

**The Friends of Nachusa Grasslands
2020 Scientific Research Project Grant Report
Due June 30, 2021**

1. Please save this form to your desktop with a unique file name that includes “Friends 2020 Science Grant Report” and your last name.
2. Complete the form using the headings in bold as your guide.
3. Save the file as a Word document or a PDF.
4. Attach the file to an e-mail, and send it to: nachusafriendsscience@gmail.com no later than June 30, 2021.
5. The subject of the e-mail should be “2020 Scientific Research Grant Report” and your last name.
6. After your research project is complete, please contact Friends so that we may learn from and publicize the outcomes as appropriate.

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2020 grant amount: \$2441

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

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

Research Project Purpose: The purpose of our research is to 1) pair reproductive hormone analysis with radiographs to determine the relationship between hormone levels and egg presence, 2) to determine the clinical utility of plasma lactate, and 3) to explore the use of a novel inflammatory marker (ceruloplasmin) in ornate box turtles. All research topics are part of a larger project establishing baseline health and disease risks for this species at Nachusa. This information will be used to inform management strategies for wild turtle populations.

Research Project Outcomes to date: None yet, but communication of our findings at the Nachusa Science Symposium is anticipated along with at least one publication.

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: Ornate box turtles (*Terrapene ornata ornata*) are considered near threatened by the International Union for Conservation of Nature (IUCN) and are listed under Appendix II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In Illinois, ornate box turtles are state-threatened and are considered a species in greatest need of conservation due to local range reduction and population declines since 1980. The decline of the ornate box turtle in Illinois is likely multifactorial, and contributing factors include loss of prairie habitat, road mortality,

collection of mature adults for the pet trade, and predation. We have been studying health in ornate box turtles at Nachusa for the last five years in order to identify threats to this population and design management strategies to support box turtle conservation.

Turtles at Nachusa 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.

We have previously investigated several blood tests to help characterize inflammation and wellness in ornate box turtles at Nachusa. In 2019, we found that blood lactate concentrations were positively associated with stress hormone (corticosterone) concentration. We used some grant funds to continue measuring blood lactate concentrations in 2020 and 2021 as a supplement to our ongoing health assessment study. We also used grant funds to investigate the clinical utility of ceruloplasmin, a potential inflammatory marker in ornate box turtles. Finally, grant funds were used to purchase reproductive hormone kits (testosterone and estradiol) for validation studies and individual turtle testing. These tests were paired with radiographs (x-rays) in 2020 to determine the relationship between blood hormones and reproductive performance, as measured by the radiographic presence of eggs.

Lactate was measured in 66 turtles in 2020 and 2021. Blood lactate concentrations were positively associated with both relative ($p = 0.005$) and absolute monocyte counts ($p < 0.0001$). Monocytes are markers of chronic inflammation, indicating that blood lactate concentrations do provide useful information about overall turtle health in addition to serving as a proxy measure for stress levels.

Plasma ceruloplasmin activity was measured in 71 ornate box turtles in 2020 and 2021. High concentrations of ceruloplasmin were associated with low packed cell volume ($p = 0.04$) and high creatine kinase ($p = 0.004$), alpha 2 globulins ($p = 0.002$), and beta globulins ($p = 0.03$). This means that turtles with fewer red blood cells (borderline anemia), higher levels of inflammation (elevated alpha 2 and beta globulins), and tissue damage (elevated CK) had higher levels of ceruloplasmin activity, supporting the use of this test as an adjunctive health assessment tool in ornate box turtles.

Including data from 2019, plasma testosterone and estradiol concentrations were measured in 137 ornate box turtles. Validation procedures, including evaluation of parallelism (whether the hormone levels in the turtles mirror those of a kit-provided standard curve) and recovery (whether the kit measures the level of hormone present accurately) were completed for each kit. Parallelism is assessed by measuring hormone concentrations in a kit-provided standard curve and in serial dilutions of pooled box turtle

plasma and determining whether the lines produced are parallel to each other using linear regression and R^2 values. Figure 1 confirms parallelism for both testosterone and estradiol in ornate box turtles. This procedure also helps determine the optimal dilution to measure hormones in box turtle plasma (dilution with approximately 50% hormone binding), for testosterone a 1:16 dilution is ideal, while for estradiol a 1:2 dilution in assay buffer is best.

