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The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation

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ABSTRACT

Endocrinology is an indispensable tool in threatened species research. The study of endocrinology in threatened species not only advances knowledge of endocrine mechanism but also contributes to conservation efforts of studied species. To this end, endocrinology has been traditionally used to understand reproductive and adrenocortical endocrine axes by quantifying excreted steroid metabolites. From these studies a large body of knowledge was created that contributed to the field of endocrinology, aided conservation efforts, and created a template by which to validate and conduct this research for other species. In this regard noninvasive hormone monitoring has become a favored approach to study the basic endocrinology of wildlife species. Due to the increased understanding of endocrine physiology of threatened species, breeding rates of captive population have improved to levels allowing for reintroduction of species to restored natural ecosystems. Although these approaches are still employed, advances in biochemical, molecular, and genomic technologies are providing inroads to describe lesser known endocrine activity in threatened species. These new avenues of research will allow for growth of the field with greater depth and breadth. However, for all approaches to endocrinology, limitations on resources and access to animals will require innovation of current methodologies to permit broad application for use in threatened species research.

1. Introduction

One in five vertebrate species is threatened with extinction, with threatened categorized as vulnerable, endangered or critically endangered species (IUCN, 2013). Obtaining basic biological information on these species compliments and augments other conservation efforts such as habitat assessment and captive management. Understanding endocrine activity provides unparalleled insight to animal and species biology because hormones affect all tissues in the body. The knowledge gained from species-focused endocrine research can be directly related back to in situ and ex situ programs, but also enhances the science of endocrinology and the collective understanding of animal biology. The growth of the field of comparative endocrinology is dependent on a broadening of understanding of the various mechanisms that compose endocrinology, and with threatened species representing nearly 20% of vertebrate species, studying these species is integral to this area of study. Finally, public awareness of threatened species provides an opportunity to engage a broader audience in the discipline of endocrinology and demonstrate that knowledge acquisition can have direct impact on these populations.

The benefits of a noninvasive approach to endocrinology have been reviewed previously for avian (Goymann, 2005), amphibian (Narayan, 2013), and mammalian (Hodges et al., 2010) species. The purpose of this review is to highlight application of these approaches in research of mammals threatened with extinction, and discuss emerging technologies that may contribute to species conservation and enhance the field of comparative endocrinology. Specifically, we address the rationale for the types of endocrine investigations taken on threatened species, the challenges and limitations of studying these rare species, the standard methods and validation approaches necessary to quantify hormones in threatened mammals, and finally emerging technologies that will advance the field. Throughout the manuscript we attempted to cite material from published studies on threatened species; however, when such source material was lacking we cited material that supports the potential application of the approach to threatened species research.

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2. Basic descriptive research

Although the importance and attraction to studying threatened species is evident, the reality of studying these species is much more complicated than traditional research on model and domestic species, and even non-threatened wildlife. The challenges are numerous, however the commonality stems from the fact the species are threatened and therefore access to animals is limited. However, the limitation expands beyond numbers to achieve robust power in statistical analysis: for example, investigations are further limited in ability to alter housing and management conditions to conduct controlled experiments, exogenously manipulate endocrine function to determine response to or absence of a hormone, and collection of tissue samples for cellular and molecular testing and analysis. Although the approaches described in this section are typical study methodologies, not only for endocrinology but other basic sciences as well, without a firm comprehension of the species' basic endocrine activity, such experimentation may have deleterious results. This is exemplified in wild felids where contraceptives were widely used in the 1990's, but the pervasive use caused reproductive tract pathologies in a number of species (Munson, 2006; Munson et al., 2002), and subsequent use of reproductive contraceptives has since been restricted. Therefore, working within the restrictions of studying threatened species, a wealth of information of endocrine biology via descriptive studies has been generated that not only has affected management of the species, but also added to the body of endocrine knowledge (Hesterman et al., 2008; Howell-Stephens et al., 2012; Jewgenow et al., 2006; North and Harder, 2008; Scarlata et al., 2011; Steinman et al., 2012). This review describes the common, and sometimes unique approaches employed to study threatened species.

3. Endocrine activity to study

Given the great variety of hormone roles among vertebrate, almost all aspects of endocrinology can be investigated in any species with novel findings. Practically, however, this is not a reality and the focus of most threatened species endocrine studies has been on the hypothalamo–pituitary gonadal (HPG) and adrenocortical (HPA) axes. Comprehension of these axes affords managers considerable insight into the areas of management most desirable: reproduction and stress (Pukazhenthi and Wildt, 2004). Furthermore, as whole body biology can be affected by the HPG and HPA axes, investigations of their function yields the greatest return. Thereby, determining the factors that favor reproduction and limit stress is ideal in wild or captive management. Inability to address these issues can have detrimental effects on the sustainability of a population as failure to reproduce or poor reproduction are just as detrimental to the persistence of a species as a viral outbreak.

Although HPG and HPA endocrine studies dominate threatened species research, a few wildlife studies have demonstrated that topics such as of thyroid endocrinology (Keech et al., 2010; Wasser et al., 2010), and effects of environmental toxicants on endocrine function (Guillette and Moore, 2006) have application for threatened species. In addition to these rarely broached subjects, descriptions of endocrine diseases common in other species are nary mentioned in threatened species. The lack of endocrine disease description, however, is not an oversight or that these diseases do not exist in threatened species; but rather the databases of normative hormone values to make such descriptions or diagnoses possible do not exist. To remedy these deficits in knowledge, efforts need to be made to establish species-specific baseline values for these hormones (e.g. insulin; thyroid hormones) so that proper diagnostic and treatment protocols can be developed.

