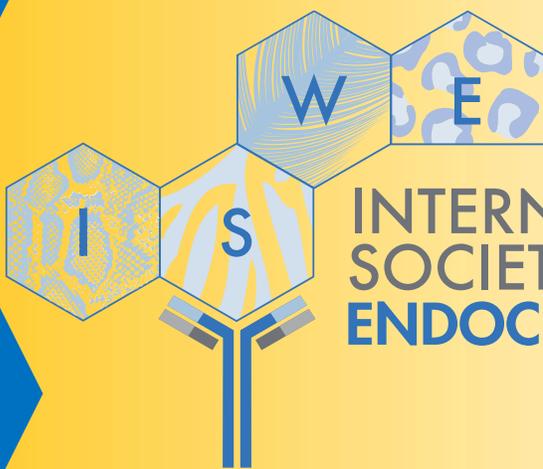


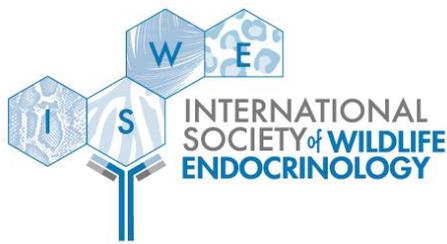


Proceedings of the 1st ISWE Virtual Event

August 16th – 17th, 2021



INTERNATIONAL
SOCIETY of WILDLIFE
ENDOCRINOLOGY



Welcome to the 1st Virtual Event of the International Society of Wildlife Endocrinology

Dear members, colleagues, and friends, a warm welcome to the first ever virtual event of ISWE. The current global challenges demand a re-imagining of social interaction and communication, and the pursuit of adaptive responses to enhance collaboration and relevance in a rapidly transforming society. We have to be innovative and proactive, and embrace new possibilities, to act as a role model and agent of local and global change, for a more sustainable world. Due to the continuous uncertainty brought about by the ongoing pandemic, we postponed our 8th ISWE Conference as you all know, but in order to fuel our enthusiasm for interaction and discussion, we decided to have a virtual event this year, which I'm sure will be an excellent opportunity to showcase new findings and developments in the field of wildlife endocrinology.

This event includes a keynote given by Dr Michael Romero of Tufts University entitled 'Glucocorticoids in Ecology and Conservation: Promises and Pitfalls'. We will have panel discussions also featuring brief talks by top Indian scientists to provide a glimpse of what to expect at our next ISWE Conference in Jim Corbett National Park, India. Further, we will have focused Q&A sessions and guided breakout rooms with selected mentors to emphasize and support our trainees. During this online event, and for two weeks after, you will have the opportunity to watch 63 pre-recorded presentations, uploaded for international viewing convenience. At your leisure, you will be able to hear about projects investigating animal reproduction, health, and welfare, as well as innovative techniques and long-term studies of wildlife endocrinology. In addition, two presenting student members have the chance to win a waiver for free-registration and first place will also receive additional funds to travel to our upcoming 8th ISWE Conference in India to present their work in a featured oral presentation. In this regard, we would like to use the opportunity to thank our long-time supporter and conference sponsor Arbor Assays for putting forward US\$2,500. Arbor Assays collaborates closely with ISWE scientists and provides essential support for ISWE through the production and sale of ISWE mini-kits, which are specifically designed for wildlife conservation research.

Like for most of us, 2021 continues to provide a bumpy road for ISWE, with some challenges that required and will require innovative solutions and ad-hoc responses. However, the first seven months of this year were also quite productive and you will hear more about that from the board members during our update session at the beginning of the event. But I would like to take the opportunity to thank all the board members for their hard and productive work and for answering all my emails and requests sent during odd hours. A very special thanks goes to our Conference Chair, Diana Koester, who has conceptualized, developed, and refined this event over the course of last year.

Finally, we thank you all for participating in this event. We currently have more than 120 colleagues from 17 countries registered, and I'm excited to see how our vibrant community will utilize the new platform to welcome new members, support students, and engage in stimulating discussions.

We wish you all a productive and enjoyable time.

Andre Ganswindt

ISWE Chair

ACKNOWLEDGMENTS

We express our sincere thanks to the following colleagues for reviewing abstracts or judging travel awards (**indicates individuals that did both*):

Cayman Adams	Elizabeth Freeman*	Anneke Moresco
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Katie Edwards*	Gabriela Mastromonaco	Karen Steinman*
Kelly Ethun	Lara Metrione	Govindhaswamy Umapathy
Kerry Fanson*	Samrat Mondol	Jella Wauters*
Ashley Franklin	Nei Moreira	Sue Walker*

Our Conference Planning Subcommittee

Diana Koester – Conference Chair
 Katie Edwards – proceedings, website, travel award
 Annie Newell-Fugate – registration, finances
 Grace Fuller – website, media updates
 Ratna Ghosal – sponsor relationships
 Lara Metrione – sponsor relationships
 Jella Wauters – trainee/mentor event planning
 Emmy Hirsch – graphic design
 Cayman Adams – graphic design

Our Student Affairs Committee

**indicates session moderators*

Allison (Allie) Case
 Breanne Murray*
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 Andrea Webster
 Kate Dutton-Regester
 Emily Bach*
 Vinod Kumar*
 Michal Zatrak

We warmly thank our invited speakers for contributing keynote sessions:

PLENARY SPEAKER

Dr. Michael Romero – Department of Biology, Tufts University, Massachusetts.
 GLUCOCORTICOIDS IN ECOLOGY AND CONSERVATION: PROMISES AND PITFALLS.

PANEL SPEAKERS

Dr. Katie Edwards – Science Department, Chester Zoo, UK
Dr. Kerry Fanson – Wildlife Endocrinology Lab, La Trobe University, Australia
Dr. Ratna Ghosal – Biological and life Sciences Division, Ahmedabad University, India
Professor Vinod Kumar – Department of Zoology, University of Delhi, India
Dr. Samrat Mondol – Department of Animal Ecology and Conservation Biology, Wildlife Institute of India, India
Dr. Nei Moreira – Biosciences Department, Federal University of Paraná, Brazil
Professor R. Sukumar – Ecology, Indian Institute of Science, India
Dr. Stacey Tecot – School of Anthropology, University of Arizona, USA
Dr. G. Umapathy – Laboratory for the Conservation of Endangered Species, CSIR-CCMB, India
Dr. Catharine Wheaton – Animals, Science and Environment, Disney's Animal Kingdom, USA



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PLENARY SPEAKER: PROFESSOR L. MICHAEL ROMERO



L. Michael Romero, Professor of Biology at Tufts University, has studied stress for over 30 years. He earned his PhD from Stanford with Robert Sapolsky and did a postdoc with John Wingfield at the University of Washington. The unifying theme to his research is the concept of stress. He has been trying to answer three main questions: what causes stress in a wild animal; what physiological, endocrinological, and behavioral mechanisms are turned on in response to those stressors; and how do those mechanisms help wild animals live in their natural environments. Professor Romero has studied with numerous avian, reptilian, mammalian, and amphibian species. His research takes an integrative approach, utilizing neuroendocrinology, endocrinology, and ecology in both the lab and the field, all with the goal of increasing our comprehension of the causes and effects of stress in wild animals. He has also recently summarized the work in this field in a book he co-wrote with John Wingfield entitled: “Tempests, Poxes, Predators, and People: Stress in Wild Animals and How They Cope.”

Indian Wildlife Research Panel

Session Description: Five of our Indian colleagues using endocrinology will share a bit about their work and research interests to give attendees an idea of what to expect to hear more about at our next, in-person ISWE conference to take place in India.



Raman Sukumar is honorary professor of ecology at the Indian Institute of Science, Bangalore. He is internationally known for his pioneering research on the ecology, behaviour and conservation of Asian elephants. His work has covered topics as varied as reproductive biology, molecular genetics, evolutionary history, population dynamics, landscape ecology, movement ecology and the cultural history of elephants. His research interests also extend to tropical forest ecology and climate change. Sukumar is a Fellow of the three major science academies in India, and the World Academy of Sciences. He has also contributed for three decades to the work of the Intergovernmental Panel on Climate Change (IPCC) that shared the Nobel Peace Prize in 2007.

Professor Vinod Kumar is a leading Avian Physiologist of India. He uses a blend of behaviour-physiology-neural-molecular approaches to understand the adaptive strategies underlying daily and seasonal processes in resident and migratory songbirds.



Dr. G. Umopathy is Senior Principal Scientist and Group leader at Laboratory for the Conservation of Endangered Species, CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India. He uses non-invasive hormone methods to study the reproductive function and stress physiology of endangered animals both in captive and free-ranging. He works on a wide range of animals and habitats, which include forest turtles in Western Ghats to Musk deer in high altitude Himalayas.

Dr. Samrat Mondol is an Associate Professor in the Department of Animal Ecology and Conservation Biology at Wildlife Institute of India, Dehradun, India. His research group combines a range of molecular and endocrinology techniques to predict the direction and degree of demographic change in free-ranging populations and understand the impacts of anthropogenic disturbances on their health and reproductive functions. His research group currently focuses on research on various animal (tiger, leopard, dhole, sloth bear, swamp deer, elephant, Indian one-horned rhinoceros, Brow-antlered deer etc.) and bird species across India and Antarctica.



Dr. Ratna Ghosal is Assistant Professor at the Biological and Life Sciences Division, School of Arts and Sciences, Ahmedabad University. Her lab group works on understanding proximate/physiological mechanisms underlying animal behaviour, and how such findings can be applied towards conservation of target species. Currently, she is also serving as the Vice-chair of Membership and Fundraising for the ISWE board.

Measuring Glucocorticoids Discussion Panel

Session Description: Experts from a diverse array of backgrounds will each spend a few minutes remarking on the topic of measuring glucocorticoids and associated difficulties from their own perspective. The panel will then open to discussion and questions from attendees, led by a moderator.



Dr. Nei Moreira is a Full Professor of Veterinary Physiology at the Biosciences Department and advisor in the Post-Graduation Program in Zoology at the Federal University of Paraná. Honorary member of the Brazilian Association of Veterinary Endocrinology (ABEV) and member of the National Commission of Wild Animals (CNAS) of the Federal Council of Veterinary Medicine (CFMV). His research group works in the area of reproduction, well-being and conservation of endangered species, with emphasis on reproductive physiology and biotechnology.

Dr. Stacey Tecot is an Associate Professor and Director of the Laboratory for the Evolutionary Endocrinology of Primates in the School of Anthropology at the University of Arizona, USA. She conducts field research in Madagascar and co-directs the Ranomafana Red-Bellied Research Project. She uses non-invasive methods to collect samples in the field to measure biomarkers related to cooperation, reproduction, and health, ultimately to help conserve species threatened by habitat disturbance and climate change.



Dr. Katie Edwards is Lead Conservation Scientist at Chester Zoo, UK, where she oversees the Wildlife Endocrinology Lab and develops and applies techniques to measure hormones and other biomarkers to understand reproduction, health and well-being of wildlife. Her work focuses both on assisting *ex situ* captive breeding programmes, and applying techniques to benefit *in situ* conservation.

Dr. Cat Wheaton is a Research Manager with Disney's Animals, Science and Environment where she develops and coordinates endocrine technology and monitoring for reproductive health and well-being in a variety of taxa residing at Disney's Animal Kingdom®, EPCOT's The Seas with Nemo and Friends, and *in situ* wildlife conservation programs.



Dr. Kerry Fanson is a Research Fellow and Head of the Wildlife Endocrinology Lab at La Trobe University, Australia. She works with a wide range of species to understand the complex relationship between glucocorticoids and reproduction.

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Non-invasive stress monitoring and temperament of tropical screech-owl (*Megascops choliba*)

Heriberto Barbosa-Moyano¹, Claudio Oliveira¹

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Abstract:

Animals constantly respond to stimuli from their environment. When these stimuli are perceived as threatening, it is possible to identify behavioral and endocrine responses that can differ at the intra and interspecific level. In order to identify differences in temperament and their possible relationship with the production of glucocorticoid metabolites (GCM) in nocturnal raptors, we examined 14 owls of the species *Megascops choliba* (males = 8, females = 6, in non-reproductive season) during three challenge tests involving novelty. Furthermore, uro-fecal material was collected during four days with an interval of 4 hours. GCM levels were assessed by enzyme immunoassay (EIA) with corticosterone serum (CJM006; C. Munro). Before measuring the GCM concentrations for each sample extract, the EIA assay was tested for parallelism ($F = 0.19$, $df = 1$, $p = 0.724$), accuracy ($95.15 \pm 2.9\%$), and intra (2.3%) and inter-assay (7.2%) precision. The behavioral traits are under study and will be evaluated by 3 judges who will identify the intensity of descriptor adjectives of temperament (active, anxious, calm, curious, among others) expressed by individuals. Preliminary results presented here show that higher levels of GCM were displayed in the night period (9:00 p.m.: 763.44 ± 287.96 ng / mL) compared to the daytime ones (minimum values at 9:00 a.m.: 402.62 ± 198.98 ng / mL; $p < 0.05$; GLM). Additionally, individual sex-independent differences were recorded in the production of GCM ($p < 0.05$; GLM). It is expected that the dynamics of daily GCM production and the temperament traits described here will help conservation programs of nocturnal raptors in order to identify potential stress behaviors.

Seasonal differences in DNA damage across tissues in house sparrows (*Passer domesticus*)

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Abstract:

Measures of glucocorticoids are included in nearly every study on the vertebrate stress response, however interpreting their levels can be challenging. It is becoming clear that measuring a suite of metrics is necessary in stress physiology. Assessing DNA damage can be a useful downstream metric. In nucleated avian red blood cells, DNA damage increases with captivity stress and acute restraint stress. We sought to understand how DNA damage varies across tissues and across seasons in house sparrows (*Passer domesticus*). In July 2019 and January 2020, we caught birds in Eastern Massachusetts and immediately took blood samples and harvested the liver, hypothalamus, hippocampus, abdominal fat, and testes (only for males in the summer). Tissues were then assayed using the Comet Assay (single-cell gel electrophoresis), stained, fluorescently imaged, and analyzed. Across tissues, abdominal fat tended to have the highest level of damage, while preliminary data show that testes were much lower. Across seasons and in both sexes, all tissues had higher damage in the summer compared to the winter. Preliminary experiments suggest that there is not a difference between juveniles and adults in the summer. The cause of these seasonal differences is not known, but may be related to other seasonally-varying metrics of the stress response, such as corticosterone and glucose.