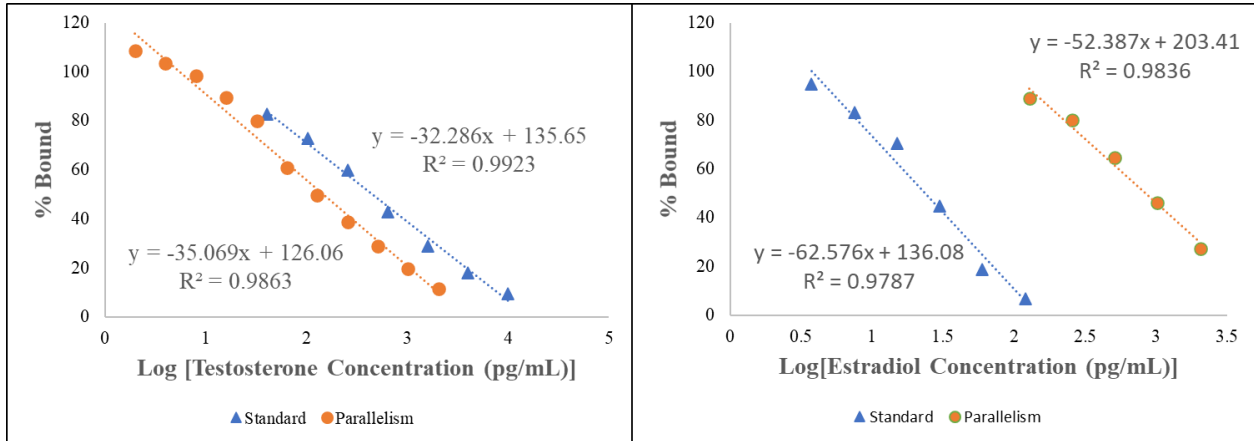


Figure 1. Percentage of hormone bound for kit-provided standard curves and serially-diluted ornate box turtle pooled plasma samples. Linear regression confirms adequate parallelism for both testosterone ($R^2 = 0.98$, $p < 0.0001$) and estradiol ($R^2 = 0.97$, $p = 0.0003$).

Recovery is assessed by spiking pooled box turtle plasma with the kit-provided standard curve and determining whether measured hormone concentrations match expected hormone concentrations. Figure 2 confirms acceptable recovery for testosterone and estradiol in ornate box turtles, with 95% confidence intervals for the slope containing one, and 95% confidence intervals for the y-intercept containing zero.

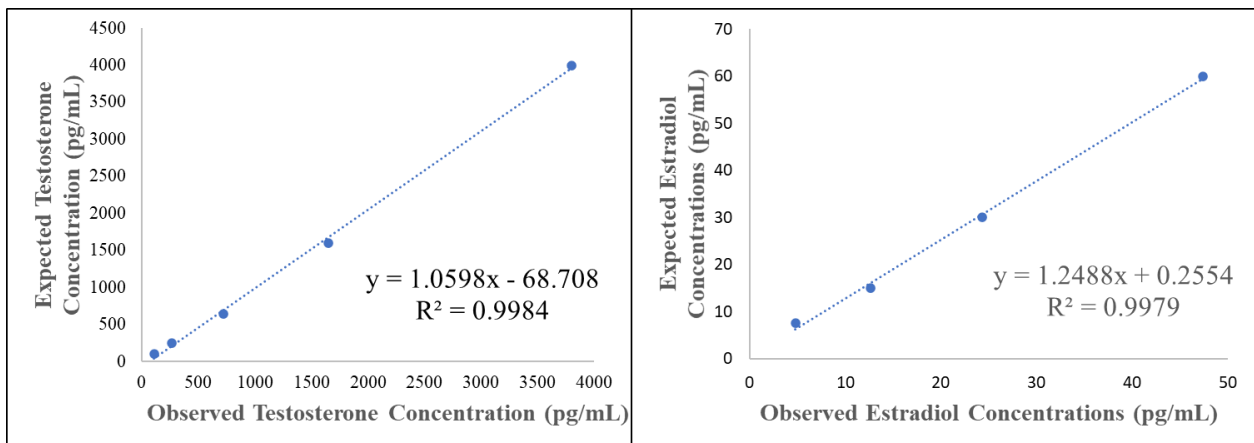


Figure 2. Expected vs. observed hormone concentrations in ornate box turtle pooled plasma samples spiked with known standards.

These kits are now validated for use in this species, and can be utilized by Nachusa and other organizations to monitor trends in reproductive status in wild and managed-care ornate box turtles.

Hormone concentrations were found to vary based on year, with higher testosterone in 2019 vs. 2020 and 2021 ($p < 0.0001$) and higher estradiol in 2021 vs. 2020 and 2019 ($p < 0.0001$). Testosterone concentrations were higher in May vs. June, potentially due to differences in spermatogenesis ($p < 0.0001$). Testosterone concentrations were also significantly higher in males than females ($p < 0.0001$). Testosterone concentrations were positively associated with packed cell volume (0.0004), relative monocyte count ($p = 0.02$), relative ($p = 0.0005$) and absolute heterophil counts ($p = 0.02$), and the heterophil/lymphocyte ratio ($p = 0.003$) and negatively associated with relative ($p = 0.0005$) and absolute lymphocyte counts ($p = 0.002$). These findings are generally consistent with bloodwork changes previously identified in sexually active male turtles.