Finally, rarely are endocrine studies of threatened species solely focused on hormones. Rather, endocrine data should be paired with other disciplines and measures, including environment, behavior, husbandry, nutrition, ecology, and veterinary medicine. These interdisciplinary efforts, sometimes under the umbrella of field endocrinology (Walker et al., 2005) or conservation physiology (Wikelski and Cooke, 2006), provide needed context for appreciating hormone events (e.g. estrual behaviors with increased estrogen measures), and relevance for managerial and conservation considerations (e.g. increased glucocorticoid [GC] concentrations in response to logging activities).

4. Study populations

4.1. Captive populations

With more than 1200 mammalian species threatened with extinction (IUCN, 2013), there is not a shortage of species to study; however, accessing a population to study is very limited. The degradation, fragmentation and overall loss of habitat limits observations of animals in native habitats and thereby opportunities to conduct endocrine studies in situ are few. Alternatively, captive environments offer investigators the intimacy needed to collect routine endocrine data on animals with known life histories. Additionally, major factors such as disease, predation and food search are regulated to provide a safe and healthy environment. Yet despite limiting risks in the captive environment, the removal of animals from native environments will produce different biological activity than free-ranging counterparts, such as environmental conditions and experiencing novel events. To encourage naturalistic behaviors concerted efforts have been made in zoos and other captive settings to provide animals with environments that mimic natural conditions and/or provide positive stimuli (Hoy et al., 2010; Kleiman et al., 2010). To this end, standards of diet, husbandry, care, and management are drafted for species to standardize optimal conditions to maintain a given species in captivity, with special attention paid to threatened species (Dorsey, 2013). Although care is taken to provide standardized conditions for a species, the dispersion of a species' captive population among various holding institutions prevents true uniformity in care and management, and therefore careful record taking of these conditions must be considered when collecting endocrine data on animals maintained across varying facilities.

Species persistence is absolutely dependent on reproduction. The biological link between reproduction and endocrinology is intrinsic, with most major reproductive activities, both behavioral and physiological, being driven by endocrine changes. Therefore, tracking reproductive endocrine activity emerged as the primary endocrine tool in threatened species research. Because reproduction is a component of many threatened species plans (Bowkett, 2009; Conde et al., 2013; Wildt et al., 2010), generating information about basic aspects of reproductive cycles is a necessary part of the conservation effort. Characterizing basic reproductive endocrine trends has led to the creation of several self-sustaining captive populations, the giant panda (*Ailuropoda melanoleuca*) being an example of such success. For much of the captive history of this species, very little was known about reproductive endocrinology and captive breeding was unsuccessful (Zhang et al., 2006). A thawing of political tensions permitted for an expansion of research in the late 1990's and early 2000's, including reproductive endocrine studies (Czekala et al., 1998, 2003; Kersey et al., 2010a,b; Lindburg et al., 2001; Steinman et al., 2006). The expansion of knowledge enabled the creation of artificial insemination and timed breeding protocols (Howard et al., 2006, 2008) that have facilitated the growth of the captive population to sustainable

levels, with a portion of the population now available for reintroductions (Zhang, 2013).

Although the giant panda and other charismatic mega fauna serve as examples of how the creation of basic endocrine knowledge can be used to adapt assisted reproductive technologies to aid in conservation efforts (Pukazhenthi et al., 2006; Swanson, 2006; Wildt et al., 2010), outside of these few species foundational endocrine and reproductive knowledge is nonexistent or limited. This is largely due to species-specificity in endocrine biology, and the need to establish basic comprehension for each species. The conceptual framework exists to develop species-specific information (paired behavioral observations and endocrine evaluations on as many individuals possible); yet, this approach requires substantial effort on the part of the investigator and care staff, as well as monetary resources for dutiful execution.

4.2. Wild populations

Ex situ studies offer a means to intimately study the basics of an animal's biology, however observations of an animal in the wild provides opportunities to enhance our understanding of endocrine mechanisms observed under managed situations. Among threatened species, most notable success with in situ endocrine studies has been achieved in the chimpanzee (*Pan troglodytes*) (Thompson, 2013). The studies of wild chimpanzees added to the understanding of the species' biology with relation to general reproductive characteristics of females (Thompson, 2005), behavioral biology (Sobolewski et al., 2012, 2013), and adrenocortical activity (Muller and Wrangham, 2004). Yet, the success achieved with the chimpanzee was built upon previous knowledge that included known life histories of study populations (Mitani et al., 2002) and a solid foundation of the species' reproductive physiology (Zimmermann and Radespiel, 2007). Application of endocrine studies to other threatened species in situ will similarly require base knowledge of study populations and species-specific endocrinology.

In addition to providing valuable insight about the natural biology of a species, in situ studies also offer the opportunity to observe an animal's response to environmental changes. Documenting responses to environmental changes is of particular interest to threatened species as the decline of most species is due to loss of suitable habitat (IUCN, 2013) caused by human-generated disturbances (Foley et al., 2005). HPA activity, as measured by GC, has been posited as the means to assess animal stress response to human activities in the wild (Sheriff et al., 2011). Positive associations between human activities and GC have been documented in a number of wild mammals, including elk (*Cervis elaphus*) and wolf (*Canis lupus*) (Creel et al., 2002), mountain hares (*Lepus timidus*) (Rehnus et al., 2014), and European pine marten (*Martes martes*) (Barja et al., 2007); however, results are not always consistent. For example, in red howler monkeys (*Alouatta seniculus*) increased human activities were not associated with increased GC, but in the sympatric and critically endangered brown spider monkey (*Ateles hybridus*) GC increased in association with human presence (Rimbach et al., 2013). This species-specific response to human activities is further illustrated in the orangutan (*Pongo pygmaeus morio*) (Muehlenbein et al., 2012), forest elephant (*Loxodonta cyclotis*) (Munshi-South et al., 2008), and savannah elephant (*Loxodonta africana*) (Ahlering et al., 2013) whereby increased human activities were not associated with increased GC, despite the impact these activities had on the immediate environment. Rather, it is likely that some species are more likely to acclimate or habituate to human activities and therefore increased GC may not be observed. Therefore, the species and type of human activity must be considered when determining the appropriateness of GC to assess the animal's response to the activities.

