The Friends of Nachusa Grasslands
2019 Scientific Research Project Grant Report
Due April 30, 2020

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2019 grant amount: $2613

Please answer the following questions with 1- to 2- sentence summaries:

Research Project Topic: Health assessment of ornate box turtles (*Terrapene ornata*) using venous blood gas and hormone analysis

Research Project Purpose: The purpose of our research was to 1) assess the response to acute and chronic stress in ornate box turtles, 2) explore the use of commercially available kits for measuring reproductive hormones in ornate box turtles, and 3) obtain additional data to supplement an existing study on venous blood gas in ornate box turtles.

Research Project Outcomes to date: Presentation at the Nachusa Science Symposium

Describe how the grant funds you have received from the Friends of Nachusa Grasslands have been used in regard to the above topic, purpose, and/or outcomes:

Grant funds were utilized to purchase 1) one plasma testosterone and one plasma 17β-estradiol kit, 2) plasma corticosterone testing for 50 turtles (100 paired samples) at the Saint Louis Zoo Endocrinology Lab, 3) iSTAT venous blood gas cartridges for 50 turtles, 4) shipping, and 5) needles, syringes, and heparinized tubes for blood sample collection and storage.

Describe how your project has benefited the work and goals of Nachusa Grasslands:

<u>Introduction to Our Research and its Place in the Nachusa Grasslands:</u>

The Nachusa Grasslands is committed to restoring native prairie grassland and conserving the species which rely upon this habitat. The Friends of Nachusa have taken special interest in the ornate box turtle (*Terrepene ornata ornata*), a state-threatened chelonian which relies heavily upon grassland habitat (such as the Nachusa Grasslands) in order to survive. Previous studies on the ornate box turtle conducted at Nachusa have focused on population characteristics, movement patterns, and behavior. While understanding population size, structure, connectivity, and resource availability/utilization is important for conservation planning, assessing animal health may also be useful for informing management decisions. Populations challenged by disease, toxins, or genetic

abnormalities may not respond in predictable ways to management interventions and could fail to rebound successfully after perturbation. Furthermore, understanding infectious disease burden is important when considering conservation actions like translocations or head-starting (currently under consideration for the box turtles at Nachusa and elsewhere in Lee County) to prevent the introduction of novel pathogens. We are conducting a multi-year longitudinal study of ornate box turtles at Nachusa to determine how temporal, spatial, environmental, and disease factors impact health at both the individual and population levels in order to design more effective conservation strategies. The goals of this project are aligned with the holistic conservation mission of the Nachusa Grasslands.

We have found that Nachusa's ornate box turtles experience a high rate of attempted predation, and shell injuries related to predator trauma are the most common cause of poor health in individual turtles. These injuries are associated with several bloodwork changes which can persist for prolonged periods after the injuries appear healed, potentially indicating a shift of resources away from growth and reproduction and towards wound healing. It is also likely that some turtles die as a result of their injuries, though the exact mortality rate is difficult to determine due to the activity of scavengers. Understanding the effects of these predator injuries on survival and reproduction is important to determine how aggressively predator control strategies should be pursued. Thanks to the support of the Friends of Nachusa during 2019, we have started to explore these relationships using reproductive and stress hormone assays, detailed below.

Reproductive Hormone Analysis

Testosterone is the major male sex hormone, while 17β-estradiol (estradiol) is the major female sex hormone. These hormones are responsible for initiating the reproductive cycle in chelonians. Measuring these hormones can help differentiate sex in individuals with intermediary external sex characteristics, and may help determine which turtles are reproductively active in a given year. Commercial kits are available to assess these hormones in plasma and they have been previously used in other reptiles, however, it is important to confirm that they work in ornate box turtles prior to planning large studies. This year, our objective was to confirm that testosterone and estradiol were detectable in ornate box turtle plasma using two commercially available kits. We also performed a partial validation of each kit, which will be completed using additional Friends of Nachusa grant funds for the 2020 cycle.

Two hundred microliter aliquots of plasma were collected from nineteen female ornate box turtles and ten males. A standard three-round liquid-liquid steroid extraction was performed using ethyl acetate and a dry ice-ethanol bath. The ether fractions were dried in a speed-vac, and the desiccated steroid pellets were resuspended in 425μL kit-specific assay buffer immediately prior to testing. A pooled plasma steroid sample including 25μL of steroid extract from 14 females and 10 males was created, then serially diluted from 1:2 – 1:2048 to evaluate parallelism (whether the hormone levels in the turtles mirror those of a kit-provided standard curve). In addition to being necessary for kit validation, the parallelism procedure helps identify the optimum plasma steroid dilution to

enable accurate hormone quantification. Kit instructions were followed to generate a standard curve and test both the pooled parallelism samples and plasma samples from each turtle for testosterone and estradiol concentrations. Quantification was performed using 4-parameter logistic regression.