Estradiol concentrations were negatively associated with relative ($p = 0.009$) and absolute basophil counts ($p = 0.03$), but were not significantly associated with egg presence (Figure 3) or any other health parameter. Concentrations of this hormone are typically expected to peak during folliculogenesis, by the time shelled eggs are observed the peak concentration may have already passed. Sampling earlier in the year will help determine the time period associated with peak plasma estradiol concentrations. This will help us determine whether estradiol concentrations can be used to predict which turtles will reproduce later in the year.

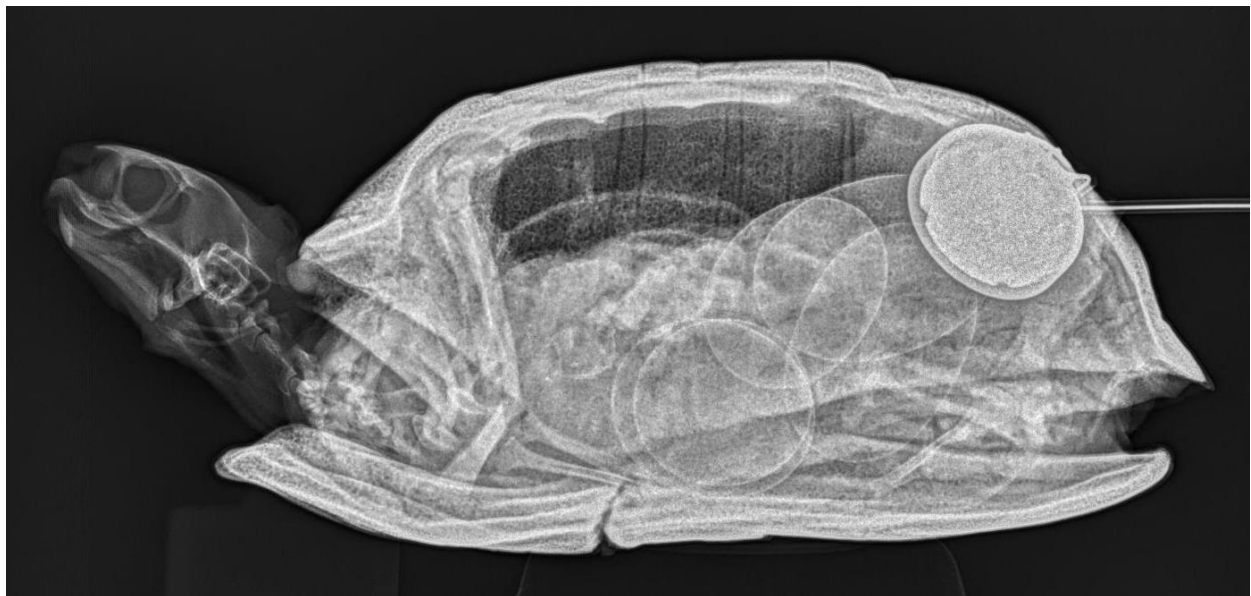




Figure 3. Lateral and dorso-ventral radiographic views of a gravid ornate box turtle at Nachusa. This turtle has five calcified eggs and a radiotransmitter affixed to the caudal carapace. Six of the eighteen females radiographed in 2020 were gravid (pregnant) with an average of 4.7 eggs (range 0 – 5 eggs).

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

This research has enhanced our ongoing health assessment study in ornate box turtles by providing complementary diagnostic testing for turtle wellness (lactate and ceruloplasmin). Our findings indicate that ceruloplasmin may be a worthwhile diagnostic test to include in future box turtle health assessment protocols due to its association with indicators of inflammation and tissue damage. Continuing to refine our health assessment methods improves our understanding of turtle wellness and promotes targeted and evidence-based management recommendations that will directly benefit the animals at the Nachusa Grasslands.

This research has also validated assays for reproductive hormones in ornate box turtles. While additional research is necessary to define associations between hormone concentrations and reproductive success, the groundwork has now been laid to work towards understanding this vital component of population stability and persistence. Ensuring healthy, robust populations of native prairie species supports the goals of the Nachusa Grasslands.

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

The findings of this research can be used to better characterize the health and reproductive activity of ornate box turtles. Continued monitoring of these analytes may help us understand the importance of predators for box turtle survival and population persistence. This information will support decisions about the necessity for and relative importance of predator control strategies. Furthermore, this research may provide tools to assess the effectiveness of predator control for box turtle conservation.

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

None yet using data generated from this work

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

We anticipate at least one manuscript will be forthcoming from this work, but none have been submitted yet.

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

Additional monitoring of ceruloplasmin activity and plasma reproductive hormone concentrations are anticipated to improve our understanding of ornate box turtle health, reproductive activity, and overall wellness.

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