Influence of visitor presence on behavior, fecal glucocorticoid metabolites, and oxidative stress in southern white rhinoceroses (*Ceratotherium simum*)

Emily Bovee¹, Jennifer Hamilton¹, Grace Fuller¹

¹ Detroit Zoological Society, Detroit, USA

Presenting author: Emily Bovee

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Abstract:

Research shows that human visitors can be an enriching, neutral, or potentially negative influence on the welfare of zoo animals, depending on species and individual. In March 2020, the Detroit Zoo was closed to visitors due to the COVID-19 pandemic, providing a unique opportunity to examine how visitors affect the two resident southern white rhinoceroses (*Ceratotherium simum*). We explored this question using behavioral observations and monitoring fecal glucocorticoid metabolites (FGM) and a measure of oxidative stress. Fecal samples were collected daily for four weeks while the Zoo was closed and four weeks following reopening. We tested FGMs using an in-house corticosterone enzyme-immunoassay (CMJ006, C. Munro) validated via parallelism (linear regression, $R^2=0.985$, $F_{1,5}=256.679$, $p<0.001$) and spiked recoveries averaging 129%. We tested for oxidative stress (OHdG) using an enzyme-immunoassay (K059, Arbor Assays) validated in fecal extracts using parallelism ($R^2=0.88$, $F_{1,5}=30.060$, $p=0.005$) and spiked recoveries averaging 105%. The OHdG assay targets 8-hydroxy-2'-deoxyguanosine, a by-product of DNA damage. Jasiri had higher log(FGM) concentrations when the Zoo was open than closed ($r=0.40$, $p=0.02$), as well as higher concentrations on days with more visitors ($r=0.59$, $p=0.003$). Jasiri also had higher log(OHdG) concentrations on days when he spent more time alert ($r=0.43$, $p=0.04$). However, log(OHdG) and log(FGM) were not correlated for Jasiri. Tamba had higher log(FGM) concentrations on days when he had higher rates of startling ($r=0.50$, $p=0.02$) and horn manipulations ($r=0.50$, $p=0.02$). Initially, Tamba did not show a significant correlation between log(FGM) and log(OHdG). However, removing apparent outliers revealed a significant negative relationship ($r=-0.39$, $p=0.02$). These outliers may represent physiological results or assay interference, and more validation is needed to establish fecal OHdG as a reliable measure of DNA damage. These results suggest that visitor presence is associated with adrenal activity for both rhinos but highlight the need to view welfare on the individual level.

The power of poo: using faecal glucocorticoid metabolite analysis to assess the impact of habitat condition on stress physiology in free-roaming waterbucks (*Kobus ellipsiprymnus*)

Jenna Greetje Boy¹, Chadi Touma¹, André Ganswindt²

¹ Department of Behavioural Biology, University of Osnabrück, Osnabrück, Germany

² Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

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Abstract:

The impact of anthropogenic factors increases since human population constantly grows. As with many wild animals, the population of free-roaming waterbucks is declining and yet, little is known about the impact of environmental stressors on this ruminant. In order to provide insight into these factors, a non-invasive monitoring technique for faecal glucocorticoid metabolites (fGCM) in waterbucks was established and biologically validated in three zoo-housed animals. Moreover, a degradation experiment was performed, revealing that samples should be collected within 30 min after defecation in order to avoid distortion of immunoreactive fGCM concentrations. Applying this non-invasive monitoring technique in a field study, two different research sites in South Africa, differing in the amount of anthropogenic pressure, were investigated and compared to gather information about the impact of habitat condition on stress physiology in free-roaming waterbucks. In total, 40 samples of male and female waterbucks from both reserves were collected and analysed using the established immunoassay. The results regarding the concentration of immunoreactive fGCM revealed no significant differences between the sexes and also between animals of both reserves, indicating that the mentioned anthropogenic factors did not significantly influence glucocorticoid output of the waterbucks. A possible explanation might be the availability of dense cover in both reserves to avoid human contact. Environmental factors such as water quality also seem to have little effect on the immunoreactive fGCM concentration, although further studies on this aspect are needed.

Glucocorticoid profiles vary with establishment history in invasive lizards

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Abstract:

Invasive species present informative case studies of the responses of animals to novel conditions and environments. Additionally, invasive species are a threat to biodiversity, human health, and economic interests, prompting a need for better understanding of invasion processes to develop effective methods for prevention. Characterizing the physiological responses of invasive animals during and after invasion may facilitate improved procedures for screening potential invaders. Glucocorticoid hormones are known to mitigate stress during translocation, prolonged restraint, and resource restriction, all of which may occur during the introduction pathway of a non-native species. Altered glucocorticoid secretion is implicated in successful avian, amphibian, and mammal invasions, but we lack information from invasive reptiles. We investigated glucocorticoid responses relating to establishment time in two species of non-native lizard established in Florida. In 2018 and 2019, we collected blood at baseline and 1 h post-handling to determine plasma corticosterone levels from 11 disjunct populations of *Leiocephalus carinatus* with estimated establishment dates from 1940 to 2018 and from 5 distinct populations of *Agama picticauda* with estimated establishment dates from 1990 to 2017. Corticosterone responses to 1 hour of handling stress were dampened in two recently introduced populations of *Leiocephalus carinatus*, but we found no pattern in *Agama picticauda*. Dampened stress responses may play an important role in the initial establishment of some invasive populations. The invasion pathway leading to establishment may also influence glucocorticoid responses. This study was approved by University of Florida IACUC.

Into the red: the effect of climate on the well-being of zoo-housed red pandas

Monika Conrad^{1,2}, Katie Edwards^{2,3}, Erika Bauer⁴, Elizabeth Freeman⁵

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Abstract:

The charismatic red panda (*Ailurus fulgens*) is commonly housed in zoos. Although they breed easily, population growth is hampered by high cub mortality and a variety of health issues. The captive population is vital for the survival of red pandas which are endangered in the wild. The aim of this study was to understand how environmental factors, such as climate and zoo visitors, impact red panda physiology to guide management and optimize welfare. Fecal samples were collected from 10 red pandas housed at four institutions, the Smithsonian's National Zoological Park (NZN) and Conservation Biology Institute (SCBI), Seneca Park Zoo, and Mesker Park Zoo & Botanic Garden, from May 2017 through June 2019. Samples were dried and fecal glucocorticoid metabolites (fGM) extracted using 80% methanol and a modified shaking technique. Concentrations of fGM were quantified using a previously validated corticosterone enzyme immunoassay (CJM006). Environmental variables, including temperature and humidity, from the day before fecal collection were taken from weather stations near each zoological facility. Visitor numbers, when available, were obtained from zoological records. Generalized linear mixed models were performed and the multi-variable model for predicting fGM concentrations consisted of month and visitor number. fGM concentrations were influenced by month, with most red pandas having significantly lower concentrations between May and August. For every 1000 fewer zoo visitors at NZN, a 2.1% increase in glucocorticoids occurred. These analyses confirm that red pandas demonstrate monthly variation in fGM concentrations, with lower concentrations in summer months, interestingly when most zoo visitations are highest. Higher fGM concentrations coincide with breeding season and may be more reflective of internal physiological changes. Understanding which environmental factors generate a stress response is important for improving reproductive success and captive management of red pandas.

An analysis of weight loss and stress physiology of California valley quail during a simulated translocation

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Abstract:

Avian translocations often result in weight loss and physiological stress, likely contributing to the low survival rate of translocated birds. However, both factors are often overlooked when translocating quail. Since quail translocations are becoming increasingly popular due to rapidly declining populations in the U.S., there is a growing need for a non-invasive assessment of stress. The objectives of this study were to (1) determine the magnitude of weight loss and corticosterone concentrations of California valley quail (*Callipepla californica*) in response to a simulated 48-h translocation, (2) biologically validate the use of fecal corticosterone metabolites (FCM) as a non-invasive measurement of stress in California valley quail, and (3) evaluate the effectiveness of dietary supplements vitamin C and E to minimize weight loss and stress during translocations. To obtain reference corticosterone concentrations, valley quail (N=13) were housed in an outdoor aviary for 3 wks. Valley quail (N=120) were weighed and sorted into four treatment groups (vitamin C, vitamin E, vitamin C + E, and control), and individually housed in a laboratory settling to simulate a 48-h translocation. Fecal samples were collected every 4 h and FCM concentrations were determined using an enzyme immunoassay. Mean weight loss for all groups was 17.68 ± 6.55 g. Dietary supplements did not reduce weight loss during the simulated translocation. Mean reference FCM concentrations were 24.6 ± 7.3 ng/g, which was lower than FCM concentrations during the simulated translocation (41.50 ± 16.13 ng/g). FCM concentrations varied diurnally during the simulated translocation with higher concentrations from 10:00–17:00 and lower concentrations from 20:00–05:00. These results verify the stressful nature of quail translocations and validate using FCM concentrations as a non-invasive method to assess stress hormone levels in California quail. Further investigation into minimizing weight loss and stress during translocation could make translocation projects more successful.

The dark side of energy-efficient lighting: investigating the impacts of LED lighting on melatonin and glucocorticoid expression in a nocturnal marsupial

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Abstract:

Artificial light at night (ALAN) is one of the most common and fastest growing forms of urban pollution and has been identified as a key threat to biodiversity. ALAN fundamentally changes the night-time environment by masking natural light cues and desynchronising the internal clock. Energy-efficient lighting, such as white light emitting diodes (LEDs) may have an even greater impact on wildlife. This is because white LEDs consist primarily of short blue wavelengths, and these wavelengths play a greater role in regulating circadian rhythm. Therefore, while there are obvious environmental benefits to white LEDs, there may also be serious ecological and physiological consequences for wildlife. However, one advantage of LED light sources compared to traditional lighting is the flexibility to change the colour and wavelengths of light. We investigated the effect of short-wavelength white LED lighting (standard urban lighting) and long-wavelength amber LED lighting (proposed wildlife friendly lighting) on melatonin and glucocorticoid expression in the Krefft's glider (*Petaurus notatus*). The amber lights were designed to exclude short blue wavelengths, and therefore are predicted to have little effect on circadian rhythm. Wild-caught *P.notatus* were randomly allocated to one of three treatments (n = 7 per treatment): 1) control (no lighting), 2) white LED lighting, or 3) amber LED lighting. Urinary melatonin and faecal glucocorticoid metabolites were monitored at regular intervals throughout the experiment to examine changes in hormone expression over 4-weeks of exposure to dim light at night. Preliminary results indicate that melatonin is suppressed by white LEDs, which is similar to results we have observed in other marsupial species. My presentation will offer a full comparison of melatonin and glucocorticoids across light treatments. Findings from this study will improve our understanding of physiological impacts of artificial light at night on wildlife and support the development of "wildlife-friendly" lighting.

The influence of social dominance and reproductive state on fecal dehydroepiandrosterone-sulfate (DHEAS) in Japanese macaques (*Macaca fuscata*)

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Abstract:

The use of the adrenal hormone dehydroepiandrosterone-sulfate (DHEAS) has been recently used as an indicator of overall stress due to its role in restoring homeostasis after increased glucocorticoid (GC) secretion. In a normal stress response, GC and DHEAS levels tend to be in balance. However, when in prolonged stress or in high energetic demands, DHEAS levels will be low (high GC/DHEAS ratio), thus negatively impacting the immune system. This difference in the response of DHEAS has led to the use of the GC/DHEAS ratio as an indicator of stress severity. The purpose of this research was to evaluate the effect of reproductive state, and social factors influencing DHEAS levels and the GC/DHEAS ratio in Japanese macaques (*Macaca fuscata*). We conducted 275.5h of focal observation in 12 free-ranging female Japanese macaques (8 pregnant/lactating, 4 non-pregnant/non-lactating) from Jigokudani Monkey Park (Nagano, Japan) during the birth season and mating season. Fecal GC and fecal DHEAS levels were validated and measured in 386 samples by enzyme immunoassay. Hormonal levels were compared with reproductive state, aggression rates, grooming time, and dominance rank using Generalized Linear Mixed-Effect Models. Our results revealed that pregnant and lactating females had higher DHEAS levels than cycling females, and that DHEAS levels were higher during the mating season. Ambient temperature was positively correlated with the GC/DHEAS ratio. There were no effects of social behavior in either DHEAS or the GC/DHEAS ratio. Dominance rank was positively associated with DHEAS levels and negatively correlated with the GC/DHEAS ratio, indicating that DHEAS levels increased with higher rank, and that high-ranking females maintained an overall lower GC/DHEAS ratio than low-ranking females. These findings indicate that the GC/DHEAS ratio is less susceptible to the effect of reproductive condition and may be used to evaluate potential long-term effects of social behavior on stress levels in Japanese macaques.

Evaluating stress-related hormones of Alaska caribou overwintering in an area with industrial development

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Abstract:

Seasonal avoidance of infrastructure has been well documented in caribou (*Rangifer tarandus granti*). Current oil and gas development in the Alaskan Arctic is expanding into an area of high use by the Teshekpuk caribou herd. This herd is an important source of terrestrial sustenance for several Arctic communities. Declines in free-ranging wildlife health and reproductive success have been associated with chronic stress and non-invasive monitoring of stress-related hormones can be accomplished by collecting fecal samples. In this study, we measured concentrations of fecal cortisol (f_C , acute stress) and fecal glucocorticoid metabolites (f_{GCM} , chronic stress) using enzyme linked immunosorbent assays (ELISAs). Samples were collected in early spring, prior to snow melt, near the northern coast of Alaska in a high-use caribou wintering area prior to development (2019) and during limited pre-construction activity (2020). Fecal pellet size, an index of caribou body size, did not significantly correlate with the f_C ($P=0.786$) or f_{GCM} ($P=0.379$) concentrations. This is likely because large pellets were absent, as most adult males overwinter south of the study area, and fecal pellets were predominantly from adult females and yearlings. Preliminary results indicate average concentrations of both acute and chronic stress-related hormones were significantly higher in 2020 ($f_C = 44.2 \pm 1.2$ pg/mg, $f_{GCM} = 84.1 \pm 1.4$ pg/mg) compared to 2019 ($f_C = 34.2 \pm 1.1$ pg/mg, $f_{GCM} = 64.9 \pm 1.7$ pg/mg), $P < 0.001$ for both comparisons. This suggests that caribou overwintering near the northern coast of Alaska were experiencing higher levels of acute and chronic stress in 2020 compared to 2019. However, industrial activity in 2020 was limited and other factors that can influence fecal stress-related hormones should also be considered, such as annual differences in environmental conditions, disease, animal density, and food accessibility. Therefore, longer-term, and broader investigations are required to clarify the influence of industrial development on caribou stress physiology.

**Validation of a fecal glucocorticoid enzyme-immunoassay for the short-beaked echidna
(*Tachyglossus aculeatus*)**

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Abstract:

This study aimed to validate a glucocorticoid enzyme-immunoassay (EIA) for the evaluation of fecal glucocorticoid metabolites (FGM) in the short-beaked echidna (*Tachyglossus aculeatus*). An adrenocorticotrophic hormone (ACTH) challenge was conducted in sexually mature captive echidnas (n = 3 males; n = 3 females). Blood samples were collected immediately before, 30 and 60 minutes after the ACTH injection and fecal samples were collected daily for 15 days starting 5 days before the challenge. Plasma samples were extracted with diethyl ether and dried fecal samples were weighed (0.2g) and extracted with 5mL of 80% MeOH; both sample types were dried under air and reconstituted in assay buffer prior to analysis. Plasma corticosterone and cortisol concentrations (CJM006 and R4866 antibody EIAs respectively; C.J. Munro, UC Davis, USA) increased significantly from 0.85 ± 0.28 ng/mL to 5.53 ± 1.66 ng/mL and from 0.25 ± 0.05 ng/mL to 0.53 ± 0.06 ng/ml 60 minutes post-ACTH injection, respectively. Serial dilutions of pooled faecal extracts failed to display adequate parallel displacement with two different cortisol EIAs (R4866, C.J. Munro, UC Davis, USA; Arbor Assays[®] cortisol ISWE002, USA). Adequate parallelism was achieved with two corticosterone EIAs (CJM006, C.J. Munro, UC Davis, USA; Arbor Assays[®] corticosterone ISWE007, USA) and a 11-Oxoetiocholanolone EIA (72a, University of Vet Medicine Vienna, Austria). The highest signal to noise ratio in four of six echidnas was recorded using the Arbor Assays[®] corticosterone EIA. The ACTH challenge stimulated an increase in FGM concentrations in four out of six echidnas. Two echidnas had increases in FGM concentrations at the beginning of the sampling period, likely a response to the translocation required to singly house these two animals for the study. These results confirm the applicability of these techniques as a tool for future studies to evaluate echidna adrenal physiology and factors influencing a stress response.

