection (12.5 mg/kg). Interestingly, higher doses of ACTH caused suppression of GCM production in females. Dexamethazone treatment resulted in an average of three-fold decreases in fecal GCM concentration. Serial dilution test also showed good results for 11,17-DOA EIA for both, males and females. Based on the successful validation of a fecal GCM EIA we are now analyzing over 1000 fecal samples collected from free-ranging Kalmykia saiga populations (Supported by RAS Program "Biodiversity", project 6.1.4. to V.V.V)

Managing data for wildlife endocrinology

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Science based data management has traditionally been viewed as the organization and processing of data to facilitate research. Previously, research projects organized results into a database based on subject tables related by function. These databases would require reorganization or reclassification to suit the needs of a new project which often requires more time and effort than the research project itself. However, the advent of the internet offers an information sharing model whereby data are viewed as a resource which are not only usable for the current project but may be used as a resource for future projects. Making data more readily available reduces data redundancy and duplication of effort, but requires an understanding of data standards in other scientific disciplines beyond the current project. The objectives of this project are to: 1) demonstrate some principles and procedures of database design which take into account the concept of creating a wildlife endocrinology data resource; 2) build a web-based wildlife endocrinology database; and 3) consider how to better manage these data in the future.

Using information on endocrine procedures and analyses gathered from the AZA's Endocrine SAG, a set of requirements (Software Requirements Specification for Endocrinology Database) have been created which will be used as a development roadmap. This project entails building a web based network of data using SQL Server to house the database, DotNetNuke as the content management system and ASP.NET to access the dataset. This resource will draw on some of the prevailing systems of nomenclature such as SNOMED for terminology, LOINC for clinical observations and ITIS for taxonomic classification. By designing a system which considers both, present and future data needs, the Wildlife Endocrinology Information Network (WEIN) will be a capable data repository for current endocrine projects and a resource for future scientific studies.

Determining factors associated with iron overload in the black rhinoceros (*Diceros bicornis*)

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Black rhinoceroses (*Diceros bicornis*) managed in *ex situ* collections are susceptible to many diseases syndromes not found in wild counterparts. Conditions such as acute hemolytic anemia, hepatopathy and ulcerative dermatopathy cause increased morbidity and mortality within the captive population, and hemosiderosis (excess iron accumulation in body tissues) is common. In humans, hemosiderosis is associated with multiple disease syndromes, including cardiac and liver disease as well as infertility, insulin resistance and diabetes. It is unclear if hemosiderosis in the black rhino is associated with other underlying disorders that may contribute to disease. Although iron overload is a well-documented phenomenon in this species, insulin sensitivity and inflammatory status have not been studied in any rhino species or population. The objectives of this study were to: 1) validate assays to quantify serum markers of body condition (leptin), inflammation (tumor necrosis factor alpha and serum amyloid A), insulin sensitivity (insulin) and iron stores (ferritin) in the black rhino; 2) generate baseline information for each of these serum markers in free-ranging individuals; and 3) compare markers of metabolic disturbances between free-ranging and captive populations to identify metabolic correlates of iron overload unique to the *ex situ* population. Previously banked serum from 100 free-ranging and 60 captive black rhinos was analyzed for each of the markers and compared between populations. Serum glucose and phosphate were quantified using a standard chemistry analyzer (Vet Test 8008, Idexx). Preliminary analysis of a subset of 47 free-ranging and 10 captive individuals indicated that captive individuals had a higher ($P < 0.05$) insulin-to-glucose ratio and increased indicators of inflammation compared to free-ranging counterparts. Additionally, serum leptin, insulin/glucose ratio and inflammatory markers appeared to increase ($P < 0.05$) over time while in captivity. These preliminary data indicate that there are measurable differences in insulin resistance and inflammatory indicators between black rhinos managed in *ex situ* environments compared to free-ranging counterparts. More studies are warranted to determine if these disparities quantified in captive individuals eventually contribute to diseases prevalent in this species.