5. Sampling

To study the biology of an animal observations and measures must be taken without disrupting that biology, for which noninvasive sampling emerged as the ideal means to assess without perturbation. However, done improperly, noninvasive sampling may perturb the animal (e.g. shaving hair from a nontractable animal), and conversely invasive sampling may not perturb a well-trained animal that accepts blood draws as part of routine care and management. Therefore, sampling is not so much about invasiveness, or lack thereof, but more about non-perturbation. Below we discuss the benefits and drawbacks for the common sampling techniques to assess endocrine activity.

5.1. Blood

Blood is the predominant sample substrate to assess endocrine activity in the field of endocrinology. Hormones utilize the vascular network for transport, therefore blood is the ideal substrate to track endocrine changes in an animal. This method of sample collection is the common approach to assess hormone values in humans, and domestic and model species, and was the approach taken during initial studies on threatened species (Lasley, 1980); however, taking blood has fallen out of favor with threatened species investigators. Although blood draw by definition is invasive, blood samples can be obtained from tractable species without perturbation, as is exemplified in the Asian (*Elephas maximus*) and savannah elephants (Brown, 2000), obtaining blood samples from nontractable species is impractical. Drawing blood from wild animals (in situ or ex situ) often requires chemical or physical restraint, all events that can affect biology and consequently endocrine observations. In addition to handling and restraint imposed on animals, repeated samples are necessary to track endocrine activity. Aside from the dangers imposed by repeated restraint, repeated venipuncture can damage vascular beds, particularly on small animals. Vascular damage can be overcome via venous cannulation, but this too imposes limitations as the animals must be constrained or confined to prevent loss of the cannula (Monfort et al., 1993). Finally, costs associated with the equipment and trained personnel necessary for blood collection all but make this approach obsolete for threatened mammal species research . Rather, the advent of noninvasive sampling for hormone quantification (see Section 5.2.) proved an ideal alternative for monitoring endocrine activity threatened species.

5.2. Urine and feces

The metabolic pathway of hormones involves inactivation and/or excretion. Metabolites of lipophilic molecules (e.g. steroids, prostaglandins, thyroid hormones) are frequently hepatically conjugated to water soluble moieties (e.g. sulfate, glucuronide) for excretion in the feces or urine (Hodges et al., 2010; Palme et al., 2005). Additionally, peptide hormones can be filtered through the renal glomerulus and excreted with the urine (Hodges et al., 2010). Furthermore, because the rate of excretion is proportional to the amount of hormone in circulation, endocrine values derived from the excreta are a reflection of endocrine changes in the body, with the added advantage of hormones pooling in the gut and bladder producing more of an average than a point in time value. Finally, because these products are routinely voided by the animal, collection of urine and fecal samples requires no, or minimal, contact with the animal, obviating the need for veterinary or trained personnel to collect samples (as is the case with blood) and permitting for routine sampling over long periods of time.

Among the excreted hormones, steroids are the most resistant to degradation and abundant for quantification. The adaption of fecal and urinary hormone assessment to threatened species endocrine studies (Lasley and Kirkpatrick, 1991) opened up a new opportunity to research biological mechanisms that otherwise would have gone undiscovered (Schwarzenberger and Brown, 2013). As a result, the reproductive endocrinology of numerous species were characterized from the excreted metabolites of circulating steroids (Brown, 2006; Lasley and Kirkpatrick, 1991; Schwarzenberger, 2007). In addition to characterizing the HPG axis with reproductive steroids (e.g. estrogens, progestagens, androgens), the HPA axis, via GC metabolites (GCM), also has been extensively investigated (Palme et al., 2005; Touma and Palme, 2005; Sheriff et al., 2011). Although this technology is beyond its second decade of use, the approach of utilizing excreted hormones to advance knowledge of threatened species endocrine function still has tremendous value and potential. Therefore, what was once considered a daily 'waste product' now has become a valuable 'research resource', a virtual pool of biological information on reproductive cyclicity, the time of ovulation, pregnancy and impending parturition, and adrenocortical (stress) status.

5.3. Saliva

Analysis of hormones in the saliva has been posited as an additional noninvasive sampling method for assessing endocrine activity in threatened species. Unlike hormones found in the excreta, steroids found in the saliva tend to be in the parent form, and even some peptide hormones (e.g. insulin) are transported unaltered into salivary glands (Gröschl, 2008). With the presence of unaltered steroids and whole peptide hormones, saliva is suitable sample material for tracking endocrine activity. Yet, the methodology has some drawbacks, particularly in terms of sample collection. Samples may be obtained from either drool collection or swabbing the interior of the mouth with a collection pad or scoop. The dangers of such intimate contact with a wild species limits the application of this approach, and salivary hormone analysis has only been reported in two threatened species; the black (*Diceros bicornis*) and Indian (*Rhinocerosceros unicornis*) rhinoceros (Czekala and Callison, 1996; Gomez et al., 2004).