Hair hormones of newborn kittens as a valid tool to estimate their physiological status

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Abstract:

Hair cortisol became a reliable tool to estimate the stress of mammals. The other steroid hormones (progesterone, testosterone, etc.) may be measured in hair as well. This non/low invasive tool may be used for small young mammals where blood-sampling is difficult. We tried to estimate the validity of this method for different felids. The aim of this study was to estimate the level of three steroid hormones (cortisol, testosterone and progesterone) in hairs of newborn kittens. This study was conducted at the Tchernogolovka station near Moscow. Hair samples were collected of the hindquarters by trimmer cutting. The extraction was conducted with 100% methanol (100:1). After centrifugation the supernatant was evaporated at 50C, reconstituted with PBS (1:5 v/v) and stored at -18C till measurements. We measured steroid hormones concentrations by ELISA. The level of all hormones was significantly higher in newborn kittens (including progesterone) than in pregnant females. This can likely be explained by the exposure of embryos to placental hormones. It is possible that this phenomenon is common for many mammalian species. Sex and litter size did not affect hairs' cortisol/progesterone level of newborn kittens. However, kittens born in litters with multiple paternity had higher cortisol levels than in litters sired by one male. The neutrophils/lymphocytes ratio supported the hypothesis that embryos in litters with multiple paternity were exposed to higher stress than in litters sired by a single male. Cortisol levels in newborn domestic kittens were significantly lower than in wild felids (Amur wildcat and lynx). It coincides with the data on serum cortisol concentration in these species. This study was supported by the Russian Science Foundation (18-14-00200).

A zoo-based post-occupancy evaluation of snow leopards (*Panthera uncia*) combining physiological and behavioral indicators of welfare

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Abstract:

Long-term post-occupancy evaluations (POEs) in zoos are uncommon, and even less common are those that monitor physiological indicators of welfare. Our study had two objectives: 1) to validate the use of an EIA to monitor fecal glucocorticoid metabolite (FGM) concentrations in zoo-housed snow leopards (*Panthera uncia*), and 2) monitor FGM concentrations, behavior, and space use of 1.1 snow leopards over 20 months before and after transfer to a more complex habitat at Cleveland Metroparks Zoo (CMZ). For Objective 1, we tested for precision, specificity (by parallelism test), accuracy, and a biological validation event (transport of 2.1 snow leopards from CMZ to new institutions). Serial dilutions of fecal extract yielded a displacement curve parallel to the standard curve (corticosterone: $y = 0.9096x + 5.119$, $R^2 = 0.993$, $F_{1,5} = 711.237$, $p < 0.001$), and there was no evidence of matrix interference on our EIA (corticosterone: $y = 0.566x + 14.241$, $R^2 = 0.965$, $F_{1,6} = 167.317$, $p < 0.001$). During the biological validation event, all three individuals displayed an initial increase over pre-transport FGM concentrations 1-4 days post-transport, reached a peak (2-8-fold) increase 5-7 days post-transport, and exhibited a sustained increase for at least three weeks post-transport. For Objective 2, we examined if FGM concentrations differed among the pre-transfer (P1), novel summer (S), and post-transfer (P2) phases. There was an observed elevation of FGM concentrations in S phase compared to the following P2 phase ($p = 0.013$), but neither S nor P2 phase differed from P1 phase. Along with the decrease in FGM concentrations from S to P2 phase, there was an increase in vigilance behaviors ($p = 0.001$). This increase in natural predatory behavior, combined with decreased FGM concentrations, suggested the more complex habitat promoted long term behavioral stimulation without adversely affecting the snow leopards' overall welfare state.

Energy balance under stress: corticosterone, thyroxine and growth rate in cold-stunned juvenile Kemp's ridley sea turtles

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Abstract:

Elevated corticosterone suppresses growth in many vertebrates, whereas thyroxine can sometimes stimulate growth, such that these two hormones are often negatively correlated. Effects on growth could be particularly important for juvenile sea turtles, in which body size is thought to influence risk of predation. Many juvenile Kemp's ridley turtles (*Lepidochelys kempii*) strand on Cape Cod, Massachusetts (USA) each year due to "cold-stunning", a physiological state characterized by low body temperature, various metabolic abnormalities, elevated corticosterone and depressed thyroxine. Both hormones typically return to presumed-normal levels after approximately two weeks of rehabilitation, yet some individuals have persistent, mild elevations in corticosterone or depressions in thyroxine that can last for several months, leading to the hypothesis that such turtles might have depressed growth rate. We used radioimmunoassays to assess corticosterone and thyroxine at approximately two-week intervals across three months of rehabilitation for 71 juvenile Kemp's ridleys (all of whom survived to be released), comparing hormone data to feeding, activity and growth rate. All research was approved by the New England Aquarium Animal Care and Use Committee. Measures of feeding (g of food eaten per week) and activity (as assessed by clinical staff) both correlated negatively with corticosterone and positively with thyroxine. Growth rate (% increase in mass) was significantly and negatively correlated with corticosterone and positively correlated with thyroxine, with endocrine status at day 18 predicting growth rate over the following 2-3 months. Though causal relationships of these measures remain unclear, attention to reducing stress in clinical settings shows potential for helping to restore normal growth rate in juvenile sea turtles during rehabilitation after mass stranding events.

Biomarkers of stress and heavy metals in the Morelet's crocodile (*Crocodylus moreletii*) in Campeche and Tabasco, Mexico

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Abstract:

The Morelet's crocodile (*Crocodylus moreletii*) under captive conditions could be exposed to various types of stressors such as capture, handling or transportation and as in wildlife to the environmental ones e.g. pollutants. These challenges are faced by individuals through the physiological stress response. However, this response (chronic or acute) has a metabolic cost that is reflected in oxidative stress (OS). The relationship between corticosterone (B) and OS has been very studied experimentally in animal models such as mammals, birds, fish and little in reptiles. About reptiles, in field studies less is known of this mechanism and about the relationship with pollutants and their effect on the hypothalamic-pituitary-adrenal axis (HPA). The aim of this research is to address the modulatory role of B in oxidative stress, generated by the environmental exposure to essential (Cu and Zn) and non-essential metals (Hg and Cd) on the Morelet's crocodile under captive conditions and in wildlife. All of this by the measurement of thiobarbituric reactive substances (TBARS), total glutathione (GSH), and metallothioneins (MTs) as OS biomarkers, and B as physiological stress biomarker. So far in our study we know that in captive individuals plasma, kidney and liver show significant differences in TBARS concentrations. On one hand the liver was the tissue with major oxidative damage and the plasma was less. And on the other hand the liver and kidney those with the smallest difference between them and liver and plasma the most. Nowadays, we continue measuring the GSH, B and MTs, and the concentration of metals in the three tissues. All the information generated in this study will improve management practices in farms and in wildlife will strengthen the conservation of the species.

Validating the use of dermal swabs in the marine sunfish (*Mola mola*)

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Abstract:

The marine sunfish (*Mola mola*) is the heaviest bony fish in the world. This slow-moving fish often is injured by fishing boats that use drift gillnets attributing to its listing as Vulnerable by the IUCN. The Monterey Bay Aquarium (California, USA) has a program, which brings in smaller *Mola* from the ocean and acclimates them for exhibit. When they grow too large for the million-gallon Open Seas exhibit, they are returned to the Bay through a “reverse” acclimatization. Our goal was to use dermal swabs to evaluate *Mola* stress physiology to better understand the effects of this program. Our objectives were to validate this method by taking opportunistic swabs throughout acclimatization and during stressful events. We swabbed each individual (n=10) in three different body locations. Swabs were analyzed using a cortisol (Munro R4866) enzyme immunoassay. We averaged the three swabs and examined the change from baseline in cortisol during treatments and these different acclimation stages, from treatment pool behind the scenes, to the exhibit, Animal Research and Care Center (an off-site holding facility) and back to the Bay. Overall, mean (\pm SEM) cortisol varied ($P < 0.05$) among individuals (476.9 ± 106.4 pg/mL). Cortisol tended to increase ($P > 0.05$) from admittance (169.5 ± 77.8 pg/mL) to 1-week in the acclimation process (307.6 ± 57.3 pg/mL) and during transportation (890.4 ± 581.6 pg/mL). However, cortisol significantly increased ($P < 0.05$) when the *Mola* were being treated for an illness (1697.8 ± 825.8 pg/mL). Although dermal cortisol varied among individuals, we did observe an increase during times that were considered stressful events. This is the first step in validating the use of dermal swabs in *Mola*. Further biochemical analysis is needed to determine the specific steroids that are being measured to ensure that the changes in cortisol are a true reflection in *Mola* stress physiology.

The plight of the penguin: the physiological stress response of African penguins to anthropogenic activity

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Abstract:

Despite the importance of ecotourism in species conservation, little is known about the effects of the industry on wildlife. In South Africa, some African penguin (*Spheniscus demersus*) colonies have become tourist attractions. The species is globally endangered, with population sizes decreasing over the last decade. As African penguin chicks are altricial and unable to move away from anthropogenic stressors, it is important to evaluate the effect of tourist activities on the physiology stress response of this age group. Chicks at three study sites within two breeding colonies (Robben Island, Stony Point), with varying levels of exposure to tourism (low/moderate/high) were monitored. Urofaecal samples were collected to determine urofaecal glucocorticoid metabolite (ufGCM) concentrations as an indication of the stress experienced. Morphometric measurements were taken to compare body condition between sites. Penguin chicks experiencing low, infrequent human presence had significantly higher ufGCM levels (1.34 ± 1.70 $\mu\text{g/g DW}$ standard deviation (SD)) compared to chicks experiencing both medium (0.50 ± 0.40 $\mu\text{g/g DW}$, $p = 0.001$) and high levels (0.57 ± 0.47 $\mu\text{g/g DW}$; $p = 0.003$). There was no difference in chick body condition across sites. These results suggest that exposure to frequent human activity may induce habituation/desensitisation in African penguin chicks. Acute, infrequent human presence was likely an important driver of elevated ufGCM levels in these chicks, though several other environmental stressors may also play an important role in driving adrenocortical activity. Nevertheless, as unhabituated chicks showed significantly elevated ufGCM levels to infrequent anthropogenic presence, managers and legislation should attempt to minimise all forms of activity around important breeding colonies that are not already exposed to regular tourism. Although the results of this study are crucial for developing enhanced conservation and management protocols for the species, additional research on the long-term effect of anthropogenic activities on African penguin physiology is required.

Assessment of an effective faecal marker for individualized endocrine function monitoring in African lions (*Panthera leo bleyenberghi*)

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Abstract:

Non-invasive faecal monitoring of endocrine function has been extensively used in zoo and wildlife species. Despite that, individualized sampling of animals is often difficult to accomplish in social groups housed together. This study aimed to evaluate the effectiveness of using shredded wax crayon as an indigestible marker for individual identification of faeces while validating a commercial enzyme immunoassay (EIA) for faecal glucocorticoid metabolite (FGM) determination in African lion (*Panthera leo bleyenberghi*). An African lion pride consisting of three fifteen-year-old females, a fifteen-year-old male and a three-year-old male was studied. Every morning, before feeding, a piece of meat covered with shredded wax (Giotto be-bè®) of the assigned colour was given to each lion. Faecal samples were collected daily and frozen at -20°C. Macroscopic examination of all frozen samples was conducted to evaluate the presence of shredded wax and ascribe each sample to the proper individual. A methanol-based extraction technique and a competitive cortisol EIA kit (Neogen®, Ayr, UK) were used for FGM determination. Shredded wax was detected macroscopically in 89.83% of the samples (n=106/118), being evident in faeces within the first 24-48 hours after ingestion. Analytical validation of the EIA was conducted successfully. The intra-assay coefficient of variation obtained was 6.98%. In the linearity-of-dilution test, observed FGM concentrations correlated with expected values ($r>0.99$, $p<0.05$). The average recovery percentage from spike-and-recovery test was $90.07\pm 8.90\%$. Sensitivity of the method was 1.94 ng/g. The group FGM mean concentration was 18.45 ± 6.11 ng/g. Individual FGM mean concentrations ranged from 15.92 ± 5.39 ng/g to 21.96 ± 7.58 ng/g, with significant higher values in the dominant male in relation to the subadult male ($p=0.01$). Results demonstrate the effectiveness of shredded wax crayons as indigestible markers for individual identification of faeces in group-housed animals and highlight the importance of monitoring endocrine function individually rather than as a collective sample.

Validation of DHEAS in feces of three species of neotropical primates (*Alouatta caraya*, *Sapajus apella* and *Aotus azarae infulatus*)

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Abstract:

Due to the importance of welfare in the quality of life of the animals, currently it has been chosen for the association of glucocorticoids (GCs) with dehydroepiandrosterone sulfate (DHEAS), as a better indicator of chronic stress. Despite many studies with DHEA (S) in old world primates, few studies have evaluated this hormone in neotropical primates. Thus, we aim to validate the DHEAS immunoenzymatic assay in feces of three neotropical primates (*Aotus* sp., *Sapajus* sp., and *Alouatta* sp.) Using the ArborAssay EIA kit. We use one female of each species, adults and healthy. The physiological challenge was performed with DHEA capsules. Parallelism and accuracy tests were performed. The dilutions performed in the three species (1: 2 to 1: 128) were parallel to the standard curve ($p > 0.05$). The general mean of recovery in the accuracy test was 114% for *A. caraya*; 110% in *S. apella* and 107% in *A. a. infulatus*. We observe adrenal response by oral administration of DHEAS, with increased concentration of this hormone in all individuals evaluated. The mean baseline (before challenge) DHEAS concentrations for *A. caraya* was 13111.69 ± 3564.30 ng/g of feces, with peak 24 after administration and an increase of 681% (89276.27 ng/g); in *S. apella* it was 375.90 ± 223.71 ng/g, peak 24h after administration and an increase of 396% (1227.95 ng/g) and in *A. a. infulatus* it was 1064.16 ± 653.56 ng/g of feces, with two peaks, one 24h, with an increase of 352% (3753.84 ng/g) and 72h after administration, with an increase of 867% (9227.58 ng/g). Our results validated the DHEAS assay in the feces of the three primates evaluated. Future studies may evaluate the efficiency of the DHEAS/cortisol index as a biomarker of chronic stress in these species.