The parallelism procedure was fully successful for the plasma testosterone kit, as the “neat”, or undiluted plasma pool had a testosterone concentration (8,162pg/mL) near the maximum standard concentration (10,000pg/mL) and linear regression of the parallelism curve revealed a similar slope (-35.07) compared to the standard curve (-32.3) (Figure 1). The optimal dilution for measuring plasma testosterone concentrations in ornate box turtles was identified as 1:16. This information will facilitate future use of this kit in ornate box turtles, and completes half of the assay validation procedure.

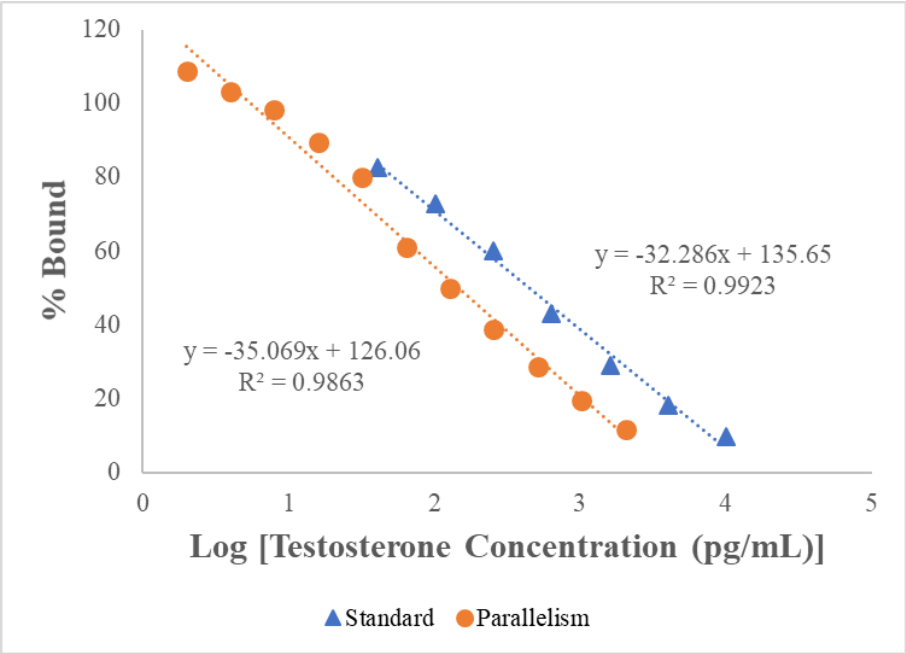

Figure 1. Plasma testosterone parallelism in ornate box turtles from Nachusa.

The concentrations of plasma testosterone observed in ornate box turtles were slightly higher than those previously identified in eastern box turtles, especially for female turtles. Despite this, plasma testosterone concentrations were significantly greater in males than females ($p < 0.0001$, Figure 2), indicating that this may be a reliable test to distinguish sex for ornate box turtles.