5.4. Hair

Hair has been recently recognized as a biomaterial that may accumulate steroids over weeks to months. Estradiol, progesterone and testosterone levels measured in healthy human adult hair positively correlated with the levels measured in their serum (Yang et al., 1998). Steroids are incorporated into the hair shaft during the growing phase of the hair follicle, and therefore steroid values in hair are believed to reflect average HPG and HPA activity over the respective growth phase. Yet, cortisol levels in hair may not exclusively reflect adrenocortical synthesis and secretion, as local synthesis in the hair follicle can be a significant source of cortisol measured in the hair (Keckeis et al., 2012). Despite these limitations, a recent study evaluated the usefulness of hair as an indicator of chronic stress in the Canada lynx (*Lynx canadensis*) (Terwissen et al., 2013). The authors demonstrated a qualitative increase in hair cortisol concentration following a five week duration adrenocorticotrophic hormone (ACTH) challenge. However, a radiometabolism study performed by Keckeis and colleagues (2012) in guinea pigs showed that only negligible amounts of systemic cortisol were incorporated into hair four days after the final radiolabel injection. Additionally, hair sampling requires a clipping/reclipping protocol, for which animals must be handled to obtain the samples (Sheriff et al., 2011), a process that may perturb unwilling animals. Therefore, the analysis of GC in hair is a potentially useful technique to evaluate chronic (long-term) exposure to stress, or potentially stressful conditions, but is likely insensitive to acute adrenocrotical stimulation and may not accurately reflect HPA activity.

6. Assay methodology

6.1. Antibody production

To establish an immunoassay, an antibody is required via immunization of an animal, usually rabbits or sheep, against a foreign antigen. Because steroids are too small to act as an immunogen, they must be linked to a protein to elicit an immune response. Commercial development of immunoassays are generally to support the largest demand, which in endocrinology is the measurement of parent hormones in the blood. However, for the analysis of fecal steroid metabolite levels, the use of the widely available immunoassays that use highly specific anti-steroid antibodies should be avoided as parent hormones are usually not present in the fecal samples. Furthermore, to develop antibodies against the main steroid metabolites voided in the excreta would be too time-consuming and costly because fecal steroid metabolites vary substantially between species, thereby it would require establishing a new assay for each species.

Therefore, the ideal antibody for measuring steroid metabolites should be group-specific antibodies that recognize an array of structurally similar steroids (Möstl et al., 2005). However, the production of group-specific antibodies necessary to quantify this array of steroids in excreta is currently limited and unlikely to increase. Additionally, most research institutions, zoological facilities and wildlife agencies do not have the mandate to maintain rabbits or sheep necessary to raise the antibodies. Therefore, researchers use commercial or custom-made antibodies for the original hormone or similar compounds, hoping that these antibodies cross-react with one or several of the hormone metabolites present in the excreta. Nevertheless, the adaption of enzyme immunoassay (EIA) systems for metabolite quantification permitted many non-traditional research institutes to perform routine hormone analyses, and even conduct endocrine-based research studies. Unfortunately, the availability of commercial EIA kits for hormones metabolites is still limited, largely due to the research demand of radioimmunoassays (RIA), which still hold many advantages (e.g. ease of repeatability, precision, less expensive kit prices), despite the associated cost for maintain a radioactive license, purchasing radioactive measuring equipment , and waste handling fees.

6.2. Chemiluminescence immunoassay (CLIA)

The principle of an immunoassay involves the competition between the antigen, which is the analyte (e.g. steroid) to be measured, and the labeled form of the same antigen tagged with radioisotopes (RIA), enzymes (EIA), or luminophores (CLIA) for binding sites on the antibody. Chemiluminescent immunoassays (CLIA) are more sensitive than the conventional colorimetric methods (e.g. EIA), and do not require long incubations or the addition of stopping reagents, as is the case in some colorimetric assays. Among various enzyme assays that employ light-emitting reactions, one of the most successful assays is the enhanced chemiluminescent immunoassay involving a horseradish peroxidase (HRP) labeled antigen and a mixture of chemiluminescent substrate (luminol derivatives), hydrogen peroxide, and enhancers (Dodeigne et al., 2000). Lanthanides are an emerging alternative to luminols in CLIA systems and can be coupled to ligands to provide strong luminescent signals that are detectable using time-resolved fluorescence (TRF) methods. This approach takes

advantage of the long fluorescence lifetime of the lanthanide and can detect less than one attomole of europium in a multi-well plate (Hagan and Zuchner, 2011).

A variety of immunoassay analyzers exist in the market designed to measure specific analytes through their properties as antigens or antibodies. Modern analyzers can run an assay in multiple steps with various reagents being added and washed away so that multiple results can be obtained from one sample. The IMMULITE® assays from Siemens Healthcare uses polystyrene beads coated with anti-hormone antibody. The sample and a known amount of hormone conjugated to alkaline phosphate are simultaneously introduced into the reaction tube containing the bead. After an incubation step, the test units undergo a centrifugal wash step, whereby residual sample and unbound analytes are removed. Then chemiluminescent substrate is added and undergoes hydrolysis in the presence of alkaline phosphate yielding the sustained emission of light, which is measured by the Immulite instrument. The limitation of the Immulite assays in application for wildlife and threatened species is that they are designed to measure parent steroid hormones, not the metabolites voided in the excreta. Although not ideal for tracking changes of the major metabolites of parent hormones, this system has been reported with some degree of success in the Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx (Jewgenow et al., 2006) and the Florida manatee (*Trichechus manatus latirostris*) (Tripp et al., 2011). Further validation of this approach for use in wildlife may be beneficial for zoos and research institutions, not only because of the increased sensitivity, but the analyzers are capable of measuring multiple analytes with great sensitivity and are widely available. Yet, to increase application in threatened species research current CLIA systems will have to be adapted for use with group-specific antibodies.

6.3. Radiometabolism studies

Because the analysis of hormone activity in threatened species requires a non-traditional approach to endocrinology, extensive validation procedures are necessary to ensure the hormones being analyzed are a true reflection of biological changes in the animal. Almost all steroids undergo some form of alteration prior to excretion. The best way to investigate the metabolism and excretion pattern of steroid hormones are studies using radiolabeled ($^{14}C/^{3}H$) hormones.