Relationships between anthropogenic chemical pollutants and fecal hormone metabolites across four primate species in Kibale National Park, Uganda

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Abstract:

Despite the thousands of anthropogenic chemicals on the market today and an increasing awareness of the hazards associated with their discharge into the environment, our understanding of physiological health effects of wildlife exposure to chemical pollutants is limited. The application of wildlife biomonitoring provides an alternative to laboratory-based toxicity tests, integrating ecological factors and real-world complexities unattainable in controlled settings. In this study, we use noninvasive biomonitoring to examine relationships between fecal concentrations of over 100 chemical pollutants, including pesticides and flame retardants, and fecal hormone metabolites of the HPA (e.g., cortisol), HPG (e.g., testosterone, estradiol), and HPT (e.g., T3) axes across four species of wild nonhuman primates inhabiting Kibale National Park in western Uganda. Our results indicate significant differences in concentrations of pollutants and hormones between species, and overall positive relationships between fecal concentrations of pollutants and steroid hormones across species. We discuss the implications of these relationships in terms of biological significance, as well as the use of immunoassays for fecal matrices and the potential for “cross-talk” between measures of endogenous hormones and exogenous chemicals, such as pollutants or endocrine-active phytochemicals in plant material, that have passed through the gastrointestinal system.

Non-invasive quantification of cortisol in critically endangered Livingstone's fruit bats (*Pteropus livingstonii*)

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Abstract:

There are presently only 1200 Livingstone's fruit bats (*Pteropus livingstonii*) world-wide due to acute threats from an increased frequency of extreme weather events and chronic land encroachment onto roost sites on their native Comoros archipelago. To safeguard this species, a captive breeding population was established by the Durrell Wildlife Conservation Trust in conjunction with the Comorian government. A critical component of successfully conserving this species *ex situ* is understanding the physiological underpinnings of their welfare. In order to use cortisol (which has been linked with stress in other species) as a reliable indicator of welfare in the Livingstone's fruit bat, it is first essential to examine the variation in baseline hormone levels between demographic groups. To meet this aim without disturbing the captive population, we have developed and validated a novel hormone extraction procedure and faecal glucocorticoid assay for this species. To date, 86 faecal samples have been non-invasively collected from the Livingstone's fruit bat breeding group at Jersey Zoo. Preliminary analysis of these samples suggests that males, older individuals, and females with dependent pups possess higher than average cortisol, while more highly social animals (as defined by their weighted degree centrality, using social network analysis) possess lower levels. Future analysis will seek to link the frequency of specific behaviours with cortisol levels with the goal of identifying pragmatic behavioural indicators of more complex physiological states linked to welfare, as well as examine in greater detail potential correlations between sociality and individual cortisol levels. The results of this research will therefore lead to practical and applicable insights for the welfare and conservation of the Livingstone's fruit bat in captivity and improve our understanding of their physiology through the implementation of a non-invasive, novel methodology for extracting and measuring cortisol in this species.

Feather corticosterone and fluctuating asymmetry as indicators of early-life stressors in juvenile birds of prey

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Abstract:

Though multiple studies have explored the effects of stress on symmetrical growth in birds over short periods of time, there have been comparatively fewer long-term measurements of how stress affects asymmetrical growth, particularly in birds of prey. Fluctuating asymmetry (FA) is known to occur as a product of poor early-life conditions and experience with persistent stressors. One way to quantify stress in birds is to measure corticosterone (CORT) in feathers. Birds deposit CORT into their feathers when first growing the feathers as nestlings and also during molt, but they cannot deposit CORT after that feather is grown. We examined FA in juvenile birds, from four species, admitted to the Illinois Raptor Center for rehabilitation and concurrently measured CORT in feathers. Finally, we explored additional downstream consequences, such as parasite prevalence in birds with varying degrees of asymmetry. We found that for juvenile birds, feather CORT showed a positive, linear relationship with asymmetry. However, there was no significant relationship between CORT and asymmetry in adult birds. Overall, our findings add to the existing body of evidence that early-life stressors can have long-lasting consequences for birds. The study was approved by the Millikin University Institutional Animal Care and Use Committee (Protocol 8-4-18) and all sample collection and animal handling was completed within the provisions of Illinois State Scientific Permit R-15-135 and R-15-136 and United States Fish and Wildlife Service Rehabilitation Permit MB783453-0.

Evaluating the impact of transport and relocation on marine mammal welfare through measurement of hair and salivary cortisol

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Abstract:

As the field of animal welfare science continues to grow, there is an increasing need for valuable welfare indicators. Among these is the study of glucocorticoids as potential indicators of 'stress,' measured through a variety of biomarkers. In this study, The Ohio State University's Center for Human-Animal Interactions Research and Education (CHAIRE) and the Columbus Zoo and Aquarium (CZA) partnered for a longitudinal study which aimed to assess the welfare of their California sea lions (*Zalophus californianus*) throughout relocation from a temporary facility to permanent habitat at CZA, by measurement of cortisol. Given the non-invasive nature of this experimental design, this study was exempt from IACUC approval. Cortisol concentrations in hair (HCC) were evaluated throughout the duration of the study (34 months) using a shave/re-shave method, and salivary cortisol (SCC), collected via oral swab, was measured throughout the transport process itself (~1 week). In our assessment of the long-term relocation, HCC was significantly lower ($P = 0.0053$) during baseline (2.69 ± 0.351 pg/mg) than acclimation (4.75 ± 0.568 pg/mg). From analysis of salivary cortisol during transport, we found that SCC was highest post-transport (21.503 ± 3.318 nmol/L, $P < .0001$), although only significantly different from pre-transport ($P = .0003$) not the transport period ($P = .0856$). We also established a species-specific range of HCC and SCC for California sea lions to be 3.256 ± 1.837 pg/mg and be 12.37 ± 18.88 nmol/L respectively, a novel contribution to marine mammal physiology. These findings indicate transport and relocation may have implications on sea lions' welfare, although cautious interpretation is necessary given factors such as the animals' prior experiences and the delicate nature of interpreting cortisol concentrations. While somewhat preliminary, we hope these findings will contribute to the growing field of marine mammal welfare science, particularly regarding transport and relocation.

Don't leave me howling! Separation from pack mates is linked to increased urinary glucocorticoid metabolites in wolves but not dogs

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Abstract:

Wolves live in tightly-knit packs whose survival depends on cooperative behaviour. Pack cohesion is reinforced by affiliative interactions, often accompanied by group vocalizations (howling). In contrast, dogs rely less on conspecifics likely due to selection during their domestication process. Domestication is thought to have affected endocrine secretion patterns, specifically of oxytocin (OT), a neuropeptide hormone released during social interactions, and glucocorticoids (GC), steroids secreted in stressful situations. To compare hormonal correlates of wolves' and dogs' conspecific social behaviours, we measured OT and GC metabolites (OTM; GCM) in non-invasively collected urine from 9 wolves and 10 pack-living dogs. Samples $N=180$ (78 dog and 102 wolf samples) were collected following two group activities (howling, 'mock' territorial defence), separation from the pack, and a baseline (resting) control condition. We predicted higher OTM concentrations following the group activities (compared to the baseline) and higher GCM concentrations after separation (compared to the baseline) in wolves than dogs. Urine samples were extracted and analysed using validated enzyme immunoassays. We fitted linear mixed models with OTM or GCM concentrations as the response and condition interacting with species as the test predictor. Group activities did not affect OTM concentrations but dogs had higher OTM and GCM concentrations than wolves ($P<0.01$), regardless of condition. Separation from the pack increased GCM concentrations, relative to baseline, in wolves but not dogs ($P<0.05$). Generally elevated hormone levels in dogs might be indicative of selective changes due to domestication. Wolves' higher GCM concentrations after separation from their pack indicate that such a situation is perceived as stressful and is in line with greater dependence on pack members whereas dogs might have adapted to rely more on humans than conspecifics as primary social partners.

Assessing fecal steroid and thyroid hormone metabolites as indicators of stress in the Northeast Pacific southern and northern resident killer whale (*Orcinus orca*) populations

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Abstract:

Fecal hormone metabolite analysis is increasingly used as a non-invasive method to evaluate physiological stress in free-ranging animals, especially those of conservation concern, including marine mammals. It is important, however, that assays are carefully selected and validated for each species, to be physiologically meaningful. In this study, we validate four commercially available enzyme-linked immunosorbent assays for quantifying fecal steroid and thyroid hormone metabolites in killer whales (*Orcinus orca*), then compare concentrations of fecal glucocorticoid metabolites and thyroid metabolites between the Northeast Pacific northern resident and southern resident populations as indicators of stress. Fecal samples were collected opportunistically from both populations, in critical foraging areas along the coast of British Columbia, Canada, from June to August of 2018-2020. We hypothesize that, despite sharing a similar diet and environment, the southern residents are more physiologically and nutritionally stressed than the northern residents due to anthropogenic threats of decreased prey availability, vessel disturbance, and environmental contaminants. If the southern residents are experiencing greater stress than the northern residents, it will be demonstrated by higher concentrations of fecal glucocorticoid metabolites and lower concentrations of fecal thyroid metabolites in the southern population relative to the northern. Results may, additionally, lend insight into sources of physiologic stress for the two populations; with thyroid hormone more strongly associated with nutritional status. These results will advance our knowledge of marine mammal physiology and assist conservation management in understanding the threats affecting the health and status of the endangered southern resident killer whale population.

How does the environment impact fecal glucocorticoids and gut microbiota in the critically endangered red wolf (*Canis rufus*)?

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Abstract:

The red wolf (*Canis rufus*), a critically endangered canid species, resides mostly in captivity with only one small ($N \sim 10$) wild reintroduced population in North Carolina. Gastrointestinal (GI) disease has caused mortality in 21% (32/151) of the population. Exposure to stressors can suppress the immune system, alter gut microbiome composition and lead to pathological conditions like GI disease. The objective of this study was to understand the relationships between fecal glucocorticoid metabolite (fGM) concentrations, captive environmental factors (e.g. housing group, enclosure type and size) GI microbiota and GI health in red wolves. Fecal samples ($n=979$) were collected from 14 red wolves three times a week for 5 to 12 months. Hormones were extracted from lyophilized samples using 90% ethanol and evaluated via enzyme immunoassays using polyclonal cortisol R4866 antiserum (CJ Munro, University of California, CA) and horseradish peroxidase coupled to cortisol-CMO (Sigma-Aldrich, St. Louis, MO). Surveys were completed for each individual to assess environmental stressors and a canine inflammatory bowel disease activity index (CIBDAI) was completed monthly to estimate individual GI health. Diet influences gut microbiome composition in red wolves, with captive wolves eating a kibble diet having the most distinct profile compared to whole meat or mixed (kibble+meat) diets. Microbiome analysis was conducted on the selected samples to assess the link between fGM concentrations and gut microbiota. Linear mixed models demonstrated the relationship among fGM concentrations and host and environmental factors to shed light on possible factors that may drive GI disease in red wolves. This research will enhance the management, health and well-being of captive red wolves and other large canids in captivity, especially ones that suffer from suboptimal GI health. These red wolves or their offspring could be a participant in the reintroduction program, therefore it is imperative that healthy individuals are maintained in captivity.

Stress-induced reproductive suppression: are elevated glucocorticoids really associated with lower fertility?

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Abstract:

Glucocorticoids (GCs) are often equated with stress, and therefore thought to inhibit reproduction. Most evidence for this comes from hormone manipulation studies in a limited number of species. However, GCs fluctuate in a predictable manner throughout the day and across the ovarian cycle. Studies that artificially increase GC levels (e.g., injections or implants) disrupt normative GC rhythms, and consequently the results of these studies may reflect altered GC patterns, rather than elevated GC levels *per se*. Our goal was to examine the relationship between endogenous GCs and reproductive success in females. We conducted a systematic review of studies that reported unmanipulated GC levels in successful vs unsuccessful breeders in captive and wild populations. To get a broad taxonomic perspective, we included all vertebrate species. Results varied depending on when in the reproductive cycle GC measurements were taken. This suggests that there may be a more complex, nuanced relationship between GCs and reproduction that requires more attention in order to better understand the effects of stress.

Age-changes in fecal dehydroepiandrosterone-sulfate (DHEAS) in Hylobatidae

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Abstract:

Evaluating age-related patterns of steroid hormone secretion can provide useful information on animal physiology and can aid in monitoring animal welfare. In particular, the steroid hormone dehydroepiandrosterone-sulfate (DHEAS) have attracted considerable interest due to its role as anti-stress and its association with longevity. Previous studies in primates reported interspecies difference in the age-related pattern of DHEAS. In great apes and humans, there is a pre-pubertal increase in DHEAS levels, which is known as adrenarche. But in old world monkeys, this increase is not apparent. Instead, DHEAS follows a linear decline with age throughout their lifespan. However, the age-related pattern in the only lesser apes (gibbons and siamangs) has not been determined. This project aimed to evaluate the age-related pattern of DHEAS in captive gibbons and siamangs housed in U.S zoos to determine whether adrenarche is apparent in these species. Fecal DHEAS levels were validated and quantified from 118 fecal samples from 38 individuals (age range = 1 to 39 years old) from the family Hylobatidae (*Hylobates* sp., *Symphalangus* sp., and *Nomascus* sp.) by enzyme immunoassay (EIA). To avoid the possible effect of stress in DHEAS levels, glucocorticoid (GC) levels were co-measured by EIA, and the DHEAS/GC ratio was compared between pre-pubertal (< 5 years old), adolescents (6 to 10 years old), and adults (> 11 years old) using a generalized linear mixed model, taking in consideration sex and species. The results revealed a non-significant increase in the DHEAS/GC ratio between pre-pubertal individuals and adolescents, and a subsequent decline in the adult category. The DHEAS/GC ratio was significantly higher in males than in females. These findings suggest that adrenarche is apparent in lesser gibbons, similarly to the pattern reported in great apes. This work brings insights to our understanding on the role of DHEAS and adrenarche in human evolution.

Validation of commercial assays to measure salivary insulin and salivary glucose in western lowland gorillas (*Gorilla gorilla gorilla*)

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Abstract:

My research investigates the correlation between insulin resistance and regurgitation and reingestion in zoo-housed gorillas (*Gorilla gorilla gorilla*). As some of our biomarkers of insulin sensitivity, we are looking at salivary insulin and salivary glucose levels using commercial kits already validated for accuracy, precision, and specificity (by parallelism test) in our lab for gorilla salivary insulin. Results from a preliminary study measuring gorilla salivary insulin in response to the consumption of applesauce indicated the insulin assay detected an increase in levels between 15 min ($\mu = 12.43 \pm 2.88 \text{mU/L}$) and 30 min ($\mu = 21.4 \pm 3.49 \text{mU/L}$) post consumption in four gorillas. Though these results were expected, this assessment only included samples taken at 15 and 30 minutes after consumption. To biologically validate insulin measurements, we need to capture the expected postprandial increase and subsequent decrease in insulin. For such validation, we will conduct an oral sugar test on 1.4 gorillas residing at Cleveland Metroparks Zoo in early summer 2021. This test will compare fasted salivary insulin and salivary glucose levels to samples taken at predetermined time points up to 120 min following an oral dose of Karo syrup. Based on results from oral glucose tests in humans and horses, we predict salivary insulin and glucose levels will gradually increase and peak approximately 60 to 90 minutes post consumption. The biological validation of these assays will provide additional feasible means for zoos to assess the health and physiology of their residing gorillas.

