Application for Graduate Research and Creative Activities (GRACA) Funding

Student: Chelsi Marolf
Faculty Mentor: James A. Wilson, Ph.D.

Pregnancy in zoo-managed female African elephants (*Loxodonta africana*): Investigating possible hormonal and metabolic indicators of successful and unsuccessful pregnancies.

**Project Description:**

The population of African elephants (*Loxodonta africana*) in United States zoos is declining rapidly. At the current rate, these populations will be non-viable in approximately 50 years (Morfeld and Brown, 2014). Moreover, the current population of females is aging, and many have irregular reproductive cycles, making every mating and pregnancy crucial (Brown, 2000). In order to slow population decline, it is important to investigate the underlying causes of failed zoo pregnancies. The overlying issue of reproductive health could stem from various metabolic and reproductive factors, including hormone imbalances, metabolic syndrome (often defined as obesity, high blood pressure, insulin resistance, and glucose intolerance), as well as stress (Day, 2007). This study will examine at least 20 successful and 9 unsuccessful pregnancies within United States zoos (success defined as the calf being carried to term and surviving the birth). Reproductive hormones (progesterone and prolactin) and metabolic markers (leptin, insulin, and glucose) will be measured in serum samples throughout each pregnancy, and the data will be analyzed to identify trends or differences in each group. Progesterone and prolactin are hormones important for maintaining pregnancy, whereas leptin (associated with fat) and cortisol (associated with stress) are important health indicators that could provide measurable markers for metabolic syndrome. By comparing hormones and sugars between successful and unsuccessful pregnancies, it may be possible to develop reproductive markers associated with miscarriage and stillbirth and allow zoos to proactively care for mother elephants and increase the success rate of captive elephant births.

In human pregnancy, the ability to pinpoint baseline levels of hormones needed to maintain a pregnancy has aided in clinical analyses to prevent complications. Monitoring hormones from serum samples during an elephant’s pregnancy could be used in similar ways. Though information surrounding African elephant pregnancy is mostly unknown, reproductive cyclicity has been extensively studied. Reproductively “normal” African elephants exhibit a cyclical progesterone pattern (Plotka et al, 1988), while non-cycling females exhibit flat-line levels (Brown et al, 2004). Key differences between cyclic and acyclic elephants include varying levels of prolactin and progesterone (Brown 2016, Brown et al, 2004). In conjunction, ovarian acyclicity is often accompanied by hyperprolactinemia (Brown et al, 2016). In humans, these reproductive problems are usually treatable, giving hope that similar treatments might be possible for elephants. For example, pregnant women that suffer from recurrent pregnancy loss can be treated with progesterone to increase the likelihood of pregnancy success (Stephenson et al, 2017). Treatments have also been created for human patients with hyperprolactinemia, including pregnant women (Verhelst et al 1999). While this project is preliminary, it marks the first steps in identifying potential treatments that could be devised for elephants.

In addition to changes in progesterone and prolactin, decreased insulin sensitivity is known to cause reproductive and pregnancy complications in humans (Catalano 2010). This sensitivity is often due to hyperglycemia, a high blood glucose level (Dandona et al 2004). Glucose, which enters the body via food, causes insulin to be released from the pancreas. Insulin then aids cells in the uptake of glucose, which is necessary for energy. This process, and the levels of these two biomarkers, can be disrupted by obesity. In turn, obesity, insulin resistance, and diabetes can lead to polycystic ovarian disease and other...
metabolic disorders in women (Kahn and Flier 2000). Metabolic syndrome, defined by insulin resistance, obesity, elevated blood pressure, elevated biomarkers, and other abnormalities, has also been linked to polycystic ovarian disease in horses (Johnson et al 2012). Similarly, in elephants, the glucose and insulin concentrations, as well as leptin, are indicators of metabolic health and can affect ovarian cyclicity (Morfeld, Brown 2017). These factors provide potential metabolic indicators that may be associated with successful and unsuccessful pregnancies in elephants. If these three biological factors show high correlates in unsuccessful pregnancies, this would suggest that metabolic factors play a key role in elephant reproduction.