Radiometabolism studies are extremely useful, because they provide necessary information about steroid secretion and excretion (Palme et al., 1996; Touma et al., 2003). These studies provide evidence of the excretion route of a steroid (whether a species excretes hormones preferentially into the urine or feces), which can differ considerably among species (Graham and Brown, 1996; Palme et al., 1996), and also the excretory route of different steroids within the same species (Palme et al., 1996). Above all, one of the major beneficial outcomes of a radiometabolism study is the determination of the antibody cross-reactivity with the excreted hormone metabolites (Goymann et al., 1999). Common among these radiometabolism studies were pronounced species, and sometimes even sex differences, concerning formed metabolites. Also notable, was that only trace amounts, if at all, of the parent hormone were measured in the fecal extracts.

Radiometabolism studies are performed by injecting the animal with a radiolabelled form of the parent hormone and waste products are collected. Samples are analyzed via liquid chromatography separation with radioactivity detection to isolate the voided metabolites (Liu and Jia, 2007). Labeling of the hormone is with either ^{14}C or ^{3}H; however selection of one isotope over the other varies. After injection of ^{3}H or ^{14}C labelled hormone, the excreted metabolites of a particular steroid can be separated by high pressure liquid chromatography (HPLC) and quantified by EIA (Möstl et al., 2002). Due to gender-specific differences regarding the structure as well as the quantity of fecal GCM (fGCM) (e.g. in the laboratory mouse [Touma et al., 2003]) it is recommended to perform a radiometabolism study in at least one male and one female per species. Although the amount of radioactivity used to determine the metabolic pathway of a steroid is not harmful to the animal, obtaining the necessary permits and institutional agreements to conduct such studies on threatened species are very limited.

6.3.1. Assay specificity

The cross-reactivities of an antibody employed in an RIA or EIA indicate the degree to which steroids may interact and bind to the antibody. However, lists of assay cross-reactivities do not reveal which substances are really measured because other metabolites, not evaluated for their reactivity, may be present in the excreta. Therefore, a recommended procedure for assessing the presence of cross-reacting substances is the analysis of samples derived from radiometabolism studies. This requires an HPLC separation of the metabolites, and the individual fractions of the eluent collected. An aliquot of each fraction is used for measuring radioactivity, and another aliquot for detecting the immunoreactivity with the respective assay(s). Co-elution of the immunoreactive substances with radioactive peaks indicate that the assay can detect metabolites of the parent steroid whereas radioactive peaks, occurring without accompanying immunoreactivity, demonstrate that those metabolites are not measured by the assay system (Palme, 2005; Touma and Palme, 2005). Conversely, immunoreactive peaks without co-eluting radioactivity are an indicator for cross-reacting substances not derived from the hormone injected. Depending on the relative quantities in an immunogram, metabolites that, for example, are not specific for testosterone or structurally similar adrenocortical androgens will be wrongly assigned as testosterone metabolites falsifying the measurements.

Fecal steroid hormone metabolite immunoassays rely on the ability of the employed antibodies to detect the relevant species-specific metabolic products. Metabolism of adrenocortical hormones, such as cortisol, and gonadal hormones, such as testosterone, may lead to fecal metabolites with similar structures. For instance 11-oxo-etiocholanolone (3α-hydroxy-5β-androstane-11,17-dione) and etiocholanolone (3α-hydroxy-5β-androstane-17-one) are representing typical metabolites of cortisol and testosterone, respectively. Due to the shared androstane structure of the majority of androgen and several glucocorticoid metabolites, which differ only in the functional group at C_{11}, antibodies generated against C_{19} cortisol metabolites are likely to substantially cross-react with androgen metabolites. Such a pitfall was demonstrated when only one of four glucocorticoid antibodies did not cross react with androgen metabolites excreted in the feces of savannah elephants following an ACTH challenge (Ganswindt et al., 2003). Thus, immunoassays designed to assess adrenocortical GC may cross-react with other steroid metabolites, distorting the results. This knowledge is particularly important when using GC are a component in stress assessment studies, whereby the employed assay would suggest an increase in GC that may not be reflective of HPA activation and lead to false conclusions about stressors. Thereby, although approval may be difficult to obtain to conduct radiometabolism studies for threatened species, when paired with HPLC immunograms; this procedure will reduce false positive results by limiting the potential interference of non-biologically relevant cross-reactive metabolites.

6.4. Biological validation

A biological validation of an assay must be conducted to demonstrate that the assay measurement of fecal steroid metabolites is reflective of changes of steroid concentrations in the blood

(Wielebnowski and Watters, 2007). This can be done by comparing blood vs. excreted hormone levels, testing for a predicted difference/change in hormone concentrations (e.g. in males by comparing fecal testosterone metabolite levels in relation to reproductive condition and sex; in females by evaluating estrogens in the period around estrus), or assessing a cause-and-effect relationship (increase in fGCM as response to stressful events). In case of fGCM, a widely used method is an ACTH challenge or stimulation test (Palme, 2005). ACTH is the pituitary peptide hormone that regulates GC release from the adrenal cortex. The GC assay should detect a delayed increase in immunoreactive metabolites in excreta following the ACTH injection. If the predicted peaks in hormone excretion are detected, the products measured in the feces by the GC assay likely reflect biologically meaningful adrenocortical activity. Alternatively, a biological validation can be performed based on serial samples collected before and after some known stressful events such as a routine veterinary exam or transportation. In further conducting these biological validation procedures, individual validation must be considered. For example, in long-tailed macaques (*Macaca fascicularis*) ACTH stimulation yielded predictable high to moderate increases in fGCM within 32 h of infusion, whereas almost an equal number showed little to no fGCM increases with the same ACTH dosage in the same time frame (Wasser et al., 2000). This example demonstrates that a validation must include multiple individuals from a species, and perhaps even multiple individuals per sex of a species, to ensure proper matching of assessment method and species-specific biological processes. Although CRH/ACTH challenges are safe and routinely used in human (Matsukura et al., 2012) and veterinary medicine (Listings and Home, 2013), there is hesitation to perform them in wildlife and threatened species. These tests are meant to mimic stress and cause no longer term health side effects, with the benefit of providing valuable information about the stress profile of an animal that can then be used to assess truly stressful conditions in the species, providing information for better informed managerial decisions.