Socioecological predictors of fecal androgen levels in juvenile geladas

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Abstract:

Androgens offer a window into the timing of male life history in many vertebrates. Here, we examine several variables that are associated with fecal androgen metabolite levels (fAMs) in a population of wild juvenile geladas (*Theropithecus gelada*). Gelada male life history is unusual - dispersal occurs well before sexual maturation, and juvenile males experience variable social conditions as members of different “reproductive units”. Androgens therefore offer insight into variation in maturational milestones due to differences in the social environment for these juvenile males. Using long term data from 53 juvenile males, we examined the effects of social predictors representing: (1) access to maternal resources (a: maternal dominance rank, b: presence of a younger sibling), (2) access to peers (number of male peers in their reproductive unit), (3) early life adversity (maternal loss before 1.5 years of age). After controlling for age and ecological variables in multiple generalized linear mixed models, we found that: (1a) juvenile males with high-ranking mothers had higher levels of fAMs compared to juvenile males with low-ranking mothers ($p=0.002$, 95% CI={1.23,5.91}); (1b) juvenile males with a younger sibling (born before the average female interbirth interval of 2.5 years) had higher levels of fAMs than males who did not ($p=0.001$, CI= {6.28,2.89}); (2) juvenile males with male peers in their social groups experienced higher levels of fAMs than males that did not ($p=0.007$, CI= {0.63,4.08}); (3) males that experienced maternal loss had lower levels of fAMs ($p=0.001$, CI= {-21.43, -6.68}). Our results indicate that better access to maternal resources and presence of peers lead to higher fAMs while maternal loss leads to lower fAMs in juvenile male geladas. Surprisingly, presence of a younger sibling increases fAMs in males. In future analyses, we will add dispersal timing to understand the preparatory role for fAMs in gelada life history.

Perspectives to integrate non-invasive endocrine monitoring and biologging to uncover seasonal changes in physiology and daily activity patterns in free-living subterranean rodents

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Abstract:

The Anillaco tuco-tuco (*Ctenomys* aff. *knighti*) is a subterranean rodent from the semi-arid Northwest of Argentina. It is diurnal in nature but nocturnal in the laboratory. This flexibility in rhythmic patterns suggests an interaction of the circadian system with biotic and abiotic factors. To understand the interplay between changes in tuco-tuco's physiology and their daily activity patterns, we are measuring daily rhythms in free-living tuco-tucos throughout the year while also characterizing assessing endocrine markers for reproduction, stress and energetic statuses. We established the methodology to assess, in this species, fecal hormone metabolites of progesterone, testosterone, estradiol, corticosterone and T3. Assay validations (detectability, accuracy, and parallelism) were conducted to validate the use of commercial enzyme-immunoassays to measure metabolites of each hormone in fecal extracts. Furthermore, hormone challenges were performed to ensure the measurements were biologically meaningful. We performed challenges with GnRH agonist (for the sex steroids measurements), ACTH (for corticosterone) and TSH (for T3). Our preliminary results show that most individuals responded with increased levels of the measured hormone after the challenges. Lastly, we are assaying samples obtained from free-living individuals throughout one year. These animals also received a collar with activity- and light-loggers to record their activity and light exposure rhythms. There was a high degree of interindividual variation in the shape of activity patterns. Clear differences between males and females were observed during spring, when females are pregnant and lactating, thus having higher energetic demands. We expect that the future integration of these biologger data with the ongoing hormone measurements will shed light on how internal physiological statuses may shape daily activity rhythms in tuco-tucos. All procedures described were authorized by the Dirección General de Ambiente y Desarrollo Sustentable – Secretaría de Ambiente – La Rioja (00501-17) and by the FMVZ-USP Animal Care and Use Committee FMVZ-USP (2045300519).

Validation of a novel methodology for processing shed skins from western diamondback rattlesnakes (*Crotalus atrox*) for corticosterone analysis

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Abstract:

Current processing protocols for snake sheds involve clipping the entire shed into small pieces. This works for smaller snakes but becomes labor intensive for larger snakes, including the Western Diamondback Rattlesnake (*Crotalus atrox*). This study evaluated the efficacy of grinding sheds into a coarse powder using a liquid nitrogen-filled mortar and pestle (cryogrinding) compared to the traditional clipping method for four different locations of the shed. Sheds from 7 snakes were washed (ddH₂O), briefly hung to dry, sprayed with MeOH and left to dry fully overnight in a fume hood. Dried sheds were cut in half lengthwise along the sagittal plane (clipped/ cryogrind) and then into four segments of equal length (head, cranial midsection, caudal midsection, and tail). Samples processed by each method were extracted (0.2g ± 0.02g in 5mL 80% MeOH) and shaken overnight (~15 hr., 22°C). The next day, samples were centrifuged (500g, 15min) and 1mL supernatant transferred to a clean glass tube, dried under a stream of air and reconstituted in 100uL buffer. Biochemical validation of the corticosterone assay was achieved by demonstrating parallelism ($R^2=0.993$ cryogrinding, $R^2=0.992$ clipping; $P < 0.05$) between serial dilutions of samples and the standard curve. Displacement curves were similar for both processing methods, but recovery of known corticosterone concentrations was higher (73.1% vs. 67%) when samples were processed by cryogrinding compared to clipping. Corticosterone concentrations in shed from the head region tended to be higher ($0.1 > P > 0.05$) than from the cranial and caudal midsections, suggesting comparisons between animals, or longitudinal comparisons within the same animal, should utilize samples from the same shed location. Based on these results, the cryogrinding method is an appropriate substitution for the clip method. In addition to its ease of use, cryogrinding saves time and produces more homogeneous samples with less potential for cross contamination from static electricity.

Advances in hair steroid measurement in the Iberian lynx

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Abstract:

The potential of steroid measurement in hair for wildlife conservation studies should not be limited to measuring glucocorticoids. Hair levels of reproductive hormones may disclose information on reproductive status and help evaluate reproductive activity, but this application potential has not been widely explored. We performed EIA measurement of progesterone, cortisol, cortisone, corticosterone and testosterone in Iberian lynx hair ($n = 41$), cross-validated the EIA using HPLC and HPLC-MS/MS, and assessed the variation in hormone concentrations between EIA and HPLC-MS/MS and with age, sex and origin (Azevedo *et al.*, 2020). EIA progesterone values (32.6 ± 34.2 pg/mg) were highly correlated ($r = 0.86$, $p = 0.018$) to HPLC-MS/MS measurements (9.2 ± 5.9 pg/mg), but were overestimated 3.4 times due to cross-reactivity with 5α -dihydroprogesterone. Progestogens were higher in adult females (85.2 ± 17.9 pg/mg) than adult males (18.1 ± 6.9 pg/mg), juvenile males (12.1 ± 8.3 pg/mg) and juvenile females (9.6 ± 3.2 pg/mg). Our results validate the use of hair progestogens as monitoring tool for reproductive status in the Iberian lynx. Cortisol-EIA measurements (37.27 ± 13.24 pg/mg) correlated with the sum of HPLC-MS/MS values for cortisol and cortisone ($r = 0.86$, $P < 0.001$) and overestimated cortisol measured by HPLC-MS/MS (13.35 ± 7.80 pg/mg) by 2.8 times due to EIA cross-reactivity with cortisone. Comparisons using cortisol-cortisone (Mann-Whitney, $U=4$, $p=0.026$) and cortisol-dihydroepiandrosterone ($U=1$, $p=0.004$) ratios were more sensitive than cortisol ($U=6$, $p=0.065$) to differences between captive-born ($n=6$) and wild-born lynxes ($n=6$) held in captivity. Longitudinal measures of cortisol and cortisone in an individual that escaped during a wildfire showed a post-event inversion of the cortisol-cortisone ratio that later reversed to its initial status. These results suggest that a local cortisol-cortisone interconversion might influence cortisol values in hair. Our study shows that hair steroid analysis can be used to identify adult Iberian lynx females and that cortisol-cortisone and cortisol-dihydroepiandrosterone ratios could be more sensitive indicators of stress than cortisol alone.

Investigation of PRL and Allo-P4 in southern white rhinoceros gestation: is there a hormone signal that can detect pregnancy earlier than day 90?

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Abstract:

Hormone data provide guidance in understanding the reproductive physiology of Southern white rhinoceros (SWR). Using fecal progesterone (fP4; P4CL425, Coralie Munro, UC Davis; MeOH extraction) alone, we are unable to confirm pregnancy before ~90d gestation. To evaluate utility as early pregnancy indicators in SWR, commercial EIAs for allopregnanolone (Allo-P4) and prolactin (PRL) were evaluated compared to P4. Serum and fecal samples were collected during cycles and pregnancy from three SWR females at Disney's Animal Kingdom®. Serum treatments included acetonitrile (ACN) extraction, ethyl acetate (EtOAc) extraction, polyethylene glycol (PEG) precipitation, or analyzed neat (untreated). Post-mixing, ACN and PEG extract supernatants were decanted (PEG) or lyophilized and reconstituted in assay buffer (ACN). EtOAc treatment followed the kit protocol. Samples were analyzed using Arbor Assays' Allo-P4 (K061) and PRL (anti-mouse PRL; K040) EIA kits. Serum EtOAc, serum ACN, and fecal extracts demonstrated parallel displacement curves to the Allo-P4 standard curve. Preliminary results indicated serum EtOAc extracts gave inflated Allo-P4 values and did not provide meaningful information. However, serum ACN and fecal extracts showed comparable patterns to P4. For two females, serum Allo-P4 correlated with serum P4 ($R^2 \geq 0.997$). Fecal Allo-P4 and fP4 were correlated in all three females ($R^2 = 0.837-0.999$). Extracted, PEG-treated, and untreated serum dilutions were not parallel to the PRL standard curve and results suggested possible matrix effects and assay interference. However, in one of 3 females, untreated serum PRL was elevated at 33d post-conception (81.25% of late-pregnancy PRL concentrations). This was not evident in the other two females at day 31. Overall, Allo-P4 mirrored P4 and did not provide an earlier marker of pregnancy. Our results suggested we need 1) timely collection of serum samples <90d; 2) testing of PRL assays with antisera raised against alternative-species to determine use as an early pregnancy indicator in SWR.

Innovation in wildlife endocrinology: LA-REIMS metabolic fingerprinting for early pregnancy testing in giant pandas

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Abstract:

Pregnancy diagnosis in giant pandas is challenged by a delay in the luteal phase (diapause in pregnant cycles) and pseudopregnancy (similar progesterone profile in bred and non-bred animals). Nevertheless, optimal management for pregnant animals and timely planned logistics to monitor birth are crucial for successful breeding. A straightforward pregnancy test is so far not existing: with current state-of-the-art methodologies, pregnancy can only be confirmed in the last three weeks. Our aim was to investigate the potential of laser-assisted rapid evaporative ionization mass spectrometry (LA-REIMS) as an early hands-on rapid method for pregnancy testing. Samples of 7 cycles (3 pregnant, 4 non-pregnant) of 3 giant pandas were included with 12 samples per cycle equally distributed over 4 stages: anestrus, late corpus luteum dormancy phase (CLD), early active luteal phase (EAL) and late active luteal phase (LAL). 50 µL of undiluted urine was loaded without sample pretreatment on a 96-well plate for automated LA-REIMS analysis using an Nd:YAG laser and Xevo G2-XS QToF-MS in negative scanning mode. A total of 2791 molecular features were obtained after preprocessing the data with Progenesis. Subsequently, data was transferred to SIMCA for multivariate analysis, showing good repeatability based on QC clustering (PCA-X). Significantly different models (7-fold validation) between pregnant and non-pregnant cycles were demonstrated by OPLS-DA for the first time during EAL ($Q^2(Y)=0.711$, $R^2(Y)=0.971$, $p<0.01$) and confirmed during LAL ($Q^2(Y)=0.742$, $R^2(Y)=0.998$, $p=0.01$). Moreover, after exclusion of bred, but non-birth cycles (potential fetal losses, $n=2$), discriminative fingerprinting between pregnant and pseudopregnant cycles was shown during CLD ($Q^2(Y)=0.81$, $R^2(Y)=0.99$, $p=0.01$). In conclusion, this study clearly illustrates the applicability of LA-REIMS as an alternative fast diagnostic tool in wildlife endocrinology. Further research should reveal whether there is potential for pregnancy confirmation in the early stages of diapause.

A novel field method for preserving African lion (*Panthera leo*) fecal samples for noninvasive hormone analysis

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Abstract:

Immediately freezing samples to suspend hormone-degrading bacteria is not always an option during remote field work; therefore, our goal was to develop a field method for preserving African lion feces. Our objectives were to determine the stability of fecal hormone metabolites: 1) when exposed to the natural environment, and 2) in silica beads at ambient temperatures. We collected several fresh feces from lions at the Lincoln Park Zoo (LPZ; Chicago, IL, USA) and combined them into two (male and female) homogenous samples. Each was divided into eight samples (~45g) to undergo a cross-designed treatment. One sample was immediately lyophilized to serve as the control. Seven samples were exposed outside in July for 0, 12, 24, 36, 48, 60, or 72 hours. After exposure, the sample was placed in a mesh bag in a plastic jar filled with 25g of silica beads for 5, 7, or 10 days. We stored samples at -20°C until extraction (0.2g feces with 5mL 90% ethanol). We analyzed samples using enzyme immunoassays for corticosterone ("CC"; Arbor Assays #K014) and testosterone ("T"; antibody R156/7, Coralie Munro, UC Davis, USA). A randomized block ANOVA was used to determine the effects of exposure time and silica bead treatments. The silica desiccation time had no effect ($P>0.05$) on CC or T for male or female samples. For males, exposure time was similar ($P>0.05$; CC: 117.0ng/g; T: 527.4ng/g) to the control (CC: 119.2ng/g; T: 580.3ng/g). For females, 0 hr exposure was similar ($P>0.05$, CC mean: 62.7, T mean: 145.1 ng/g) to the control (CC: 59.9, T: 133.2 ng/g), but longer exposure times from 12-72 hours were higher ($P<0.05$; CC: 94.6-284.0, T: 167.4-528.6 ng/g). Our results highlight the importance of validation and indicate that fresh lion fecal samples are stable in silica beads for up to 10 days before freezing.

Developing a new extraction method to minimize interference in immunoassay of urinary oxytocin

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Abstract:

Methods of assaying peripheral oxytocin have been controversial, in part due to concerns about extraction techniques. Urine samples are particularly important for studies of wildlife, due to the ability to collect urine non-invasively, yet urine contains many metabolites that might interfere with immunoassay measurement. We therefore explored the question of assay interference by measuring urinary samples from oxytocin knockout (KO) and wildtype (WT) mice. On the Arbor Assays Enzyme Immunoassay (EIA) kit, unextracted urine was indistinguishable across genotypes ($W = 91$, $p = 0.589$), revealing high levels of interference. Solid phase extraction using a reversed-phase cartridge provided an improved genotype contrast, but knockout samples still measured well above the assay's lower limit of detection. Solid phase extraction with a mixed-mode cation exchange (MCX) cartridge allowed for optimization of the extraction selectivity, improving the genotype contrast and minimizing interference in the knockout samples. Similar interferences were observed in reverse-phase extracted samples with two other commercial EIAs (Enzo Life Sciences and Cayman Chemicals), although measured concentrations varied across kits; in all cases the MCX extraction method was effective at minimizing interference. Using this MCX extraction method, we conducted an analytical validation for both wildtype mice and wild Verreaux's sifaka (*Propithecus verreauxi*) on the Arbor Assays kit; sample collection from both species was approved by the appropriate IACUCs. Pooled wildtype mouse urine samples diluted parallel to the standard curve (CV of corrected concentrations = 10.1%) and exhibited excellent spike recovery (mean = 114%). Similarly, pooled sifaka urine samples demonstrated good parallelism (CV of corrected concentrations = 17.5% over full range; 5.11% for lower 5 dilutions) and excellent spike recovery (mean = 114%). This work suggests that common methods of measuring urinary oxytocin also measure compounds other than oxytocin and establishes a new method of extraction that minimizes this interference in immunoassay measurement.

Detection of steroid and thyroid hormones in mammalian teeth

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Abstract:

Endocrine techniques can provide a better understanding of mammalian life histories and predict how individuals and populations may respond to environmental stressors; however, few options exist for studying long-term trends in endocrine function in individuals. Here, we aimed to (1) determine whether steroid and thyroid hormones (progesterone, testosterone, estradiol, triiodothyronine, aldosterone, corticosterone, and cortisol) could be measured in teeth from four marine mammal species: narwhal (*Monodon monoceros*), beluga (*Delphinapterus leucas*), killer whale (*Orcinus orca*), and walrus (*Odobenus rosmarus rosmarus*); (2) validate commercially available enzyme immunoassay kits for use with tooth extracts; and (3) conduct biological validations for each species to determine whether hormone concentrations in teeth correlated with known life histories, such as sexual maturity. Teeth were collected from harvested or stranded individuals, pulverized, and hormones were extracted using methanol. Acceptable parallelism and accuracy results were observed for each hormone and species; however, cortisol was undetectable in all tooth extracts. Biological validations involved measuring progesterone and testosterone concentrations in one or both sexes and comparing concentrations between different age classes or growth layers for each species. Patterns in the narwhal and walrus tusk were consistent with those exhibited by other species; however, patterns in beluga and killer whale teeth were inconsistent with predictions. Here, we have validated the use of enzyme immunoassays for tooth hormone analyses and have shown that hormones can be reliably measured in marine mammal teeth. Additional biological validations are needed to gain insight into correlations between tooth hormone concentrations and life history characteristics.

A method for storage, preservation and transportation of anuran urine samples using filter paper for hormone analysis

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Abstract:

Urine sample provides crucial insights into the physiological status of captive and free-ranging amphibians. However, storage and preservation of urine samples in field conditions is always a difficult task due to limited facilities. The present study aimed to standardize and validate a field method for short-term storage and preserve of anuran urine samples using Whatman filter papers. About 30 anuran urine samples were collected and pooled to experiment with short term storage on the filter papers. The filter papers were stored for ten weeks, with six replicates each week at room temperature. Hormone metabolites were extracted from filter papers at one-week interval. To examine the efficacy of storage conditions, urinary-based enzyme immunoassays were used to measure the reproductive (progesterone and testosterone) hormone metabolites. High-Performance Liquid Chromatography was performed and revealed the presence of immunoreactive progesterone and testosterone metabolites in the urine samples. No significant difference was observed in the hormone metabolites concentration between samples stored in filter paper at room temperature and control samples stored in -20 C for the same period. Further, Whatman filter paper grade 50 was found to be more consistent and stable for storage and preservation of anuran urine samples compared to Whatman filter paper grade 3. No significant difference between the overall weekly (Day0-Week10) hormone concentrations over at room temperature was observed. The results indicate that anuran urine could be stored, preserved and transported at ambient conditions without significant changes and loss in steroid hormone concentration in field conditions in the absence of refrigeration. The finding of this study would facilitate endocrine monitoring of anurans in remote areas where limited logistics on the sample collection and preservation.

Validation of non-invasive hormone monitoring in the field to assess wildlife-livestock interactions: a case study from Patagonia, Chile

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Abstract:

Throughout large parts of the Patagonian Steppe, guanaco (*Lama guanicoe*) share much of their habitat range with domestic sheep. Due to overhunting, competition with domestic sheep for foraging resources and the resultant persecution, guanaco have seen large declines in population and habitat range and have become increasingly reliant on private sheep ranch lands. Guanaco-sheep competition has been readily investigated, mainly in Argentine Patagonia, however has been limited to studies of habitat or dietary overlaps. As far as we are aware, this is the first study to validate and use non-invasive hormone monitoring of an ungulates' stress hormones, to assess the impact of sympatric domestic livestock on the physiology of wild ungulates. Here we introduce the early validation results for a non-invasive field-based method to store and quantify levels of faecal glucocorticoids from guanaco faecal samples. We collected 25 faecal samples from Pali Aike National Park and 21 samples from the surrounding ranch lands. Our aim was to determine whether glucocorticoid concentrations in guanaco faeces varied between protected areas, where sheep are excluded, and neighbouring ranch lands where sheep coexist with guanaco. After previously validating a novel method in extraction and analysis in the field, we are hopeful this information will later assist researchers working under challenging conditions or at remote field sites and research stations.

The validation of a field friendly faecal extraction method for the analysis of thyroid hormone

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Abstract:

The non-invasive monitoring of thyroid hormones such as Triiodothyronine (T3), is becoming a widely appreciated technique as a valuable tool for assessing an animals overall health and wellbeing. The aim of this study was to validate methods for the processing and storage of faecal samples for thyroid analyses, either in the lab where samples can be frozen, or in the field where access to electricity can be limited. Following a similar approach used for steroid hormones, the method utilised in this study would allow samples to be loaded and stored on solid-phase extraction (SPE) cartridges and eluted when convenient for analysis, offering the potential to monitor these biomarkers within *in-situ* populations. The recovery of a synthetic T3 standard from C8 HyperSep™ SPE cartridges was assessed by loading onto pre-conditioned cartridges before elution with MeOH and subsequent concentration by evaporation. Analysis of T3 was performed using a commercially available enzyme immunoassay (EIA). Preliminary results indicated that a 40% methanol loading solvent was suitable for retaining T3 on the SPE for storage, meaning the same field extraction as for steroids could be used. However, low recovery of the standard led to further investigation of areas that could be effecting analyte recovery. The use of polypropylene tubes during the drying process significantly improved recovery of synthetic T3 compared to glass tubes typically used. Using methanol acidified with either hydrochloric or formic acid to elute from SPE cartridges did not improve the mean total percentage recovery. The same EIA has been biochemically validated for both black rhino and African savannah elephant, using both the lab and SPE extraction methods. This study demonstrates the importance of validating extraction techniques and how these methods should be carefully considered in relation to different compounds of interest.

Corticosterone and metabolomics as a multimodal approach to studying stress in waterfowl

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Abstract:

Waterfowl populations will likely decline because of anthropogenic and environmental changes (stressors). In response to stressors, the hypothalamic pituitary adrenal (HPA) axis releases corticosterone (CORT), initiating physiological processes to provide energy, restore homeostasis and increase survival. Corticosterone has been used to monitor the impacts of stressors. However, as CORT fluctuates amongst life-history stages and is impacted by acute stressors, such as blood sampling, it may not be a reliable indicator of adverse conditions. A better approach may be to examine CORT along with the metabolic responses to identify physiological changes associated with stress. Metabolomics is a comprehensive measurement of endogenous metabolites that participate in metabolic reactions. The objective of this study was to utilize CORT and metabolomics to recognize stress in ducks. We predicted that fecal CORT and metabolomics could be used to differentiate ducks that were subjected to a stressor from unstressed ducks. To test this prediction, we surgically implanted CORT (n=15) or placebo (n=10) pellets into mallard ducks (*Anas platyrhynchos*). Fecal samples were collected prior to and during implant-release of CORT. Blood samples were collected from a subset of birds to examine the relationship between blood and fecal CORT. ¹H Nuclear Magnetic Resonance (NMR) spectroscopy was used to analyze metabolites, and CORT was analyzed by radioimmunoassay. Corticosterone concentrations were significantly elevated during CORT implantation (Friedman Test $P > 0.001$). However, CORT concentration peaked at day 1 and 2 post-implantation then dropped on day 4 before increasing for the remainder of the active period. This may indicate a negative feedback response of the HPA axis to elevated CORT followed by a possible rebound effect. Fecal metabolite profiles distinguished CORT implanted ducks from control individuals. Fecal metabolomics shows promise as a non-invasive novel tool in identifying and characterizing physiological responses associated with large-scale environmental changes in wild birds.

Menses, genital swellings, and behavior can be used to accurately track reproductive cycling and fertility windows in zoo-housed lion-tailed macaques (*Macaca silenus*)

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Abstract:

While *ex situ* populations of lion-tailed macaques are rigorously managed, relatively little information on the relationship between behavior and reproductive physiology is available to ensure successful management. In 2016, Disney's Animal Kingdom® received 4 lion-tailed macaques (1 male, 3 females). With goals of successful social management and reproduction, we tracked social/reproductive behaviors and visual signs and hormone patterns of ovarian cycle stage in this group. We rated genital swellings of females, used all-occurrence group sampling to assess social dominance and reproductive behaviors, and recorded copulations and menses. We collected fecal samples daily from females, extracted samples using methanol, and assayed extracts using in-house enzyme immunoassays measuring progesterone (P4 CL425, C. Munro, UC-Davis, CA) and estradiol (E2 R008). We compared hormone patterns of ovarian cycles with genital swellings and behavior relative to menses. Cycle lengths averaged 30 ± 3.5 days. Progesterone concentrations declined preceding first day of menses in 84% of cycles. Estradiol peaks preceded maximum swellings (lasting 1-5 days, average=2 days) by 1-3 days. In two females, copulations occurred during rising estradiol concentrations, preceding or during peak swellings (no peak swellings in third female after starting birth control mid-study). Behavior varied between females, but was indicative of ovarian cycle stage and dominance status. In one, non-contact aggression towards other females and mounting by the male were correlated with low progesterone. In another, mounting by the male correlated to high estradiol. In the last, affiliation towards group members was correlated with low progesterone, and genital inspection of others correlated to high estradiol. As estradiol values were variable between and within each female's cycles, other fecal estrogen metabolites, or a potential role of social dominance in reproductive suppression of subordinates, will be investigated in the future. Results indicate observations of menses, genital swellings, and behavior can be used to track ovarian cycles in zoo-managed lion-tailed macaques.

Ovulation induction in African lions (*Panthera leo*) using the GnRH agonist buserelin-acetate

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Abstract:

Ovulation induction (OI) is prerequisite for most assisted reproduction techniques in felids. Most OI protocols require repeated doses of exogenous gonadotropins, which often trigger side effects that may reduce fertility. Hormonal protocols that induce safe ovarian responses in non-domestic felids are thus still needed. This study examined the effect of one single dose of buserelin-acetate (BA; 20 mg, im, Receptal[®]) to induce ovulation on days 4 (n=3), 5 (n=5), and 6 (n=2) of oestrus in African lions. For 12 months, daily monitoring of five captive lionesses enabled detection of females in natural oestrus (high frequency of purring, flirting run, lordosis, allowing mount, and rolling behaviours; and >90% of superficial cells, absence of neutrophils, large number of bacteria, and clean background in the vaginal smears). In parallel, blood sampling (n=188; 37.6 ± 4.07 samples per female; range: 23-47) took place 1-7 times per week, during positive reinforcement training. A competitive enzyme immunoassay utilizing antibodies against 5 β -pregnane-3 β -ol-20-one-3HS:BSA was used for serum progesterone (sP) quantification. Transrectal ultrasounds of the reproductive tract were performed on day 6. Ovulation was confirmed by absence of oestrous signs, a predominant proportion of parabasal and intermediate epithelial cells associated, or not, with neutrophils and a dirty background in the vaginal smears, a rise in sP concentrations, and presence of ovarian *corpora lutea* (0.8 ± 0.84 CL; n=8 complete examinations; range: 0-2 CL). Induced ovulation occurred in all cases (40% of ovulations occurred about 72h after BA administration, 20% occurred about 24h after BA, 20% about 48h after BA, and 20% about 96h after BA). Induced pseudopregnancy was about 59 days in length (n=10; range: 56-65 days). BA proved to be a valuable tool to induce ovulation in African lions. Its use may help to improve assisted reproduction techniques for this and other threatened large felids.

Predictors of testosterone in zoo-managed African elephants (*Loxodonta africana*)

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Abstract:

Reproductive complications for both male and female zoo-managed African elephants (*Loxodonta africana*) contribute to the rapidly declining population. In captive bull elephants, few studies have explored the potential physiological, physical, social, and environmental factors that influence bull fertility, particularly, androgen production. Testosterone is the essential steroid hormone for male sexual maturation and inadequate concentrations can be detrimental for spermatogenesis. For this study, testosterone, cortisol, leptin, glucose, insulin, and triglycerides were analyzed from weekly fecal and blood serum samples taken over six months in six zoo-managed African elephant bulls (10-19 years of age). Testosterone levels were compared to endocrine factors, weekly social and environmental variables, daily musth signs, and body condition scores (BCS). The glucose-to-insulin ratio (G:I) was the only physiological biomarker found to be positively associated with testosterone. Predictive physical variables included Musth Score (+) and Moderate Exercise (+). Bulls with body condition scores signifying overweight (BCS 4) had lower testosterone (36.6 ± 1.6 ng/g feces) than bulls with healthy body condition scores (BCS 3; 51.2 ± 4.9 ng/g feces). Numerous social variables influenced testosterone concentrations, including Total Contact Day (+), Female Interaction Day (+), Indirect Contact Day (+), Indirect Contact Night (+) and Total No Contact (-). Both percentage of Time Outdoor and Time Mixed positively influenced testosterone, whereas, testosterone decreased for percentage of Time Indoors. Each additional daily browse opportunity increased testosterone by approximately 7ng/g feces. In managed care, the emphasis should be placed on optimizing these markers of testosterone production to promote bull reproductive health.

Use of a combination of synthetic oral progesterone and prostaglandin F2-alpha for ovulation induction protocols in *Chinchilla lanigera*.

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Abstract:

Altrenogest is a synthetic oral progesterone agonist used in farm animals as pre-treatment for suppressing the natural estrous cycle, thus mimicking an extended luteal phase for as long as the progestin is administered. Because progesterone has an inhibitory effect on the release of LH, the rationale is to artificially maintain an elevated concentration of progesterone until the corpora lutea regresses. Following withdrawal, estrus and ovulation should occur at a predictable time. The effectiveness of this synthetic progestagen was evaluated in an ovulation induction protocol for *Chinchilla lanigera* applied together with prostaglandin-F2 α (PG-F2 α). Two i.m. injections of PG-F2 α (100 μ g cloprostenol-sodium; Ciclase-DL) were applied 7 days apart to sixteen females. During those 7 days, 0.1 mg/kg/day of altrenogest (Progestal) was orally administered to each animal. Half of the females also received 100 U.I. of eCG (Novormon-5000) at time of the second PG-F2 α dose. All urine samples produced by the animals were collected one week before, during and one week after the injections. Samples were analyzed for creatinine (CRT, Sigma #C3613), pregnanediol glucuronide and estrone conjugates (PdG-R13904, EC-R522-2, C.Munro) by EIA. The results obtained in both protocols showed that altrenogest applied with PG-F2 α successfully suppressed ovarian activity evidenced by the basal EC and PdG concentrations observed during the days of administration, with the exception of a slight increment in PdG levels in one of the protocols, attributed to oral administration of the synthetic progestagen, which is excreted mainly as PdG. After altrenogest withdrawal, an increase in EC and PdG levels as sign of ovarian activation, was observed only in the protocol that also included an eCG injection. It is likely that the altrenogest dose administered was too high and probably produced an excessive reduction in ovarian activity and therefore, the progesterone withdrawal alone was not enough to induce ovulation. The study reported here was approved by the IACUC of the Faculty of Medicine.