Development of an anti-Müllerian hormone assay for veterinary use: Assessing the fertility of older females

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Anti-Müllerian hormone (AMH), also known as Müllerian inhibitory substance (MIS) is produced and secreted by the ovaries, specifically by the granulosa cells of primary, secondary, and early antral follicles. Determination of AMH concentrations in the circulation is commonly performed in human infertility clinics as part of an assessment of follicular reserve and as a predictor of gonadotropin responses in preparation for *in vitro* fertilization. A commercially available AMH ELISA (Beckman Coulter) has been effectively used for research purposes in a substantial number of mammalian species (e.g., dogs, cats, horses, cows, mice, rats, and hamsters), but the cost of the assay has precluded its use in clinical veterinary practice. The Diagnostic Endocrinology Laboratory within the New York State Animal Health Diagnostic Center at Cornell University is working to develop an AMH assay using antibodies generated against canine AMH. Given the high cross reactivity of the human-based assay with a wide array of species, a canine-based ELISA is likely to detect AMH in samples from a variety of species. Such a test could be used to help assess the fertility potential of older females in zoo and wildlife settings. Whereas chronological age is a good predictor of fertility at the population level, it may be a poor indicator of fertility potential at the individual level, because the rate of reproductive aging is highly variable between individual females. In an older female with high genetic value an assessment of AMH concentration, in combination with other tests (e.g., ovarian ultrasound), may help to determine if that animal warrants the investment of resources to breed her or to produce offspring through assisted reproductive technologies. Other potential applications for an AMH test include the determination of spay status when presence or absence of the ovaries is uncertain, as in cases of suspected ovarian remnant syndrome, and in the evaluation for granulosa cell tumors. Serial AMH testing might also be used to monitor for unusually rapid declines in fertility due to either genetic or environmental reasons, which would alert zoos of the need to mate certain females sooner rather than later.

Decreased baseline fecal glucocorticoid concentrations associated with skin and oral lesions in black rhinoceros, *Diceros bicornis*

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Ulcerative skin and oral lesions are a health concern for black rhinoceros (*Diceros bicornis)* populations. To examine possible relationships between adrenal activity and the presence of lesions, fecal samples were collected twice weekly for 1 year for glucocorticoid metabolite analyses for 25.20 (male.female) black rhinos at 18 zoos. During the collection period, 5.1 rhinos exhibited skin lesions, 1.0 had oral lesions and 1.0 had both. A double-antibody ^{125}I corticosterone radioimmunoassay (RIA) was used to quantify corticoid metabolite concentrations in feces. There were no differences ($P > 0.05$) in fecal glucocorticoid variability (± SEM) between rhinos with (CV = 57.1 ± 7.2 ng/g) and without (53.8 ± 2.3 ng/g) lesions, or in overall mean (± SEM) glucocorticoid metabolite concentrations between rhinos with (45.1 ± 4.0 ng/g) and without (34.6 ± 2.8 ng/g) lesions. However, baseline mean (± SEM) glucocorticoid metabolite concentrations were lower ($P < 0.05$) in rhinos with lesions (n = 5.1, baseline mean = 29.9 ± 3.3 ng/g) than without (n = 19.19 baseline mean = 40.0 ± 2.4 ng/g). For a male rhino that developed lesions during the study, the mean glucocorticoid concentrations were lower ($P < 0.01$) when lesions were present (n= 12, overall = 30.1 ± 2.4 ng/g, baseline = 28.7 ± 2.2 ng/g) than prior to lesion onset (n=75, overall = 36.5 ± 1.0 ng/g, baseline 35.3 ± 0.8 ng/g). These results suggest that ulcerative lesions may be associated with changes in adrenal activity, although it is not clear if this is a cause or effect of disease. In the broader sense, occurrence of lesions may only be one manifestation of an underlying health problem. Therefore, it might provide more insight to consider this problem in conjunction with the other disease syndromes that affect black rhinos. Such a meta-analysis might reveal physiological and/or socio-environmental variables that are common among rhinos with varying health issues. A clear understanding of these potential relationships is needed so that effective mitigating strategies, such as medicinal treatments and alterations in management practices, can be developed.