6.5. Chemical identification of metabolites

From a practical perspective, it is not always necessary to know the chemical identity of the fecal metabolites in each species. Particularly for initial characterization of hormone trends, it is more important to demonstrate that an antibody can track fluctuations in metabolites that provide biologically relevant information (see Section 6.4.). However, even if biological validation succeeded, the chemical identity of the fecal metabolite(s) detected by the antibody is unknown in most cases.

6.5.1. Gas chromatography–mass spectrometry (GCMS)

To identify steroid metabolites mass spectrometry currently provides the highest specificity in steroid analyses. For example, noninvasive urinary GCMS steroid profiling techniques allows for the diagnosis of almost any adrenal enzyme defect in steroid biosynthesis (Wudy and Hartmann, 2004), and the detection androgen-based performance enhancing drugs by athletes (Parr and Schanzer, 2010). Before analysis, analytes of interest must be extracted from the matrix into a liquid solvent phase following derivatization, as GCMS requires volatile substances. The analytes are carried through a narrow separation column by the carrier gas and separated from each other by virtue of their competitive distribution between the two phases, the moving gas stream and the stationary liquid phase on the inside wall of the column. In cases where measuring conjugated forms of metabolites are desired, further steps, such as hydrolyses procedures, are required. Although hydrolysis and extraction procedures are time consuming, the ability to analyze a multitude of substances with high sensitivity highlights the advantage of GCMS. Yet despite these advantages, GCMS is not common in noninvasive steroid monitoring and has only been used on occasion in threatened species. For example, from the fecal extracts from the black rhinoceros 33 different pregnanes were identified (Lance et al., 2001). The most abundant progestagen, 5α-pregnan-3β,20α-diol, contributed to 44.5% of the total amount, did not possess any significant cross-reactivity towards the progesterone antiserum; whereas 5α-pregnan-3β-ol-20-one (10.7% of total amount) showed 97% cross-reactivity towards the antibody. This implies that an antibody against 5α-pregnan-3β,20α-diol would be the more appropriate antibody to analyze fecal progestagen metabolites in this species. Therefore, if an EIA designed to analyze fecal steroid metabolites failed to produce reliable results, GCMS-based identifications might help to choose an appropriate antibody that cross-reacts with one of the major metabolites or identify the candidate steroid for generating an antibody against.

6.5.2. Liquid chromatography mass spectrometry (LCMS)

LCMS combines the separation technique of HPLC with those of a coupled mass spectrometry to yield similar chemical analysis results as GCMS without the laborious derivatization. For use in endocrinology, LCMS is particularly powerful in separating and quantifying steroids, and steroid conjugates and derivatives from a single sample (Krone et al., 2010; Shackleton, 2010), a capacity attractive to noninvasive assessment of endocrine activity (Murtagh et al., 2013). In a recent study, simultaneous LCMS measurement identified an array of HPG and HPA steroids and related metabolites from the feces of white-faced capuchins (*Cebus capucinus*) (Weltring et al., 2012). Utilizing LCMS for identifying urinary metabolites is equally powerful, and is now the preferred means for the detection of illegal use of performance enhancing drugs in human athletes (Danaceau et al., 2008). In research and clinical endocrinology LCMS is rapidly supplanting immunoassays because of the capacity of LCMS to quantitate steroids from a single analysis (Stanczyk and Clarke, 2010). For example, the analysis of 200 μl of urine collected from primates produced the quantitative measurement of 23 steroid hormones and their metabolites (Hauser et al., 2008). This advantage not only reduces the quantity of sample needed to yield an array of steroid values, but also obviates the need to generate a specific antibody to measure a single steroid (Stanczyk and Clarke, 2010). Despite the advantages offered by LCMS, challenges exist that limit broad application in endocrinology (Möstl et al., 2002; Weltring et al., 2012). Yet, the greatest limitation of LCMS in application to threatened species research is the feasibility of costs involved in purchasing, maintaining, and operating the equipment (Murtagh et al., 2013). Although application of LCMS has been limited in studying endocrine mechanisms of threatened species, the approach warrants further exploration not only to enhance resolution of trends in hormone production, but also changes in steroid metabolism during different biological states and steroid-related diseases (Krone et al., 2010).

7. Novel noninvasive analytes

7.1. Thyroid hormones

Noninvasive measurement of thyroid hormone in feces of avian and mammalian species creates an enormous potential for understanding environmental physiology at individual and population levels. Thyroid hormones are involved in regulating growth and metabolism, and measuring these hormones in the feces has recently been described (Wasser et al., 2010) opening opportunities for noninvasive investigations of the hypothalamo–pituitary thyroid axis in animals. Validation of this approach was further

demonstrated by tracking changes in fecal triiodothyronine and thyroxine concentrations in response to injection of thyrotropin (thyroid-stimulating hormone) in Stellar sea lions (*Eumetopiajsu batus*) (Keech et al., 2010). Based on triiodothyronine measures it was determined that inadequate prey (salmon) availability jeopardizes the threatened population of southern resident killer whales (*Orcinus orca*) in the inland waters of British Columbia, Canada (Ayres et al., 2012). Results from this study suggested that a recovery of strategic salmon populations would promote the recovery of the inland water killer whale populations off British Columbia. Although a new approach in noninvasive endocrinology, fecal thyroid hormone analysis offers promising insight into general metabolic processes that affect health and well-being in wildlife species. However, more extensive validation procedures, including scrutiny of excretory metabolic pathways, the metabolites present in the excreta, and the stability of the hormones post-excretion are warranted before broad application across species.