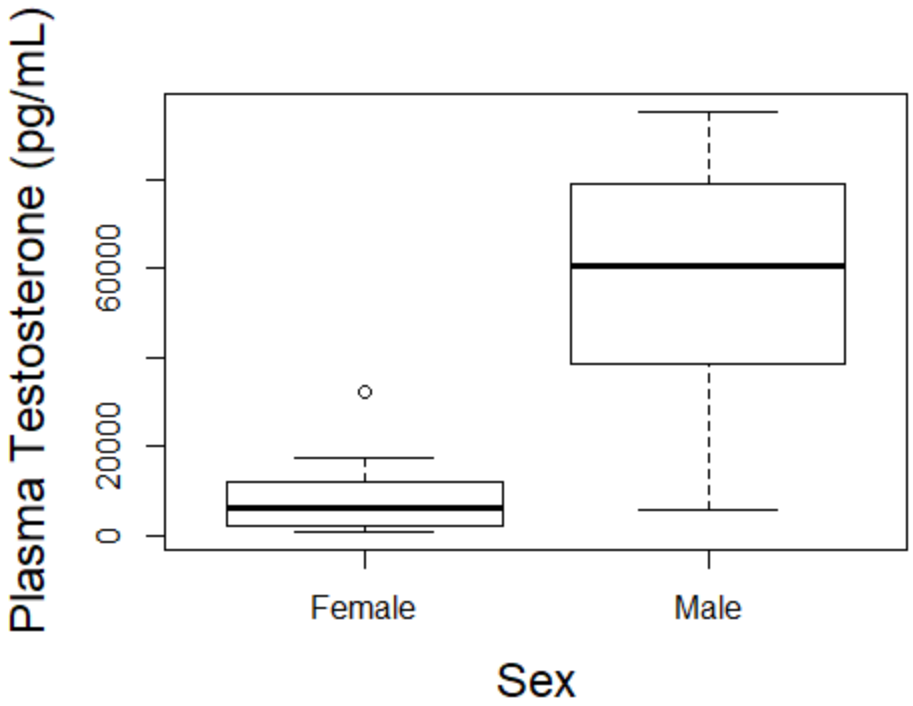

Figure 2. Plasma testosterone concentrations in ornate box turtles at Nachusa.

The parallelism procedure was only partially successful for the plasma estradiol kit, as the "neat" plasma pool had an estradiol concentration (155pg/mL) significantly lower than the maximum standard concentration (10,000pg/mL). This precluded completion of a parallelism curve, but did help determine that dilution of plasma steroid extracts is not necessary to measure estradiol concentrations in ornate box turtles, at least not for samples collected during May. Inclusion of plasma samples collected earlier and later in the year and increasing the number of female turtles in pooled samples may help overcome this issue for next year. Plasma estradiol concentrations were low, but quantifiable in both male and female turtles, and there were no differences identified between sexes ($p > 0.05$). This likely has to do with the timing of sampling relative to the reproductive cycle, and additional research is needed to determine when estradiol concentrations peak in reproductively-active females.

These findings are a useful first step towards using plasma hormone assays to understand reproductive activity in ornate box turtles. In 2020, we plan to complete assay validation procedures for both hormone kits, and to measure hormone levels in more turtles in conjunction with radiographs. This will allow us to determine whether hormone levels correlate to important measures of reproductive success such as egg presence. Following this, we intend to determine whether the presence of shell injuries impacts reproductive activity, which may indicate a greater need for management intervention.

Stress Hormone Analysis

Corticosterone (cort), the reptilian stress hormone, is likely different in turtles with predator injuries compared to those without. Chronic elevations in cort from injuries can suppress testosterone release and potentially interfere with reproduction in turtles. Chronic stress can also blunt the normal physiologic response to acute stressors and negatively affect immune function, contributing to poorer overall health. Our main objective for this study was to compare cort levels between turtles with and without shell injuries both at baseline and in response to an acute stressor (handling) in order to better characterize the effects that injuries have on ornate box turtle physiology.

To address our research objective, we collected a small blood sample from seventy-nine turtles from the Orland Track within two minutes of capture, then collected a second sample following transport to our field station. Cort levels were measured at both time points using an existing assay designed for eastern box turtles. General linear mixed models were used to investigate the relationships between cort and handling time, and between cort and health status.

Plasma corticosterone levels were associated with time as a quadratic function ($p < 0.0001$), with concentrations rising following capture, then falling off again (Figure 3). Peak plasma cort concentrations were reached 157 minutes after capture.

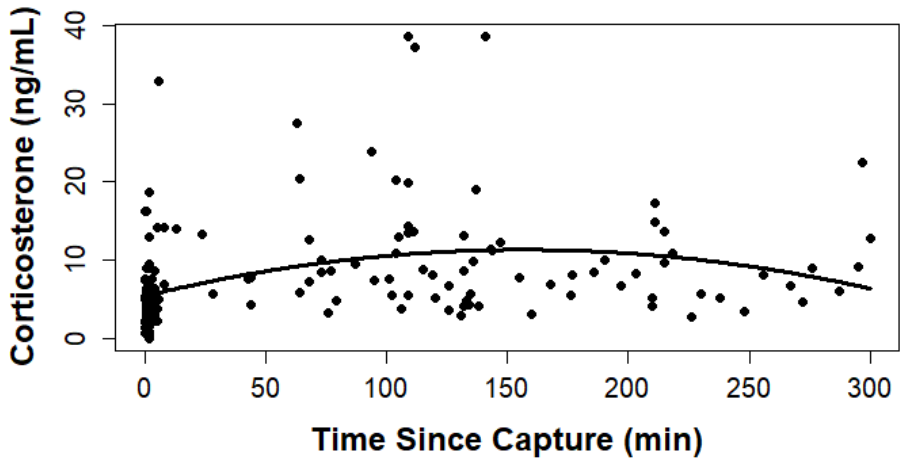

Figure 3. Plasma corticosterone concentrations over time in ornate box turtles at Nachusa.