Using fecal hormone monitoring and behavioral observation to investigate factors that influence breeding success in pied tamarins

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Pied tamarins (*Saguinus bicolor bicolor*) are a critically endangered Amazonian primate that have had limited breeding success in zoos compared to other Callitrichid species. Unfortunately, little is known about their reproductive biology and adrenocortical activity, which presumably may influence breeding success. Our goal was to determine if fecal hormones could be utilized to monitor gonadal and adrenocortical activity in the pied tamarin. Objectives were to: 1) characterize male and female gonadal and adrenocortical hormones and 2) determine if there were differences between adrenocortical activity and behavior in an on-exhibit pair compared to an off-exhibit pair of pied tamarins. Feces that were not cross-contaminated with urine (n=727) were collected three to seven times/week for 9 months from four (2 male; 2 female) individuals. Hormones were analyzed for progestin (FPM), androgen (FAM) and glucocorticoid (FGM) metabolites by enzyme immunoassay. Behavioral observations were conducted three times per week for 6 months. An ethogram was used to collect data on instantaneous behavior (n=12), location (n=9) and all occurrences of intra-specific behaviors (n=8). FPM was validated by pregnancy (mean±S.E.M., 28.8±16.0 µg/g; range, 4.1 to 59.6 µg/g) versus FPM non-pregnancy values (mean, 8.4±4.0 µg/g; range, 2.5 to15.0 µg/g). FAM value was similar ($p>0.05$) between the breeding male (963.71 ± 91.06 ug/g dry feces) and non-breeding male (901.57 ± 122.39 ug/g dry feces). FGM was validated by response to veterinary procedure with elevated values (mean, 7.3±1.5 µg/g; range, 5.8 to 8.8 µg/g) seven times the baseline (mean, 0.2±0.01 µg/g; range, 0.04 to 20.5 µg/g) at 24 hr post-physical for 48 hrs. Baseline FGMs differed (ANOVA; $F_{1,430}=257$; $p<0.05$) between on-exhibit pair (0.84±0.03 µg/g; range, 0.06 to 27.44 µg/g) and off-exhibit pair (0.32±0.01 µg/g; range, 0.03 to 72.45 µg/g). Behavioral data revealed the presence of stereotypical behavior in the on-exhibit pair (tail biting and repetitive jumping at exhibit glass) but no stereotypical behaviors were observed in the off-exhibit pair. Pied tamarins may be sensitive to the presence of other species and the public resulting in increased adrenocortical activity. Therefore, fecal hormone monitoring and behavioral analysis may provide insight on the limited breeding success of pied tamarins in zoos.

Use of urinary biomarkers of ovarian function to enhance captive breeding success in the Indian rhinoceros (*Rhinoceros unicornis*)

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The captive Indian rhinoceros breeding program has been successful in terms of the number of offspring produced. However, the frequently aggressive interactions exhibited by males and females during introductions for mating have limited the number of breeding pairs and resulted in a captive population over-represented by a few highly prolific founders. Several established biomarkers of ovarian function are now available for assessing reproductive function in Indian rhinoceros. The aim of this study was to use urinary estrone conjugate (EC) and progesterone metabolite (PdG) analysis to predict estrus and time mating introductions for Indian rhinoceros at remote facilities to enhance breeding success.

Urinary hormone analysis was conducted on two adult female Indian rhinoceros that exhibited minimal or no estrual behaviors traditionally used to time breeding. Urine was collected throughout two consecutive estrous cycles to establish preliminary data on each individual's pattern and concentration of EC and PdG during follicular and luteal phases. The number of days urinary EC remained elevated above baseline was determined and the pattern of PdG excretion during the late follicular phase was examined for any transient rise indicative of ovulation. Finally, the concentration and excretion pattern of PdG and EC during the follicular to luteal transition was determined. Following initial endocrine analysis, urine samples were shipped on a frequent basis to verify when each female was off baseline in EC. Estrus and breeding dates were then predicted. Females were introduced to fresh male rhinoceros fecal samples daily during the follicular phase to potentially stimulate estrous behaviors. Introductions were planned accordingly.