7.2. PGFM

Prostaglandins are small, lipid soluble hormones produced for local control of tissue function (e.g. smooth muscle activity), and are highly involved in reproductive physiology in some species (De Rensis et al., 2012). Given the small size and tight paracrine regulation of production, in often species-specific manners, quantifying these hormones to determine their biological importance in wildlife has been difficult. In the Iberian and Eurasian lynx, it is impossible to use fecal and urinary estrogen and progestagen metabolite analyses as a reliable means for differentiation between pregnancy and pseudo-pregnancy. Both hormones rise during pregnancy, decrease periparturition, and increase again during lactation. As an alternative, the placental hormone PGF2α was considered as a specific pregnancy-related signal. Prostaglandin F2α is rapidly metabolized to PGFM (13,14-dihydro-15-keto-PGF2α) and is established as a useful analytical marker of PGF2α in many species. In these lynx species the use of both urine and fecal samples favors PGFM as a reliable indicator of pregnancy if determined in samples collected 45 days post-mating. The use of PGFM as a gestational biomarker was further proven in 18 other species of felids (Dehnhard et al., 2012), and a semi-quantitative PGFM-based fast assay has been developed for pregnancy diagnosis of the critically endangered Iberian lynx.

7.3. Oxytocin

The neurohypophyseal hormone oxytocin has been implicated in many aspects of reproduction including sexual behavior. In contrast to other peptide hormones highly involved in reproduction (e.g. LH; FSH) oxytocin is relatively small (9 amino acids) and can pass into the renal filtrate with minimal metabolic alteration (Seltzer and Ziegler, 2007). Noninvasive measures of oxytocin levels in urine of wild chimpanzees revealed that oxytocin levels were higher after grooming with bond partners compared with non-bond partners or after no grooming (Crockford et al., 2013). Thus a new tool is available that could measure changes in urinary oxytocin levels in relation to changes in specific social events in social mammals in their natural environment. However, because the hormone is a neurohypophyseal in origin, systemic, and thus urinary, concentrations are very low, thereby assay sensitivity is crucial to obtain measures. Furthermore, diligence in sample handling is required, as alterations in pH, temperature, and time exposed to room temperature can alter the protein structure and affect ability of immunoassay antibodies to reliably bind the hormone (Thomas et al., 2010).

7.4. Relaxin

In urine several peptide hormones such as LH, hCG and relaxin have been detected that can be related to sexual activity or pregnancy status. de Haas van Dorsser and colleagues (2006) showed that relaxin could be quantified from the urine of pregnant domestic cats and leopards (*Panthera pardus*) and serve as a pregnancy biomarker. In domestic cats, the placenta is the major source of relaxin and an increase in urinary relaxin at the beginning of the second trimester (days 20–25) is detectable via a bench top kit (Witness® Relaxin) for pregnancy diagnosis (de Haas van Dorsser et al., 2007). However, this approach requires the concentration of urine by ultrafiltration prior to assaying samples, thereby limiting the practicality of this approach in research application. Furthermore, the reliability of relaxin as an absolute indicator of gestational state is questionable as among wild felids tested to date, relaxin is was only certain for pregnancy detection in the Iberian lynx (Braun et al., 2009), the Arabian leopard (*Panthera pardus nimr*), and the Pallas'cat (*Otocolobus manul*) (Steinetz et al., 2009), but not the lion (*Panthera leo*), cheetah (*Acinonyx jubat*us), or sand cat (*Felis margarita*) (Steinetz et al., 2009). Therefore, although relaxin may provide a valuable diagnostic tool for pregnancy status, its broad application across an array of species may be limited.

7.5. Ceruloplasmin

The study of reproduction in bears is obscured by two atypical reproductive strategies: delayed implantation and pseudopregnancy demonstrated in six and four of the eight species of Ursidae, respectively (Spady et al., 2007). Both phenomena apply to two species of high conservation concern the polar bear (*Ursus maritimus*) and the giant panda. The combination of delayed implantation and pseudopregnancy have complicated efforts to diagnose pregnancy in these species as all tests utilizing steroid hormones and their metabolites have proven ineffective for distinguishing between pregnancy and pseudopregnancy (Curry et al., 2012; Kersey et al., 2010b). However, as traditional biomarkers of pregnancy described in other mammals are largely based on gestational proteins of humans and domestic animals, it may be that gestational proteins in bears are different, particularly upon excretion. For example, applying two-dimensional in-gel electrophoresis and mass spectrometry five unique proteins were identified to be significantly more abundant during pregnancy in the polar bear (Curry et al., 2012). This study demonstrated that fecal biomarkers other than steroid metabolites may be useful for monitoring reproduction in wildlife species and provide a strong motivation to develop a noninvasive pregnancy assay for use in bears.

Recently, the evaluation of a specific urinary acute phase protein (ceruloplasmin [CP]) has shown potential for pregnancy detection in giant pandas. However, this requires facilities designed to collect urine samples which are feasible for captive giant pandas but not for captive polar bears and wild bears, where the acquisition of urine is impossible. CP measurements indicate that in term pregnancies, levels of urinary CP were elevated the first week of pregnancy and remain elevated until 20–24 days prior to parturition, while no increase was observed during the luteal phase in known pseudopregnancies (Willis et al., 2011). Although the biological rationale behind the CP is still unenlightened, in recent years CP has been applied for pregnancy diagnosis in giant panda breeding.