Stress is another important factor in the reproductive health of animals. Prolonged stress is thought to suppress or inhibit reproduction and may come in the forms of metabolic, immunological, and psychological stressors (Tillbrook et al, 2002), all of which are possible in a captive setting of zoo elephants. Glucocorticoids, including cortisol, act directly on the uterus and are important in early human pregnancy (Whirledge and Cidlowski, 2013). Similar associations have been observed in elephants as well, indicating significance of cortisol levels in both reproduction and possibly during pregnancy (Fanson et al, 2014). Due to a variety of stressors on zoo-managed populations, including housing arrangements (with social and physical space implications), transfers to different zoos, and the obvious human interaction, cortisol cannot be overlooked. While precise determinants of stress can be difficult to quantify, recent studies have evaluated the social and housing environments of elephants (Meehan et al, 2016) in relation to stress. If elevated stress is a common factor of unsuccessful pregnancies, this will only strengthen the argument to improve the wellbeing of the elephant population in zoos.

The main goal of this study will be to identify ranges of hormone and glucose levels throughout a typical successful pregnancy, and to compare these data to unsuccessful pregnancies. In the very least, preliminary data on a typical pregnancy can be obtained. This may consequently provide insight into miscarriages and stillbirths. Of the 58 pregnancies that will be analyzed for this project, 8 calves died in utero or during the birth, and one mother miscarried. While the birthrate has increased based on previous studies, the issue is still large enough to cause concern. African elephants are a vital part of many zoos in North America, and therefore failed pregnancies have huge implications on public opinion and funding. This provides great potential for future research in investigating pregnancies in the species.

**Methods and Materials:**

**Study Population**

This study will utilize serum samples from 35+ female African elephants sampled between 2000 and 2017. All serum will be obtained upon request from banked samples from the corresponding zoos taken at the time of the pregnancies. Serum has been and will be stored at -80°C until analyzed for each of the hormones and glucose in this study.

**Hormone Analyses**

Prolactin, progesterone, and cortisol will utilize multi-species enzyme immunoassays, according to each protocol (Arbor Assays, Inc., Ann Arbor, MI, USA, K040-H1, K025-H1, K003-H1). Assays have been previously validated for use in elephants. For each assay, 50 µL of each serum sample will be added in duplicate to a 96-well plate, along with standards and controls specific to each hormone. A plate is then incubated for one to two hours on a plate shaker, washed 4 times, and 100 µL of TMB Substrate is added to each well. The plate is incubated for a further 30 minutes and Stop Solution is added to halt reaction.

Insulin will be analyzed using a bovine enzyme immunoassay (Mercodia, Inc., Uppsala, Sweden, 10-113-01). Methods will be followed according to the accompanying manuals. 25 µL of samples, standards, and controls will be added to each plate in duplicate. The plate is incubated on a plate shaker.
for 2 hours at room temperature, washed by hand 5-6 times with wash buffer, and 200 μL of TMB substrate is added to each well. The plate will then be incubated on the plate shaker for a further 15 minutes and finally, 50 μL will be added to each well. Leptin will be analyzed using an equine immunoassay, according to the kit instructions (MyBiosource, Inc., San Diego, CA, USA, MBSO12684).

For all five hormones, plates are read on a plate reader at 450 nm using 4PLC software (Dynex) and concentration values are produced using computer software (Revelation Quicklink). The duplicate wells of each serum sample, standard, and control are analyzed, and a mean of the two calculated. Samples with a coefficient of variation (CV) greater than 15% will be retested. All assays have been previously utilized for elephant serum samples and high rates of success are expected.

**Glucose Analysis**

For each serum sample, 15 μL will be pipetted onto a sterilized workbench and tested for glucose concentrations using a blood glucose meter (One Touch Ultra, LifeScan, Inc., Milpitas, CA, USA). Each sample will be measured twice and the mean of the two results calculated.

**Product of Funded Project/Contribution to Graduate Studies:**

I am currently working towards a Master’s in Biology with Dr. James A. Wilson as my academic and project advisor, in conjunction with Dr. Kari Morfeld, an elephant endocrinologist based at Omaha’s Henry Doorly Zoo and Aquarium. Approval of funding for this project will allow me to complete my thesis, which is a requirement for graduating. The stipend and laboratory funds would allow me to complete most of my sample analysis this summer. The remainder of funds needed for this study will be provided by collaboration with the Henry Doorly Zoo. I will then prepare my paper and presentation to be presented at the 2019 Student Research and Creative Activity Fair and potentially a national conference.

**Contribution to African Elephant Endocrinology:**

Results of this study will provide insight into reproductive disorders that plague zoo-managed populations of African elephants. Current research in the field of elephant endocrinology is primarily focused on metabolic health such as obesity as well as cyclicity, or the capability for females to become pregnant. This project will utilize the efforts of that research and apply it to case studies of successful and unsuccessful pregnancies to reveal connections. This could potentially affect future welfare and reproduction for zoo-managed African elephant populations, which are facing extinction.

**Project Timeline:**

Request serum samples from AZA institutions, purchase materials .............................................Spring 2018
Test samples ..........................................................................................................................Summer/Fall 2018
Statistical analyses, prepare thesis ........................................................................................Spring 2019
Present thesis, prepare paper for submission to research journal ........................................Fall 2019

**Student/Advisor Roles:**

The student’s role is to carry out all laboratory procedures, maintaining scientific integrity by keeping a laboratory notebook. At the conclusion of the project, the student will present the findings and prepare a paper to be submitted to a scientific journal. Dr. Wilson will serve as the primary advisor, overseeing project progress and use of funds. Dr. Morfeld, of the Omaha Zoo, will serve as a research advisor and aid in purchasing of supplies.
Budget Justification:

ELISA Test Kits (Arbor Assays, Mercodia) ................................................................. $2,300
Laboratory Supplies ................................................................................................... $200
Student Summer Stipend ......................................................................................... $2,500
**Total Amount Requested** .................................................................................. $5,000

Laboratory supplies for this project will include latex gloves, pipette tips, test tubes, glucose test strips, as well as the ELISA kits which will be purchased from Arbor Assays, Mercodia, and MyBiosource. The remaining budget will serve as a salary to offset costs of living between June and August to allow the student to allot the required time to complete the project.

Literature Cited:


10 February 2018

Dear Grant Committee,

I am writing on behalf of Chelsi Marolf, who is a graduate student working in my lab. Chelsi is in the first semester of her Master’s degree in Biology and is working on a collaboration project between my lab and the Henry Doorly Zoo. Chelsi is the first student in a long collaboration between me and Kari Morfeld who is associated with the zoo. I have been interested in physiological ecology since my graduate program and have published several papers in this subject. This collaboration is new and in the fall I anticipate taking a second student to work on the elephant reproduction project. I am also working to expand this collaboration to include a new course for undergraduates at UNO that would include taking students to Africa to perform fieldwork collecting data for use in future research projects. Chelsi’s work marks the beginning of this long process and is instrumental in understanding the basics of successful and unsuccessful elephant reproduction.

With funding from the GRACA program we will be able to obtain these plasma samples from elephants in zoos around the United States, including the Henry Doorly Zoo and perform hormone, metabolic, and stress measurements from elephants who successfully birthed a calf and those who did not. Chelsi’s project is imminently publishable and we fully anticipate publishing her work when she is finished. Chelsi has already shown me that she is an excellent science writer and is able to work independently. In addition Chelsi has the benefit of Dr. Morfeld, who is a leader in the field of captive elephants. I am confident Chelsi will be able to maintain the time table she presents in her GRACA. In fact, she has already started her proposal, has her thesis committee, and is making plans to obtain the plasma samples she needs. I hope that you find her proposal worth funding and I look forward to having Chelsi present the results of her findings at the funding colloquium when she is finished. Thank you for your support.

Sincerely,

James A. Wilson, Ph.D.
Assistant Professor
Tel: (402)-554-2585