Changes in fecal androgen levels and agonism rates in the mating versus post-mating seasons in black-and-white ruffed lemurs

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Abstract:

Agonism, the suite of displacement-causing and submissive behaviors that often occur among group-living species during times of conflict, has been identified as an evolutionary strategy that confers food and fitness-related benefits to high-ranking, dominant individuals. The “challenge hypothesis” predicts androgen levels and incidents of agonism to be enhanced in dominant individuals in order to maintain their social status and combat the challenges associated with mating season competition. The objective of this study was to examine mating versus post-mating season differences in androgen levels and agonism rates of male black-and-white ruffed lemurs, a female-dominant primate species endemic to Madagascar. We evaluated fecal androgen levels, agonism rates, and dominance ranks during the mating (May-July) and post-mating (August-September) seasons to test the following predictions: in male ruffed lemurs, we expected 1) higher androgen levels and agonism rates to be exhibited in the mating season compared to the post-mating season, 2) high-ranking individuals to exhibit greater agonism rates than low-ranking individuals, and 3) androgen concentrations to be associated with male dominance rank. We used a series of two-sample and paired t-tests to evaluate differences within and between males inside and outside of the mating season. We observed non-significant trends indicating that male androgen levels ($p = 0.06$) and agonism rates ($p = 0.08$) were higher, on average, during the mating season compared to the post-mating season. Pearson correlation coefficients indicated a significant, positive relationship between male dominance ranks and agonism rates ($p < 0.05$). However, dominance ranks and androgen levels were unrelated ($p > 0.05$), suggesting that mating-season increases in androgens overall are unlikely to have strong effects on an individual’s social status; rather, heightened androgen levels during the mating season may be contributing more towards essential reproductive processes, such as spermatogenesis, as opposed to facilitating ruffed lemur agonism.

Characterising the reproductive physiology of *ex situ* Owston's civets (*Chrotogale owstonii*)

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Abstract:

Zoos house important insurance populations designed to support threatened *in situ* populations. However, owing to low rates of reproductive success in recommended pairs, many captive breeding populations are unsustainable. A striking example is the Endangered Owston's civet, *Chrotogale owstoni*, in which captive populations have been plagued by low rates of reproductive success and are currently too small to be sustainable. *Ex situ* conservation of the species is further complicated as little is known about the species' life history or biology. Using a combination of non-invasive endocrine and behavioural data collection, we characterised the reproductive biology of the Owston's civet. Monoclonal progesterone (P4) enzyme linked immunoassay (EIA) CL425 was biochemically validated for Owston's civets. Preliminary analyses of faecal samples collected from 5 individual females (n=456 samples, mean \pm S.E. = 91.20 \pm 43.45 samples per female) has shown a clear breeding season with elevated, variable P4 concentrations from late January to mid-April in two individuals, a parous 6 year old and a nulliparous 10 year old. Increased concentrations during the breeding season were more pronounced in the parous female; with higher average P4 concentrations over the period. To determine whether this observed difference in concentration may correlate with reproductive success, further samples and data from additional females are currently being added to this dataset. These data will inform management practices in the *in* and *ex situ* captive populations, as well as the species' conservation strategy.

Plasma progesterone secretion during gestation of captive short-beaked echidnas (*Tachyglossus aculeatus aculeatus*)

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Abstract:

This study describes the progesterone profile and changes in pouch development during pregnancy in captive short-beaked echidnas (*Tachyglossus aculeatus aculeatus*). Key reproductive behaviour was monitored daily by video surveillance. Plasma samples were collected and pouch morphology examined three times a week. The female echidna pouch only develops during gestation and it was possible to create a four-stage grading system using the most distinguishable characteristics of development. Maximum pouch development was associated with declining progesterone concentrations with the pouch closing in a drawstring-like manner at egg laying. Control of pouch development in pregnant echidnas is unclear but pouch closure may be under mechanical influences of egg or young presence in the pouch. Plasma progesterone peaked to 10.5 ± 0.9 ng/mL within 12 days of mating, declined to baseline levels within 1 day of egg laying and remained basal throughout incubation, confirming that progesterone is elevated throughout pregnancy and that gestation does not extend beyond the luteal phase. The length of pregnancy was 16.7 ± 0.2 days with a 15.1 ± 1.0 days luteal phase followed by an incubation period of 10 days in the pouch. Eggs could be detected *in utero* at least 4 days before laying. After the loss of an egg or pouch young, most females entered a second oestrous cycle and ovulated, suggesting echidnas are seasonally polyoestrous. The association of luteal phase and progesterone elevation is similar to that seen in marsupials and eutherians suggesting that this process has been conserved for over 184 million years of mammalian evolution.

Cortisol correlates positively with TGF β 2 and negatively with INF- γ in amniotic and allantoid fluid in wild boar: a case report

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Abstract:

Wild boar populations (*Sus scrofa*) are expanding and intruding into urban areas over the world. The urban food sources and the habitat structure of the cities promote their expansion in these areas. Their reproductive cycle is characterized by early puberty, high fertility, and a short gestation period. Jointly with embryo development, appropriate homeostasis and extraembryonic environment are essential for the fertility outcome. Among other components, glucocorticoids and cytokines play crucial roles in several biological functions during the maternal-fetal interface. This study aimed to identify (i) cortisol concentrations, (ii) pro- and anti-inflammatory cytokines, and (iii) correlations between them in the amniotic and allantoid fluids of wild boar ($n=4$ fetuses). The pregnant female, of 33 months of age, presented concentrations of hair progesterone and fecal progesterone metabolites (Progesterone ELISA KIT; Neogen Corporation) of 640.88 pg/mg and 4598.17 ng/g, respectively. The cortisol concentration (Cortisol ELISA KIT; Neogen Corporation) was 1.97 ± 0.63 ng/mL and 2.33 ± 0.27 ng/mL, for amniotic and allantoid fluid, respectively. Cytokine results (Luminex® xMAP® technology) showed an increase of the pro-inflammatory cytokines IFN- γ , IL-18, IL-8, and TNF- α in the amniotic fluid. However, IL-12, IL-1 α , and IL-6 were lower in the amnios. IL-12 was absent in amnios, and IL-1 β , IL-2, and IL-10 were absent in both fluids. The anti-inflammatory cytokines IL-4, TGF β -2, and TGF β -3 were higher in the amniotic fluid, whereas IL-1RA and TGF β -1 were lower in the amniotic fluid. Cortisol showed a negative correlation with INF- γ in both fluids. However, a positive correlation was found in TGF β 2 and IL-6 (allantoid fluid); and TGF β 1, TGF β 2, TGF β 3, and IL-1 α (amniotic fluid). The anti-inflammatory predominant cytokine profile and the confirmed correlations with cortisol concentrations, require further analyses to define suitable biomarkers for pregnancy maintenance in this species. Supported by the Research Council FORMAS (Project 2019-00288), Sweden; and PID2019-108320RJ-I00, Spain.

High dietary fiber enhances fecal androgens levels excretion: experimental analysis in brown brocket deer (*Mazama gouazoubira*)

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Abstract:

Non-invasive endocrinology is an important tool for animal conservation, but its success depends on many factors (e.g. adequate hormonal extraction, diet). Dietary fiber is one of the main sources which can confound the interpretation of the reproductive status provided by EIA analysis. This study aimed to evaluate the effect of dietary fiber on the excretion of fecal androgen metabolites (FAM), on the daily defecation rate and fecal production, as well as to analyze the gastrointestinal passage and retention time of the experimental diets. Eight brown brocket deer (*Mazama gouazoubira*) males were randomly assigned to two groups and submitted to both isocaloric and isoproteic experimental diets for 10 days, in a crossover system: low fiber percentage feed (LF, 7% fiber) and high fiber percentage feed (HF, 19% fiber). Such groups were alternated in the middle of this period, with an interval of 10 days between them. In addition, there was a five-day adaptation phase at the beginning of each diet. Fecal collection for FAM measurement was performed during 10 days of treatment, whereas, the rate of defecation and fecal production were performed every two hours, for 6 days. Utilizing a polyclonal anti-testosterone antibody (R156/7), the mean FAM concentration in the HF group was 5038.0 ± 1529.1 ng/g, while for LF, 2178.7 ± 824.9 ng/g ($p < 0.05$). The mean HF fecal production was 182.6 ± 36.2 g DM/day and 117.5 ± 12.6 g DM/day for LF ($p < 0.05$). There were no differences in terms of mean defecation rate, passage, and retention times between groups. The results suggest that dietary fiber affects the fecal androgens metabolites excretion, and this should be taken into consideration before conducting experiments using fecal samples as a source of reproductive hormones profiling.

An investigation of ovarian and adrenal hormone activity in pregnant and non-pregnant luteal phase cheetahs (*Acinonyx jubatus*) after natural breeding or artificial insemination

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Abstract:

Although cheetahs have been the subject of reproductive study for over 35 years, ovarian and adrenal hormone activity are poorly described in pregnant and non-pregnant animals after natural breeding and artificial insemination (AI). Utilizing fecal samples collected at the Smithsonian Conservation Biology Institute (SCBI), our objective was to measure and compare fecal progesterone (fPM), estrogen (fEM), and glucocorticoid (fGM) metabolite concentrations and patterns in females that were either pregnant (PREG) or experiencing a non-pregnant luteal phase (NPLP) following natural breeding or exogenous hormone stimulation and AI. Fecal samples were collected a minimum of 3x/week from 12 females for at least two weeks prior to (PRE) through 92 days post-breeding/exogenous hormone injection. Samples were divided into trimesters for statistical analysis. Fecal profiles were classified as 1) PREG (n=15), confirmed by birth of cubs; 2) Natural NPLP (n=7), categorized by a luteal response after natural breeding, but no cubs born; and 3) AI NPLP (n=5), those with exogenous hormone administration and AI, but no cubs resulting. Fecal samples were assessed for steroid hormone concentrations using enzyme immunoassays with antibodies previously validated by SCBI in cheetahs (fPM: CL425; fEM: R4972; fGM: R4866). Measured fPM followed expected patterns from previous studies for pregnant and non-pregnant groups. For all groups combined, fEM were lower in the first trimester ($p < 0.01$) and higher in the third trimester ($p < 0.01$) than PRE concentrations, but second trimester were not different ($p = 0.8668$). There were no differences among groups in fGM, but in PREG females, concentrations were higher ($p < 0.01$) in the last trimester than any other time. Interestingly, for PREG females that gave birth to singletons, fGM were higher ($p = 0.0205$), but fEM tended to be lower ($p = 0.0626$) than those with multi-cub litters. Our results provide additional insight into the physiological events surrounding natural and artificially stimulated luteal activity in the cheetah.

**The use of the anti-müllerian hormone as a biomarker of ovarian function in wild maned wolves
(*Chrysocyon brachyurus*)**

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Abstract:

In the last decades, the Anti-Müllerian hormone (AMH) has aided studies aiming to evaluate reproductive potential, gonadal function, and ovarian aging of domestic mammals, with great potential to improve assisted reproduction techniques. However, its use in wildlife is still in its infancy. Our study aimed to biologically validate and measure AMH concentrations in a threatened canid species, the maned wolf (*Chrysocyon brachyurus*), in order to describe its variation throughout different ages and reproductive states. We used 29 banked serum samples from wild females between 4 months to 10-years-old. AMH was assayed using a commercially available ELISA kit (AMH Gen II ELISA, Beckman Coulter, Inc., Brea, CA, USA). Baseline concentrations found in our research (0.586 ± 0.517 ng/ml) were similar to those reported in bitches. Linear regression analysis did not find a significant relationship between AMH levels and age progression. However, there was a significant difference between serum concentrations of immature females (0.299 ± 0.150 ng/ml) and matures ones (1.142 ± 0.301 ng/ml). Additionally, we also identified significant differences between values during the breeding season (1.718 ± 0.650 ng/ml) and non-breeding season (0.772 ± 0.243 ng/ml). The difference in concentrations between immature and mature females suggests that AMH is a valuable ally in evaluating pubertal changes in gonadal function. Our findings also point to the breeding season effect on AMH production, which is not common to all seasonal breeders. Ultimately, our data represent a first step towards the usage of AMH as a biomarker of ovarian function in this species, with potential applications in the study of follicular dynamics, improvement of ARTs and in the assessment of female's fertility on captive breeding programs.

Steroid hormones as biomarkers for reproduction and stress response in blubber of gray whales from the Eastern North Pacific

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Abstract:

The goal of the present study was to provide updated insights on reproductive and stress response physiology of gray whales from the Eastern North Pacific, using steroid hormones as biomarkers. Concentrations of the reproductive hormones, testosterone and progesterone, and of the stress-related hormones, corticosterone and cortisol, were validated and measured using enzyme immunoassay techniques in blubber tissues from 106 individual whales sampled from March to November over a span of 12 years (2004 – 2016) between California and Alaska. Testosterone concentrations in males increased significantly with age ($p = 0.03$) and adult males had significantly elevated hormone concentrations when sampled in the fall compared to the summer ($p = 0.01$), likely indicating physiological preparation for mating. Progesterone concentrations were significantly higher in pregnant females compared to non-pregnant groups of females and adult males ($p < 0.001$), indicating this hormone is a valid biomarker for pregnancy. A mixture of two normal distributions was fit to progesterone concentrations of females to estimate the probability of being pregnant for whales of unknown reproductive status. Both female and male calves had elevated progesterone concentrations, suggesting maternal offload via lactation. Corticosterone was found to be significantly higher in females than males ($p = 0.003$) and calves had significantly elevated levels compared to adult ($p = 0.05$) and immature ($p = 0.008$) gray whales. Reproductive status was found to be a significant factor in determining cortisol concentrations in females of known state (i.e., immature, lactating and pregnant) ($p = 0.02$), with lactating whales showing higher concentrations than pregnant and immature whales ($p = 0.01$). With the increasing interest in developing markers for monitoring wildlife stress responses, it is important to understand variation of hormone profiles in relation to naturally occurring processes such as reproduction.

Determining sex steroid hormone concentrations in adult and juvenile male and female zebra sharks (*Stegostoma fasciatum*)

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Abstract:

Managed breeding programs can play an important role in the conservation of elasmobranch species. However, for breeding programs under managed care to be successful, it is important to understand the basic reproductive physiology of the species. Although there is some information on endocrine parameters of adult female zebra sharks, there is no information for adult male or juvenile zebra sharks. The purpose of this study was to examine hormonal differences between juvenile and adult male and female zebra sharks (*Stegostoma fasciatum*) in aquaria. Previously collected plasma samples were used from nine male (two mature and seven immature) and twelve female (three mature and nine immature) individual, stored frozen (-20°C) until analysis. A double diethyl ether extraction method was utilized to extract the steroid hormones from the plasma. Samples were analyzed using a competitive double antibody enzyme immunoassay using antibodies and corresponding horseradish peroxidase (HRP) conjugates (C. Munro, University of California, Davis, CA) to progesterone (11 α -hemisuccinate progesterone; R4859;), estradiol (1,3,5(10)-estratrien-3, 17 β -diol; R0008) and testosterone (testosterone-6-carboxymethyl oxime; R156/7). Serial dilutions of pooled extracted plasma yielded curves parallel to the standard curve. Preliminary results show higher testosterone concentrations in male versus female ($p < 0.05$) and juvenile ($p < 0.05$) zebra sharks, and higher estradiol and progesterone in adult female ($p < 0.05$) than juvenile female zebra sharks. These findings provide biological validation for the assays used and set a baseline that will help to understand the reproductive biology of this species that will allow zoos and aquaria to achieve healthy managed breeding programs.

Seasonal endocrine changes in female sand tiger sharks (*Carcharias taurus*)

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Abstract:

Sand tiger shark offspring production under managed care has been limited to 27 individuals in seven aquaria worldwide, with most pregnancies resulting in premature or still births. Wild sand tiger sharks experience seasonal changes of temperature and light, previously demonstrated to influence male plasma testosterone concentration and semen production. Females have a two to three year reproductive cycle, with an active ovary in one year where numerous eggs are produced, followed by one or two resting years, presumably to recoup energy after egg production. To better understand female reproductive endocrinology, lithium-heparin plasma was collected from aquarium female sand tiger sharks (n=26) throughout the year and stored frozen (-20°C) before double-ether extraction. This project received IACUC approval. Plasma was analyzed using a competitive double antibody enzyme immunoassay using antibodies and corresponding horseradish peroxidase (HRP) conjugates (C. Munro, University of California, Davis, CA) for progesterone (11 α -hemisuccinate progesterone; R4859) and estradiol (1,3,5(10)-estratrien-3, 17 β -diol; R0008). Serial dilutions of plasma and hormone standard for progesterone and estradiol gave parallel dose-response curves. Results (mean \pm SEM) showed lowest concentrations in January-March, followed by increasing estradiol concentrations to highest concentrations in April (0.58 \pm 0.23 ng/ml) and sustained elevated concentrations that declined in August to reach nadir concentrations in January (0.08 \pm 0.002 ng/ml). Progesterone concentrations were low in Jan-Mar and increased starting in April to highest concentrations in May (1.26 \pm 0.27 ng/ml), before declining gradually to October (0.19 \pm 0.04 ng/ml). Results show a seasonal increase in estradiol that preceded an increase in progesterone, coinciding with the known breeding season (March-July). Further investigations will compare wild and aquarium females and examine effects of aquarium water temperature on hormone concentrations.

Determining the relationship among estrogen, estrus, and behavior in the female black-footed ferret

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Abstract:

Endangered black-footed ferrets (*Mustela Nigripes*) have experienced a continued decrease in reproductive success in captivity, with pregnancy decreasing from 70% in 1996, to 34% in 2020. Behavior has not been fully assessed as a cause, even though natural mate searching behaviors are prevented during assigned breeding. We aimed to characterize the relationship among reproductive hormones, estrous state, and behavior of this olfactory dominant and fossorial mustelid. During the breeding season, females (n=20) were offered body odors of two males using a modified “T” maze, and their behaviors were observed. Fecal estradiol metabolites (FEM) were analyzed, using an enzyme immunoassay (Munro, R0008) as a proxy for reproductive state and to determine their influence on behavior. Estrus was determined by vaginal cytology analysis with 90% superficial keratinized cells indicative of estrus. We found no relationship between FEM and vaginal cytology; however individual FEM was highly variable. Mean (\pm SE) FEM was similar ($P>0.5$) between females in estrus (-4 to 6 days from reaching 90%, 3.36 ± 0.25 ug/g) and not in estrus (-21 to -5 days from 90%, 2.84 ± 0.17 ug/g). Female exploration of male odor and proportion of time spent sniffing was not driven by FEM; however, mean (\pm SE) proportion of time spent sniffing was higher ($P=0.008$) for females in estrus (67.0 ± 7.87) than females not in estrus (37.2 ± 5.91). Overall, estrous females have a higher interest in exploring the scents of potential mates and letting them display these instinctual behaviors before pairings could provide enrichment or be used to study mate choices. Next steps will involve evaluating a different estrogen EIA to determine if there is a more appropriate assay to capture pre-breeding hormone changes and their influence on behavior. Additionally, FEM evaluation could further inform on male mate choices, another future direction aimed at improving fitness in this rare species.

Hormone levels predict spatial preferences by female tiger sharks exposed to provisioning tourism

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Abstract:

Studies on the impacts of provisioning tourism on shark behavior have been growing, however how physiological condition can influence decision-making at provisioning sites remains largely uninvestigated. In the present study, we used reproductive (testosterone, 17 β -estradiol and progesterone) and metabolic (corticosteroids) hormones, integrated with ultrasonography and passive acoustic telemetry to non-lethally explore the relationship between hormone levels and decision-making by female tiger sharks (*Galeocerdo cuvier*) exposed to provisioning tourism. In total, 33 sharks were sampled and tagged at a popular location for shark diving tourism in the northern waters of Little Bahama Bank, Bahamas, but only 22 individuals were detected within 90 days. Our results showed that, while gravid females (n=7) had more varied spatial behavior, adult, but non-gravid females, spent significantly more time at provisioning sites (mean \pm standard deviation, 20.6 \pm 31.4 hours, n=9) than immature females (2.3 \pm 5.5 hours, n=6), which preferentially used non-provisioning sites (83% of individuals). Among all hormones measured, only testosterone concentrations were positively correlated with proportion of time sharks spent at provisioning stations. However, when separated into provisioning (n=7) and non-provisioning (n=9) groups, female at provisioning group had higher testosterone (276.5 \pm 221.4 pg mL⁻¹), 17 β -estradiol (204.3 \pm 213.2 pg mL⁻¹) and relative corticosteroids concentrations (6471.5 \pm 9793.9 pg mL⁻¹), compared to non-provisioning group: testosterone (89.9 \pm 39.4 pg mL⁻¹), 17 β -estradiol (50.8 \pm 72.3 pg mL⁻¹) and relative corticosteroids concentrations (394 \pm 507.0 pg mL⁻¹). These findings suggest that hormones, especially testosterone, possibly play an important role in shaping spatial behavior in female tiger sharks in the context of food provisioning, also suggesting the involvement of this androgen in dominance behavior in tiger sharks. This study highlights the importance of considering life-stage and endocrine regulation of sharks, with particular reference to within-individual variation, to improve our understanding of the impact of the provisioning tourism on apex predators. Procedures were approved by the UMiami Institutional Animal Care and Use Committee (Protocol-15-238).

The reproductive endocrinology of female mountain pygmy-possums (*Burramys parvus*): a comparison of successful and unsuccessful breeders

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Abstract:

Species extinction is a global crisis, and conservation programs are increasingly reliant on captive breeding. While widely viewed as a useful tool in conservation biology, captive breeding is often unsuccessful. Reproduction of the critically endangered mountain pygmy-possum (*Burramys parvus*) in captivity has been variable, with some animals breeding multiple times and others failing to reproduce, despite repeated attempts. Here we used non-invasive urinary hormone monitoring to investigate reproductive physiology in captive *B. parvus* females. The aims were to (1) test whether enzyme-immunoassays (EIAs) can be used to monitor biologically relevant changes in reproductive endocrinology via *B. parvus* urine, (2) describe the estrous cycle and reproductive physiology of females that successfully bred in captivity, and (3) compare the reproductive profiles of successful and unsuccessful breeders. Samples were collected over one breeding season from three reproductively successful and three unsuccessful females. The progesterone assay was successfully validated based on alignment of profiles with pregnancy and other reproductive events, but the estradiol assay failed to reflect biologically relevant changes in physiology. The three successful females each exhibited 3 estrous cycles over the course of the breeding season. The average duration of an estrous cycle was 27.4 ± 4.98 days, with an average luteal phase length of 8.4 ± 4.23 days. In contrast to the females that successfully gave birth, no estrous cycles or ovulations were detected in any of the unsuccessful females. Urinary progesterone metabolite concentrations in unsuccessful females remained low throughout the study, indicating anestrus. This is the first endocrine study of reproduction in female *B. parvus* and our findings can be used to assist in future captive breeding of this species, and also aid conservation efforts in the wild.

First antler development and its relation to fecal androgens metabolites in deer: an example with some species of *Mazama*

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Abstract:

Puberty in deer from temperate regions is defined as a period that extends from 9 to 15 months of age or as early as 6 months. In male deer, it is attained by a steady increase in testosterone secretion which its peak corresponds to the beginning of the reproductive ability, and responsible for the secondary sexual character development (e.g. antler). To date, no information regarding the puberty onset is available for the *Mazama* species. In this study, we aimed to characterize the development of the first antler and its relation to fecal androgens metabolites (FAM) profile, identify the first peak of FAM levels, which corresponds to puberty onset and the beginning of the reproductive ability, in four species of *Mazama*: *M. americana* (A, n=1), *M. bororo* (B, n=1), *M. nemorivaga* (C, D, n=2) and *M. gouazoubira* (E, F, n=2). The fecal collection was performed since the feces of the fawn were detected and the antler phase was recorded and FAM levels were measured by EIA using a polyclonal anti-testosterone antibody (R156/7). The pedicle was observed at 7-12 months and there was no increase in FAM during pedicle formation. Antler growth was associated with low levels and a steady increase in FAM (except in male E). Antler shedding occurred at 11-18 months and associated with the peak of FAM levels in *M. americana*, *M. bororo*, and male D of *M. nemorivaga*, which corresponds to the beginning of reproductive ability, while in others, antler shedding was characterized by a steady increase (male F) or steady FAM levels (males C and E). We suggest that antler development might follow an endogenous rhythm among males since FAM levels might not be associated with antler phases in some *Mazama* species and puberty onset varied between 11-18 months among deer.

Reproductive events and respective faecal androgen metabolite concentrations in captive male roan antelope (*Hippotragus equinus*)

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Abstract:

Over the past few decades, populations of roan antelope (*Hippotragus equinus*) have declined drastically throughout Africa, especially in southern Africa where the species is now classified as vulnerable. Consequently, intensive breeding programmes were developed to assist conservation efforts, but basic information on, especially male, roan antelope reproductive biology, including endocrine correlates is scarce. Therefore, the present study examined the patterns of faecal androgen metabolite (fAM) concentrations and male sexual behaviours in relation to reproductive events (mating, calving, gestation and lactation) in females. For a period of 12 months, three breeding males at Lapalala Wilderness Nature Reserve (South Africa) were observed, and faecal samples collected for hormone monitoring. FAM concentrations were quantified using, an enzyme immunoassay utilizing antibodies against testosterone-3-CMO: BSA. The assay was biologically validated by demonstrating a significant difference between fAM concentrations in non-breeding adults, breeding adults and juvenile males. Results showed that overall mean fAM concentrations were 85% higher during the breeding period when compared to the period of lactation/gestation, but only 5.3% higher when compared to the birthing period. Furthermore, fAM concentrations were lower during the wet season compared to the dry season and rose with the diminution of the photoperiod. Finally, frequencies of all sexual behaviours monitored, with the exception of courtship varied with fAM concentrations. This first detailed description of the reproductive biology in the male roan antelope could also help conservation efforts to optimise the breeding of captive roan antelope populations.

Determining the efficacy of non-invasive sampling techniques for hormone assessment of elk in East Tennessee, USA

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Abstract:

Elk (*Cervus canadensis*) were reintroduced to East Tennessee, USA in the early 2000s and have since established a population within their historic range. Due to sampling constraints, little research regarding population reproductive parameters has been conducted since reintroduction. We will assess hormone concentrations of adult female elk using invasive and non-invasive sampling at the North Cumberland Wildlife Management Area (NCWMA) in Tennessee. Pregnancy-specific protein B testing will be used as a confirmation of pregnancy in blood samples collected from captured females and compared with fecal progesterone metabolite (FPM) of feces collected concurrently. Concentrations of FPM from confirmed pregnant females will be used as reference range in comparison to samples collected non-invasively on the landscape and identified by elk genotype. Fecal and blood samples were collected from 21 captured females during the winters of 2019 and 2020. We collected 375 fecal samples from 65 collection areas during an 11-week sampling period from February to May 2019. We used DNA extracted from hair of captured females and fecal samples collected on the landscape to identify individual elk. Genetic analysis included sex determination and identified 73 individuals from 128 confirmed female samples collected non-invasively. Nine individuals had data from all three means of sampling: blood and fecal samples collected from capture, and non-invasive fecal collection on the landscape. Subsequent FPM analysis will compare hormone concentrations across all fecal samples to determine whether non-invasive sampling provides consistent results and can be successfully used on a population scale as an alternative to invasive techniques. Non-invasive techniques have been shown to provide a stress-free alternative to assess natural hormone concentrations and evaluate reproductive parameters in many species. More research on the viability of non-invasive hormone analysis for the application of population level hormone assessment of elk at the NCWMA is warranted.

The novel use of androgens to optimise estrus detection in giant pandas

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Abstract:

Giant pandas are seasonally monoestrous, with increasing estrogens in Spring for 1-2 weeks before a peak is reached, followed by 24-48 hours of fertility. Accurately monitoring the hormone profile is vital for prediction and detection of this fertile window to facilitate natural mating/insemination in a captive breeding programme. We therefore aim to consider biomarkers for advanced warning of estrus and the accurate timing of insemination. Urine samples from six females covering 16 breeding cycles (total 1010 samples), from 40 days before estrus until 10 days after estrus, were assayed for estrone-3-glucuronide (E1G) and progesterone (P4) by Arbor Assays ELISAs, and for testosterone (T) and dehydroepiandrosterone (DHEA) by validated in-house ELISAs. Hormone concentrations were corrected against urinary specific gravity (USpG). E1G increased for 11.2 days (range 9-14) before peaking, with the start of the increase detected retrospectively. Prospectively, using current methods, estrus starts when E1G concentrations become higher than P4 (9.2 days, range 7-11). Alternatively using androgens, E1G becoming higher than T gives 4-days advanced warning of impending estrus (13.1 days, range 9-17). In determining peak E1G, DHEA showed a 1.7-times increase on day -1 (NS), and a significant 3.9-times increase ($p < 0.001$) on the day of peak E1G. Thus monitoring T alongside P4 can provide 4 days additional warning of an upcoming estrus, and moreover monitoring DHEA can aid in identification of peak E1G without needing to observe a decrease in E1G itself. T and DHEA respectively enlarge the preparative window for captive breeding, facilitate panda management within the *ex-situ* conservation breeding programme, and unequivocally confirm impending estrus. Concluding, measuring androgens advances and optimises how estrus is monitored in the giant panda.