8. Molecular and cellular approaches

While much of the research on endocrine function in domestic and model species has advanced to cellular and molecular tech-

niques, such research has been slow to come for threatened species. This limitation is not imposed by capabilities of investigators, but more so from limited access to hands-on studies, potential undesirable effects of manipulating an animal's physiological status, hesitation to allow invasive (e.g. tissue) sample collection and a lack of research infrastructure at zoos and other facilities interested in threatened species research. Despite these restrictions, tissue samples and biopsies can be obtained opportunistically during routine veterinary and post-mortem exams. For example, tissues harvested from culled wild Eurasian lynx allowed for characterization of annual changes in corpora lutea quality and hormone (estrogens, progesterone) values (Carnaby et al., 2012). In a rare example of molecular approaches applied to a threatened mammal, Tubbs and colleagues (2012) characterized and cloned estrogen receptors for Indian rhinoceros, as well as the non-threatened white rhinoceros (*Ceratotherium simum simum*), by using harvested tissues from deceased animals. Using recombinant techniques, they were able to produce cells from insects with the rhinoceros species' estrogen receptor to determine effects of different estrogen and estrogen-like products ability to bind to the receptors, factors that could affect normative endocrine function. Although the research on the rhinoceros does not address the cellular outcomes of the ligand–receptor interaction in the rhinoceros species, it is one of the few examples where now common molecular techniques were successfully applied to address endocrine function in threatened species.

9. Genomic approaches

Obtaining genetic material from feces was first explored more than 15 years ago (Kohn and Wayne, 1997), but these techniques have yet to be merged with endocrinology in threatened species research. The emergence of high-throughput applications, such as expression arrays and next generation sequencing methods (NGS) offers new opportunities to investigate the genetic mechanisms facilitating adaptation in natural populations (Ekblom and Galindo, 2011). One major advantage of NGS in ecological studies is the small amounts of genetic material needed for analysis, making these technologies suitable for analyses of threatened species where tissue access is limited. By shifting the realms of genomics from laboratory-based studies of model-species towards studies of natural populations of ecologically well-characterized organisms, researchers can now start to address important conservation-based ecological and evolutionary questions. Modern molecular methods, such as NGS, are facilitating research to explain the genetic basis of adaptations at the levels of DNA and RNA (Shendure and Ji, 2008), changes that would be reflected in individual and species level differences of peptide hormones, steroidogenic enzymes, and hormone receptor sequences. Ultimately, comparative species analyses of these sequences will enhance the description of the processes that influence the persistence of extant species, emergence of new species, and determine their geographic distribution (local adaptation). Understanding the adaptive changes of wildlife under natural selection forces is thus not only a key question in evolutionary genetics (Gilad et al., 2009), but it is also essential for scientifically based biodiversity conservation. To ensure long-term survival of wildlife species it is necessary to preserve genetic diversity through the identification and protection of differentiated populations, the assessment of variation within local populations, and through a better understanding of reproductive and dispersal behavior. The application of molecular genetic techniques is helping to provide answers to some of these previously intractable questions.

10. Chemosignal detection

Animals secrete chemosignals (pheromones) outside of the body where they are used for communication among individuals. Chemosignals can code for species, subspecies, sexual identity, age and reproductive status, as well as motivational state. Animals also use learned olfactory cues to recognize group membership, kinship and individuality. Detecting, identifying, and analyzing these chemosignals would allow their application for biostimulation to support reproductive management as pheromones from males could hasten sexual maturity, reduce post-partum anestrus, and induce ovulation and mating (Dehnhard, 2011). The analysis of estrus-related signals from females also could be used as excellent analytical indicators to supplement estrus detection. This has been recently shown in the water buffalo (*Bubalus bubalis*), where two estrus related compounds, 4-methylphenol and trans-verbenol were identified in feces as reliable indicators of estrus (Karthikeyan et al., 2013). The detection of estrus related substances in species where reproductive management requires estrus detection (e.g. the seasonally monestrus female giant panda) would greatly facilitate breeding management.

However, chemosignal detection does not necessarily require sophisticated analytical tools. Dogs can be trained for various odor detection tasks, and have been used to search for explosives, drugs or cancer biomarkers. Trained dogs have been shown to detect estrous in dairy cows based on urine (Kiddy et al., 1978) and milk samples (Hawk et al., 1984). More recently, scent-detection dogs were trained to detect polar bear pregnancies with an accuracy of 97%, when provided fecal samples from pregnant and pseudo-pregnant polar bears (Barber, 2013).

11. Conclusions

Threatened species represent large portion of animals and not studying them would be counterproductive for advancing the field of endocrinology. The role of endocrine research on these species is twofold: (1) Create new knowledge of unique, species-specific endocrine mechanisms; and (2) Apply that new knowledge towards more effective management and conservation of the species. Historically, endocrine research on threatened species has been devoted to descriptions of reproductive cycles of captive populations via noninvasive methodologies. The combination of this approach with other disciplines, such a behavior, nutrition, and management has benefited the management of many charismatic megafauna. Although the approach of characterizing HPG and HPA axes by analyzing voided steroid metabolites via immunoassays is approaching its fourth decade of use, it is not antiquated and is still necessary to provide a foundation of endocrine activity in yet to be studied, or understudied species. However, the limited supply and production of immunoassays designed to recognize group-specific steroid metabolites, rather than parent steroids, will require the adaption of emerging technologies (e.g. CLIA) to track endocrine changes from noninvasively collected samples, or refinement of analytical techniques (e.g. LCMS) to increase feasibility of repeated measures and reduce associated costs.

Advances in endocrine technologies are allowing investigators to broaden and deepen their understanding of endocrine function in threatened species. Emerging descriptions of non-steroid hormones, steroid receptors, and details of steroid metabolic pathways demonstrate the applicability of previously underutilized approaches for addressing knowledge deficits. Although these methods have value in threatened species research, limited resources and animal access may not allow investigators in the field to be at the forefront of these new technologies. However, the area of threatened species endocrinology grew by adapting

then current technologies to these non-traditional model species; similarly, for this field to continue to grow it must adapt current biotechnological, genomic, and molecular and cellular methodologies despite the obvious limitations.

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