Urinary EC and PdG analysis was successful in providing a predictive tool for estrus and breeding in the Indian rhinoceros. Exposure to male feces during the follicular phase did appear to enhance the display of estrous behavior by females. Despite successful assessment of follicular phase dynamics, females sometimes failed to exhibit estrus. Both females conceived following mating introductions that were timed using hormone analysis. One female experienced early pregnancy loss and the other successfully completed a term pregnancy. These results demonstrate a science based management strategy that relies on urinary biomarkers of ovarian function can facilitate the natural breeding of captive Indian rhinoceros.

Associations between social behavior and adrenal activity in female Barbary macaques *(Macaca sylvanus)*; consequences of study design.

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The acquisition and maintenance of dominance rank is a potential source of stress in social species. Fecal glucocorticoid metabolite (fGC) concentrations have been used to evaluate relationships between social or environmental factors and stress responses in a number of primate species. However, across studies, the relationships between dominance rank, social behaviors and adrenal activity are inconsistent. We propose that these inconsistencies may partly be explained by the use of average fGC concentrations collected over a period of weeks or months, rather than fGC concentrations that are temporally-matched with behavioral data.

In this study, we used a cortisol enzyme immunoassay (R866), and compared average and temporally-matched data to determine whether particular social behaviors predict adrenal activity in eight semi-free ranging female Barbary macaques. Average rates of autogrooming were positively correlated with average fGC; however, this relationship was not robust in temporally-matched samples. Rather, specific social behaviors associated with agonism were associated with fGC in temporally-matched samples within individuals.

We conclude that it is not dominance rank itself but the relative rates of agonistic social behaviors an individual is involved in that affects adrenal activity. These results demonstrate that analysis of relationships using average fGC and temporally-matched samples does not provide comparable results, and that relationships between specific behaviors and adrenal activity vary across individuals. This work highlights the need for an individual-based approach and the importance of study design in determining associations between an individual's social behavior and the relative physiological costs involved.

The effects of management practices on cortisol metabolites in the Indian rhinoceros (*Rhinoceros unicornis*).

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Non-invasive hormone monitoring of glucocorticoids is a useful tool for making informed decisions regarding the physiological impacts of *ex-situ* animal management. For instance, zoos and conservation facilities routinely transport individuals between institutions to promote genetic diversity. Additionally, operant conditioning programs are often employed to obtain cooperative animal behaviors for advanced husbandry, medical, and reproductive procedures. However, the physiological impacts of these management practices are unknown. The aim of this multi-part study was to assess the effects of both translocation and operant conditioning on adrenal activity in the Indian rhinoceros.

An ACTH challenge was performed in a male Indian rhino to validate the efficacy of an anti-cortisol antiserum EIA (R4866, Coralie Munro) to detect immunoreactive glucocorticoid metabolites in matched urine and fecal samples. Three adult, female Indian rhinos were translocated and fecal samples collected ~ four times weekly one month prior to and two months following transport between facilities. Finally, a 5-month positive reinforcement operant conditioning program was employed to habituate two females to regular transrectal ultrasound exams in a hydraulic restraint device. Urine and fecal samples were collected 5-7 days weekly before, during and after the conditioning program to examine the physiological impacts.

The ACTH challenge demonstrated that the Cortisol EIA was effective at measuring peaks in immunoreactive glucocorticoid metabolites in urine (23-fold increase) and feces (4-fold increase). Translocation among facilities elicited a significant rise in cortisol metabolites ($P < 0.05$) which varied in magnitude and duration between the three females. In contrast, there were no significant differences in cortisol metabolites in urine or feces before, during or after operant conditioning in the two female rhinos. However, considerable variability in the glucocorticoid response to operant conditioning was observed between the two subjects.

Our results suggest that relocation to a new institution represents a significant stressor for this species with variation among individuals, suggesting that management plans should include sufficient acclimation time. In addition, operant conditioning does not appear to elicit a significant or sustained increase in glucocorticoids, suggesting that such programs can be an effective means of training behaviors for husbandry and research without negative physiological implications in Indian rhinoceros.

Evaluating the relationships between age and stress in male Belding's ground squirrels using fecal glucocortiod metabolites

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Glucocorticoids have been widely studied as important mediators of many physiological processes, including responding to stressors, impacting reproduction, and regulating development. However, adrenal activity research on how age affects stress responses is limited in free-living animals. Belding's ground squirrels (*Urocitellus beldingi*) were used to evaluate how adrenal activity changes according to age. *U. beldingi* are social, burrowing rodents living in alpine and subalpine regions of the western United States, where they hibernate for 8 months out of the year. The lifespan of male *U. beldingi* is generally 3-5 years (but up to 12 years); males are sexually active at 2 years of age but typically start breeding at 3 years. Shortly after emerging from hibernacula in April, males fight for access to females to mate, regularly resulting in injuries. Throughout their time above ground, *U. beldingi* encounter aerial and terrestrial predators. Towards the end of their active period, *U. beldingi* must prepare for the long hibernation by gaining enough weight to last the 8 months in torpor. Thus *U. beldingi* experience a range of social and environmental stressors across the active season. We will use longitudinal fecal glucocorticoid metabolite (FGM) data from 8 field seasons to evaluate how FGM change across age classes from juvenile to yearling to adult stages in male *U. beldingi*. Moreover, we are going to evaluate if the FGM change in the different age classes when encountering different stressors. Fecal samples were processed and assayed for cortisol using a radioimmunoassay (RIA). For *U. beldingi*, FGMs have been previously validated, molecularly and physiologically, for cortisol using the Corticote RIA kit from MPBiomedicals. To compare FGMs across multiple field seasons, FGMs will be standardized to z-scores. Male *U. beldingi* FGMs will be compared across different time periods within the field season (mating, post-mating, and pre-hibernation) at each age class. This study will reveal how adrenal activity changes with developmental stages across annual challenges.

Factors affecting tiger adrenal activity in the wild.

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The Siberian tiger lives in the Russian Far East and in the North-East part of China. It is a rare subspecies included in IUCN and Russian Red Lists and less than 500 animals currently exist in the wild. Tiger habitat includes mainly south Taiga forest with a high percentage of oaks and pines. This habitat is generally suboptimal for the tiger because of low prey density (necessitating large tiger home ranges) and severe winter conditions (deep snow and low temperatures). In addition a high level of poaching may further contribute to the substantial anthropogenic stress for this species. The aim of this study was to identify the best way to non-invasively assess adrenal activity in Siberian tigers, to compare adrenal activity among captive and wild tigers, and to further help pinpoint the main factors affecting tigers' welfare in the wild. We collected tiger feces from six captive animals (3 males & 3 females) in the breeding center of the Moscow Zoo from December 2008 until December 2009. The samples from the wild tigers were collected in six different study places in the Russian Far East from 2008-2010. More than 450 samples were analyzed. A physiological and biological validation of a commercially available cortisol assay ("Immunoteck", Moscow, Russia, cross-reactivity of the antibodies was 6% for prednisolone and less than 1% for other hormones) was conducted during an ACTH-challenge test (April 2008) and a transportation test (August 2008) (2 different animals). Both tests resulted in a significant (2-3 folds) increase of immunoreactive cortisol in the feces after the ACTH challenge procedure and transport. The level of adrenal activity measured in captive tigers remained stable throughout the year. In wild tigers the level was significantly higher during the entire year, and the highest levels were measured during the winter (November-January). Some differences in tigers' adrenal activity in various parts of their range will be discussed, as well as implications of the findings for tiger welfare and conservation.

Basic hormonal and behavioral patterns of reproduction in the hylobatids

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Hylobatidae, which includes gibbons and siamangs, are the least studied family of apes. Native to Southeast Asia, hylobatids are the only monogamous ape. Zoo-housed hylobatid populations are relatively small due to a limited number of breeding recommendations per year and variable reproductive success. Current information regarding reproduction in these highly-endangered species is incomplete with most data published from only one genus. Thus, a more thorough understanding of basic hylobatid reproduction is essential for management and breeding success of zoo-housed populations. Our objectives were to: 1) characterize menstrual cyclicity and gestation in female gibbons and siamangs; 2) compare temporally matched reproductive behavior (breeding and allo-groom solicitation) with hormonal metabolite patterns of menstrual cyclicity in females; 3) determine the variability of menstrual cyclicity within and among individuals and species; and 4) assess the influence of season on gonadal hormone patterns in females and males. We used non-invasive fecal hormone metabolite monitoring and a progestin enzyme immunoassay (EIA) to characterize menstrual cyclicity and gestation length, and behavioral data to characterize reproductive behavior of female white-cheeked gibbons (*Nomascus leucogenys*, n = 4), buff-cheeked gibbons (*Nomascus gabriellae*, n = 1), and siamangs (*Symphalangus syndactylus*, n = 2). We also used an estrogen and androgen EIA to determine seasonality in females and males, respectively. Mean menstrual cycle length was 21.5 ± 1.5 days (7 females: 19 cycles) and was similar across individuals and species (range, 13.5 - 25.0 days). Gestation length in the white-cheeked gibbons was 184 ± 14 days (n = 2 females). Reproductive and allo-groom solicitation behavior occurred throughout the menstrual cycle for all species. There was no effect of season on female or male gonadal hormone patterns. This was the first study to use fecal hormone metabolite analysis to characterize menstrual cyclicity and gestation length for several hylobatid species and to associate hormonal patterns with female hylobatid reproductive behavior. These results not only contribute to our general knowledge of the basic biology of hylobatids and highlight the importance of new endocrine-based species-specific information, but they can also be applied to assist management and breeding of zoo-housed populations to support overall conservation efforts.

Non cat-like endocrine pattern in female lynx – methodical miracle or physiological luteal function to ensure monoestrous?

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The genus *Lynx* includes four species: the Eurasian lynx *(Lynx lynx)*, the Canada lynx *(Lynx canadensis*), the Bobcat *(Lynx rufus)* and probably the most endangered felid species in the world listed on CITES Appendix 1, the Iberian lynx *(Lynx pardinus).* All lynx except the Bobcat are known to be strictly seasonal, monestrous breeders, starting their mating period in January. To non-invasively monitor the reproductive activity in captive animals, fecal samples were obtained from Eurasian (n = 4) and Iberian lynx (n = 9) beginning in January. Frequent sample collection was continued throughout pregnancy and lactation or for two months if no parturition occurred. Fecal samples were processed as described before (Dehnhard *et al.*, 2008) and analysed for gestagen, estrogen and prostaglandinF2alpha metabolites.

In female lynx, a typical hormone profile is characterized by an absence of a significant estrogen elevation before mating and a positive correlation between fecal gestagen and estrogen metabolites. Both steroid hormones rise during pregnancy, decrease towards parturition, and increase again during and after lactation. Pseudopregnant profiles do not differ from pregnancies, showing luteal activity throughout several months. Therefore pregnancy diagnosis based on fecal gestagens is ineffective. The Prostaglandin2alpha metabolite (PGFM) profile, however, was pregnancy specific with an increase above baseline after 5 – 6 weeks post-mating, a distinct peak around parturition and a subsequent fall to baseline after delivery.

Postpartum luteal activity was confirmed by ultrasonography (Goeritz et al. 2009) in June/July and late October/November and by elevated serum levels of progesterone with 3.56 ± 1.3 ng/ml in Eurasian and 6.1 ± 0.26 ng/ml in Iberian lynx, respectively. Ultasonographical findings suggest that the same corpora lutea persist throughout the year. Only prior parturition a functional luteolysis induced by PGF2apha was observed.

The existence of active corpora lutea found several months after pregnancy until November is unique for felid species. The physiological role of persistent corpora lutea in lynx reproduction including luteotrophic factors is unknown. We hypothesize that corpora lutea remain active to prevent a new estrous cycle. Elevated progesterone levels throughout the year may convert a normally polyestrous cycle in felid into a strong monoestrous cycle.

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Non-invasive endocrine monitoring: a unique method for studying the reproductive physiology of the critically endangered chinchilla (*Chinchilla lanigera*).

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The chinchilla, a South American hystricomorph rodent, possesses one of the most valuable pelts in the world. Intensive hunting for fur placed the species at the brink of extinction and today it is considered critically endangered by the IUCN and is included in Appendix I of CITES. Although native chinchilla are extremely rare, a hybrid produced by cross-breeding the two chinchilla taxa has been domesticated and selected for superior fur production for more than 80 years. Therefore, knowledge gathered using the domestic stock as a model likely can be applied to its endangered counterparts.

Despite its biological and economic importance, little scientific information is available about this species' basic reproductive physiology, a key aspect for the implementation of assisted reproductive techniques and captive breeding programs. Physiological measures of reproduction have typically relied upon the evaluation of steroid hormones in serum or plasma. However, attempts to obtain repeated blood samples from chinchilla were unsuccessful because of small vein size and their stress-susceptible nature. Non-invasive techniques provided a unique opportunity, allowing long-term endocrine monitoring while avoiding the stress-evoking stimuli of restraint and repeated venipuncture. With this in mind, the objectives of our studies were to demonstrate the validity and accuracy of urinary steroid metabolites quantifications for studying different aspects of the chinchilla reproductive biology. Hormones assessed in 24 h longitudinal urine samples included creatinine (colorimetric assay kit, Wiener Lab, Argentina), progesterone and estradiol metabolites (I^{125} RIA kits, Coat-A-Count, Siemens), LH and FSH (NIDDK-anti rat LH-RIA-S118rabbit AFPC697071P; NIDDK-anti rat FSH-RIA-11 AFP-C0972881, National Hormone & Peptide Program, Harbor-UCLA Medical Center, CA, USA).

We were able to establish, among others, the endocrine profile of female pregnancy (111 d gestation length) and post-partum estrus (48 hs after pups birth), sexual maturity and responses to exogenous activation of the hypothalamic-hypophysis-gonadal axis (hormonal peak 4 d after eCG injection, vaginal opening at day 7). An improved understanding of these aspects will undoubtedly help animal managers to develop more effective captive breeding programs for both domestic and wild chinchillas.

Corticosterone concentrations are higher in nulliparous than parous captive female southern white rhinoceros (*Ceratotherium simum simum*)

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As female southern white rhinoceros (*Ceratotherium simum simum*) reproduce poorly in captivity, this project examined the potential role of stress, as evidenced in fecal corticosterone concentrations, among parous, nulliparous, cyclic, acyclic, and adolescent females. Also examined were relationships between corticosterone levels and social and physical aspects of the captive environment. Dominance relationships, social group size and composition, enclosure size, and other housing characteristics were assessed though behavioral observations and review of historical and institution records. Fecal or serum samples were collected from captive-born parous, n=13; captive-born nulliparous, n=11; wild-caught parous, n=1; wild-caught nulliparous, n=7; and captive-born adolescent, n=6, females. Metabolized and circulating progesterone in fecal and serum samples, respectively, were analyzed by enzyme immunoassay (EIA) to determine luteal cycle activity, pregnancy, and onset of puberty. An ACTH challenge was conducted to test the assumption that elevated corticosterone in serum samples is evidence of an adrenal response in white rhinos. To test the assumption of no diurnal variation in fecal corticosterone metabolite (hereinafter, corticosterone) concentrations, samples were collected 2-3 times per day for 15 days at 2 institutions. Measured by EIA, serum corticosterone concentrations increased >20-fold in 6 hours following intramuscular injection of ACTH, thus demonstrating the relevance of elevated serum corticosterone concentrations to activation of the pituitary-adrenal axis. Also measured by EIA, corticosterone concentrations in fecal samples did not differ between samples collected in the morning and those collected in the afternoon ($p > 0.05$). Average (±SEM) corticosterone concentrations in nulliparous females (688.2 ± 72.9 ng/g) were higher ($p = 0.032$) than those in parous females (480.9 ± 29.8 ng/g). However, nulliparous females were not socially subordinate to parous females ($p > 0.05$), nor did subordinate females have higher corticosterone concentrations than dominant females ($p > 0.05$). Average corticosterone concentrations were not consistently elevated for females housed in any of the environmental conditions assessed ($p > 0.05$). Corticosterone concentrations did not differ between cycling and acyclic females ($p > 0.05$), and some nulliparous females exhibited estrous cyclicity. Future research should explore the possibility that the hypothalamic-pituitary-adrenal axis of nulliparous females is more responsive to stressful stimuli than that of other females.

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