Overall, corticosterone values were higher in 2018 than 2019 (effect size = 3.86ng/mL, $p = 0.004$), but did not differ based on age class ($p = 0.9$) or sex ($p = 0.6$). Turtles with active shell injuries had lower cort concentrations compared to turtles with normal shells (effect size = 3.35ng/mL, $p = 0.02$), supporting the possibility of a blunted stress response and the potential for limited resiliency in the face of additional health stressors. These findings provide further evidence for the importance of shell injuries for regulating multiple aspects of ornate box turtle physiology.

<u>Venous Blood Gas</u>

Blood gas panels provide important information about physiologic function, including measures of respiratory efficiency, tissue oxygenation, acid-base status, and metabolic stability. The health data determined by blood gas evaluation is not provided by any other non-invasive test, and could be useful for identifying pathologic changes associated with disease. We originally collected blood gas data for ornate box turtles at Nachusa in 2016, and thanks to the Friends we were able to collect additional data in 2019 to supplement our previous work and conduct a rigorous analysis.

Blood samples were collected from 93 ornate box turtles in 2016 and 2019. Immediately following collection blood was loaded into a CG4+ blood gas cartridge, which was then inserted into an iSTAT portable analyzer. Results were recorded for each turtle, corrected for the ambient temperature, then related to physiologic and hematologic values using general linear models.

Blood pH was significantly lower in turtles with quiet mentation (i.e., those that remain in their shells) compared to bright, active turtles ($p = 0.01$, Figure 4). pH was also negatively associated with packed cell volume (PCV), an estimate of the number of red blood cells present ($p = 0.02$). pCO_2, the partial pressure of carbon dioxide in the blood, was significantly higher in turtles with quiet mentation ($p = 0.006$), and was positively associated with both PCV ($p = 0.002$) and ambient temperature ($p = 0.03$). Heart rate was also significantly higher in turtles with quiet mentation ($p = 0.006$). Taken together, these results indicate that turtles with quiet mentation are experiencing a fight-or-flight response, with a spike in adrenaline contributing to higher heart rates and a transient increase in PCV. Concurrent with this fight-or-flight response, boxed-up turtles experience changes in respiratory and acid-base status, likely due to breath-holding, which elevates pCO_2 and drives pH down.

We have previously documented these trends in eastern box turtles as well, but interestingly quiet eastern box turtles typically have changes in more blood gas parameters, including partial pressure of oxygen (pO_2). Furthermore, their alterations in pH and pCO_2 are even more extreme than those of ornate box turtles (pH effect size of 0.19 in easterns vs. 0.057 in ornates; pCO_2 effect size of 15 mm Hg in easterns vs. 2 mm Hg in ornates). This may have to do with behavioral differences between the species, with eastern box turtles typically preferring to box-up during exams while ornate box turtles rarely remain fully within their shells during handling. This gives us information about ornate box turtle respiratory and acid-base physiology which could not be fully extrapolated from similar research in a closely-related species.

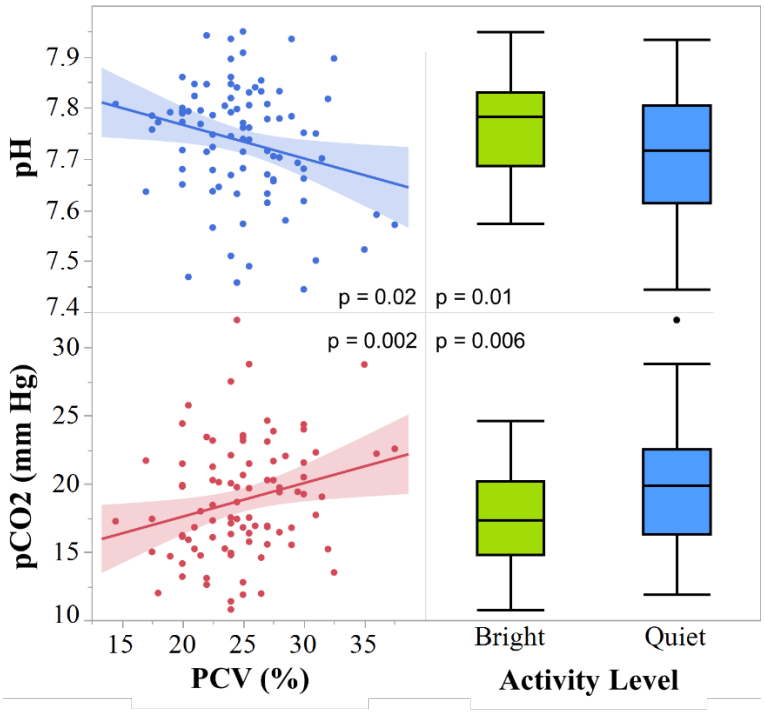

Figure 4. Statistically significant predictors of venous blood gas parameters in ornate box turtles

In addition to the above findings, we also documented a statistically significant relationship between blood lactate values and plasma corticosterone concentrations ($p = 0.009$; Figure 5). This is an interesting finding because it indicates that blood lactate values may serve as a proxy for stress hormone levels. Blood lactate can be quantified with a hand-held meter using a single drop of blood, and is therefore much cheaper and easier to measure than corticosterone concentrations. Based on this finding, we have elected to incorporate blood lactate concentrations into our health assessments for the 2020 field season. Investigating adjunctive health assessment tools can help us identify the most feasible and cost-effective means to accurately determine the wellness of a population, ultimately helping us meet the goals of the Wellness of Wildlife project and the Nachusa Grasslands.

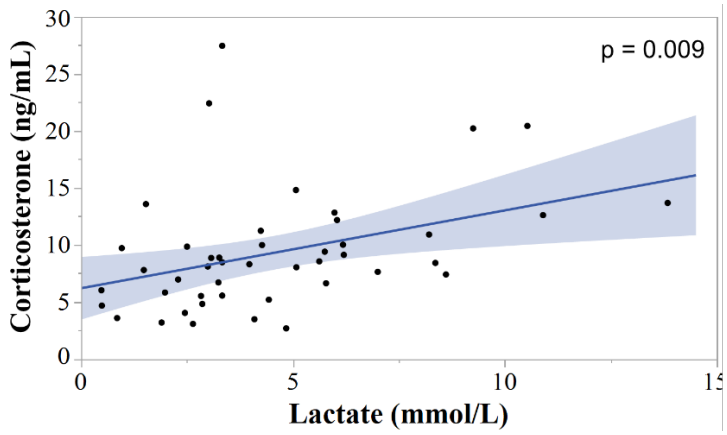

Figure 5. The relationship between plasma corticosterone and blood lactate in ornate box turtles.

Describe how your findings can be applied to challenges in management practices for restoration effectiveness and species of concern:

Our findings are immediately useful because they have provided further evidence of significant physiologic alteration in box turtles as a result of predator injuries. Instituting predator control measures may benefit the overall wellness of ornate box turtles at Nachusa. Our research has also identified a promising tool for assessing the reproductive success of turtles at Nachusa. Following further validation, reproductive hormone analyses may help us determine whether additional management such as nest protection or head-starting is necessary to support the ornate box turtle population at this site. Additional recommendations may be forthcoming following continued health assessment of Nachusa's box turtles in subsequent years.

Please list presentations/posters you have given on your research:

Nachusa Science Symposium, October 19, 2019. "Venous blood gas in free-living ornate box turtles (Terrapene ornata ornata) at the Nachusa Grasslands"

Have you submitted manuscripts to scientific journals? If so, which ones? If not, do you anticipate doing so? (Please send copies of published articles to the Friends so that we can learn from your work.**)**

The work based on this grant has not yet been published, but will produce three separate manuscripts: one focusing on venous blood gas, one on corticosterone and hematologic changes, and one on reproductive hormones, health, and egg presence. Likely target journals include the Journal of Zoo and Wildlife Medicine and Conservation Physiology.

What follow-up research work related to this project do you anticipate (if any)?

We have obtained additional funding from the Friends of Nachusa to continue investigating reproductive hormones and blood lactate concentrations in 2020. As always, we will continue to perform complete health assessments in every turtle that we encounter, and we expect additional research topics to emerge during subsequent years.

<u>Optional</u>: Suggestions for improving the application and award process for future Friends of Nachusa Grasslands Scientific Research Grants: