The Friends of Nachusa Grasslands
2017 Scientific Research Project Grant Report

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2017 grant amount: $2673

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

Research Project Topic: Measures of inflammation as markers of health in ornate box turtles

Research Project Purpose: The purpose of our research was to evaluate protein electrophoretograms and hemoglobin-binding protein (HBP) as part of a larger project establishing baseline health and disease risks for ornate box turtles at Nachusa.

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) 50 protein electrophoresis panels performed at the University of Miami, 2) Shipping blood samples to the University of Miami for processing, 3) Commercial kits and control reagents to perform HBP tests in 50 turtles, and 4) Needles, syringes, and heparinized tubes for blood sample collection and storage.

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

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 a predictable way to management interventions, and could fail to rebound successfully after perturbation. Furthermore, understanding infectious disease burden is important when considering animal movement to prevent the introduction of novel pathogens to naïve populations. Baseline health assessments performed at Nachusa in the last two years have helped identify a potential threat to box

turtle wellness: predators. We are currently conducting a multi-year longitudinal study of ornate box turtles at Nachusa to determine how environmental, population, and disease factors impact health. The products of this study include a model for population health which will identify the most important drivers of ornate box turtle wellness, the most useful diagnostic tests for health assessment in this species, and provide practical, evidence-based management recommendations for conservation of ornate box turtles at Nachusa and beyond. The goals of this project are aligned with the conservation mission of the Nachusa Grasslands.

The 2017 grant funds we received were utilized during the second year of the larger project described above. To summarize the methods: Ornate box turtles were located within the Orland Track and the South Bison Unit from May 15-18 2017 using a combination of canine and human searches. GPS coordinates and habitat data were collected at capture. Complete physical examinations were performed by two veterinarians, and abnormalities were recorded. A blood sample was collected for hematology, plasma biochemistry, protein electrophoresis, HBP, and quantitative polymerase chain reaction (qPCR) pathogen testing. Swabs of the oral cavity and cloaca were also collected for pathogen testing. Turtles were weighed, sexed, measured, and the shells were notched for permanent identification. Body temperatures were collected via thermography camera, and heart rates were determined using Doppler ultrasonography. Following the complete health work-up, each animal was released at its site of capture. Funding for hematology, biochemistry panels, and pathogen surveillance was obtained through a State Wildlife Grant, while the Friends of Nachusa funded protein electrophoresis and HBP.

For context, all study results will be summarized first, then the protein electrophoresis and HBP findings will be highlighted. A total of 88 live turtles were evaluated, 74 in the Orland Track and 14 in the South Bison Unit (Figure 1). Sixty-three turtles were classified as adults and 25 were juveniles. Four of the juveniles were found in the South Bison Unit, indicating ongoing recruitment in this population. Forty two turtles were male, 29 were female, and 17 were of unknown sex (juveniles). For the second year in a row, there was a statistically significant male bias in the Orland Track ($p=0.01$). Twenty five of the turtles evaluated in the Orland Track had been notched the previous year. The population size at this site is therefore estimated at 253 individuals, with a 95% confidence interval from 187-359 turtles using the Lincoln-Petersen method.

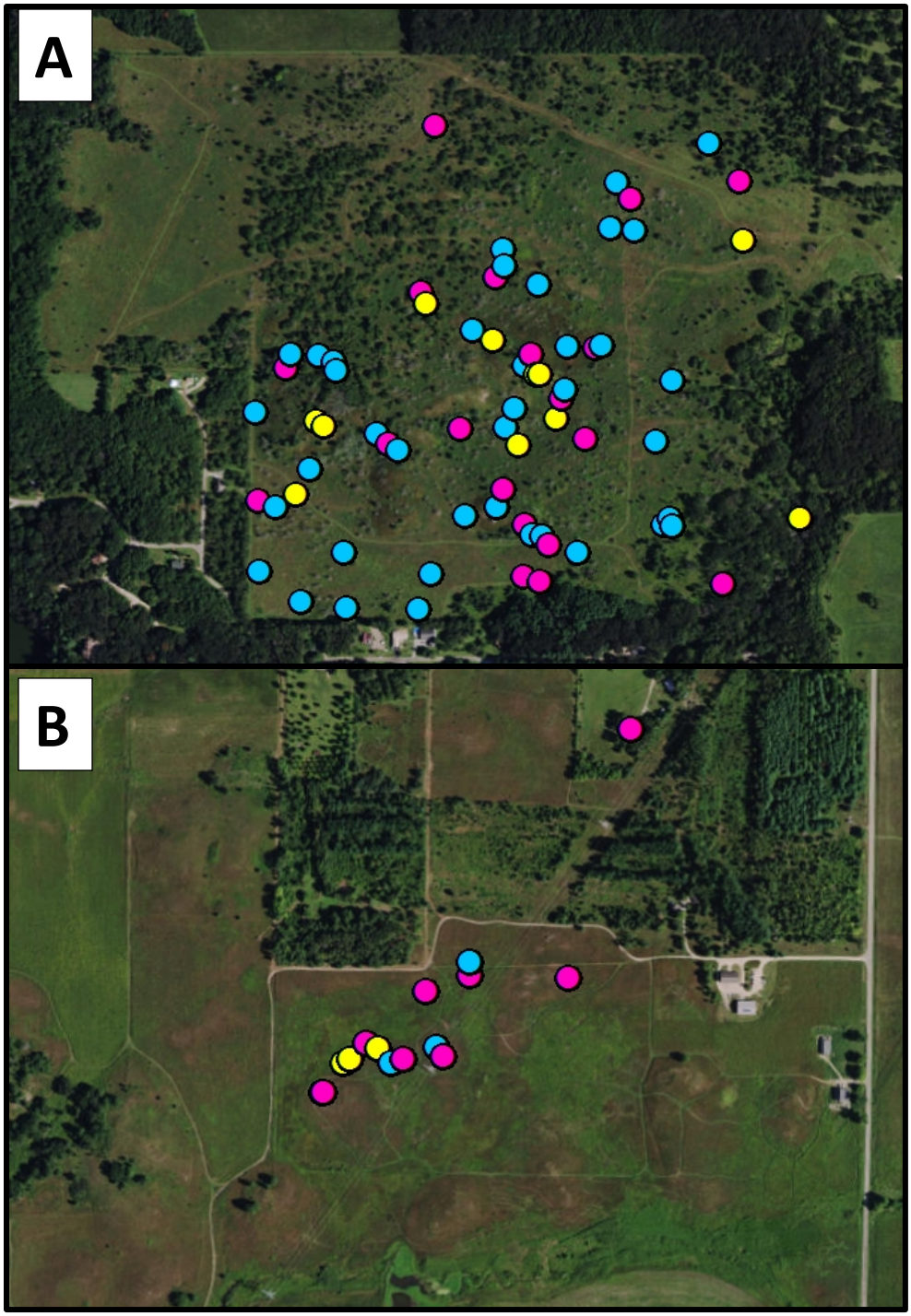

Figure 1. Ornate box turtle capture sites (circles) by sex (blue: male, pink: female, yellow: unknown) at the Orland Track (A) and South Bison Unit (B) during May, 2017.

Physical examination abnormalities included shell abnormalities (N=45), missing digits (N=5), and asymmetrical nares (N=1). The shell abnormalities consisted of mild to significant predator damage (N=36), erosions (N=23), flaking keratin (N=6), and developmental abnormalities (N=3), with several individuals having multiple abnormalities. Examples of shell abnormalities are displayed in Figure 2. The presence of all physical exam abnormalities ($p=0.0004$), specifically shell abnormalities ($p=0.004$), was significantly lower in juvenile turtles compared to adults. Body temperature was also significantly lower in juveniles ($p=0.03$), perhaps reflecting different microhabitat usage or increased sheltering behavior.

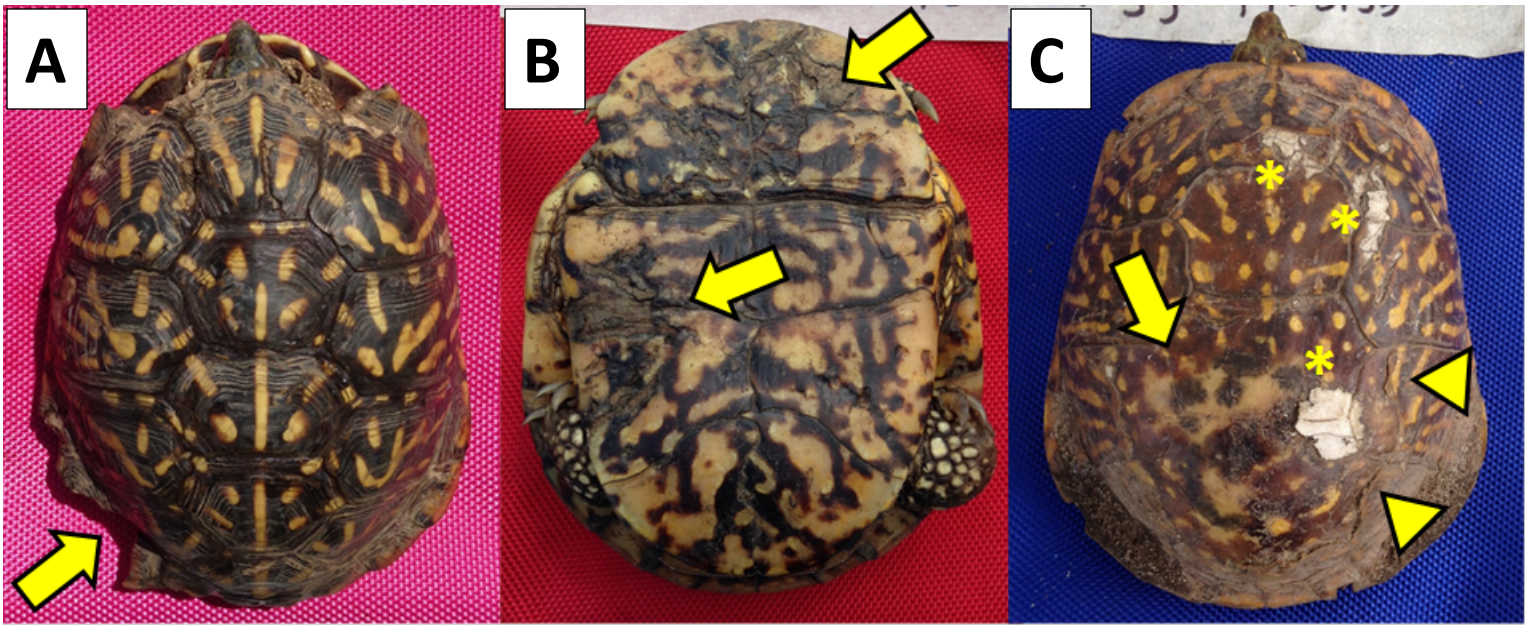

Figure 2. Examples of shell abnormalities identified during physical examination of ornate box turtles at the Nachusa Grasslands in May, 2017. A) Predator injuries to the marginal scutes, B) Erosions of the plastron, C) Healed burn injury (arrow), bone exposure (asterisks), flaking (arrowhead).

Hematology (a blood test characterizing the number and distribution of white blood cells, the percent of red blood cells, and the total amount of protein in the blood) was performed in 83 turtles. Plasma biochemistry panels (a blood test for liver function, kidney function, and electrolytes) were performed in 44 turtles. Plasma protein electrophoretograms (a blood test describing the distribution of blood proteins) were performed in 50 turtles. HBP, a test of acute inflammation, was performed in 78 turtles in 2017 due to the inclusion of excess reagents in the kits. Banked plasma samples from 2016 (N=42) were also assayed for HBP. Results were generally consistent with those reported for zoo-maintained ornate box turtles and other species of free-living box turtles.

Several significant bloodwork differences were noted between age classes, while only a few sex and site-based differences were identified. Juvenile turtles had significantly

higher absolute ($p=0.01$) and relative ($p=0.04$) lymphocyte counts, higher absolute monocyte counts ($p=0.01$), higher absolute ($p=0.00001$) and relative ($p=0.007$) basophil counts, lower relative eosinophil counts ($p=0.003$), and a lower heterophil/lymphocyte ratio (H:L; $p=0.04$) than adults. Decreases in absolute heterophils ($p=0.05$) and eosinophils ($p=0.07$) and increases in relative monocytes ($p=0.06$) approached significance. Detecting expected differences between adults and juveniles is important for informing the clinical interpretation of box turtle bloodwork. Sex-based differences included significantly higher packed cell volume (PCV) in males ($p=0.007$), and higher calcium ($p=0.0001$) and phosphorous ($p=0.0003$) in females. These changes have been documented in several other species of chelonians, and are expected. Turtles in the South Bison Unit had lower absolute ($p=0.02$) and relative ($p=0.01$) basophil counts than Orland Track turtles. The role of basophils in the reptile immune system is poorly understood, but this could represent a slight difference in health status between the Orland and SBU populations.

qPCR pathogen testing was performed in 44 turtles. Adenovirus was detected in a single adult male. This turtle did not have any clinical signs of illness, but he did have the highest H:L of all 2017 turtles. Elevations in the H:L are consistent with stress, and this may indicate a subclinical effect of the virus.

PROTEIN ELECTROPHORESIS:

The protein electrophoresis panels conducted in 2017 were evaluated in conjunction with results from 2016 to maximize information gain. Total protein ($p<0.0001$), absolute prealbumin ($p=0.004$), absolute albumin ($p<0.0001$), absolute alpha1 globulins ($p<0.0001$), relative and absolute alpha2 globulins ($p=0.0001$, $p<0.0001$), absolute beta globulins ($p=0.0001$), and absolute gamma globulins were higher in 2017 while relative albumin ($p=0.03$) and gamma globulin ($p=0.02$) values were lower. The albumin to globulin ratio (AGR), relative prealbumin, albumin, alpha1 globulins, and beta globulins were not affected by year. These changes may have been due to differences in temperature (higher in 2017), food availability, or disease burden between years. Statistical models were constructed to account for these differences when considering the effects of sex and physical exam abnormalities on EPH values.

Female turtles had lower relative albumin and higher beta globulin levels compared to both adult males ($p=0.0008$, $p=0.0007$) and juveniles ($p=0.0003$, $p=0.001$). They also had lower absolute albumin levels, relative gamma globulins, and absolute gamma globulins compared to juveniles ($p<0.05$). Finally, the AGR was lower in females compared to males ($p=0.0006$) and juveniles ($p=0.0009$). These sex and age-based differences are important to know about when considering applying EPH panels for health assessment. Statistical models evaluating the effects of physical exam abnormalities on EPH values were constructed to control for these sex and age-based differences, as well as differences in EPH values between years.

Controlling for the effects of year, sex, and age, turtles with both active and inactive shell abnormalities had significantly lower relative albumin levels ($p=0.02$, $p=0.01$) and a lower

AGR (p=0.02, p=0.01) compared to animals with normal shells. This is important because it shows that even turtles with healed injuries maintain prolonged inflammatory protein changes. This represents an investment of resources into immune function, and decreases resource allocation to growth and reproduction. Therefore, even if predator injuries do not kill ornate box turtles outright, they result in chronic changes which may affect longevity, growth, and fecundity. Interestingly, few hematologic changes were observed for animals with physical examination abnormalities. The only statistically significant change in turtles with active shell lesions was an increased heterophil count (p=0.04), consistent with active inflammation. The same turtles had a non-significantly higher HL, also consistent with inflammation and/or stress (p=0.07). Our findings support continued use of hematology and protein electrophoresis panels for health assessment in ornate box turtles.

HEMOGLOBIN BINDING PROTEIN:

HPB levels were affected by age class, with significantly lower levels in juveniles compared to adults (p=0.001). This may indicate that adults have higher levels of background inflammation related to higher lifetime exposure to immune stimulants such as injury and pathogen infection. HPB levels were also significantly higher in turtles with predator injuries involving the plastron (p=0.03). There was a non-significant trend for higher HBP levels in turtles with active or inactive lesions anywhere on the shell. These trends may become significantly different with a larger sample size. We are thankful to the Friends of Nachusa for generously funding another set of HBP testing in 2018 so that we can continue to explore its diagnostic utility in box turtles.

CONCLUSION:

Ornate box turtle health was characterized at an existing and new site at the Nachusa Grasslands in 2017. Both sites had evidence of recruitment, though a persistent male bias was identified in the Orland Track population during both 2016 and 2017. The most significant finding in these populations is a high prevalence of shell damage related primarily to predator trauma. Subsidized mesopredators are a known threat to ornate box turtles, and it is possible that mesopredator populations are higher at active restoration sites like Nachusa than they would be in a completely natural prairie setting. Measures for meopredator control are being actively discussed to improve ornate box turtle wellness at this site.

The infectious disease burden at Nachusa was low in both 2016 and 2017 and was restricted to Terrapene herpesvirus 1 (2016 & 2017) and box turtle adenovirus (2017). Clinical disease associated with these two pathogens appears infrequent, though herpesvirus detection increases in association with physical and environmental stressors in eastern box turtles. In 2016, turtles had more inflammatory leukogram and protein electrophoretic changes and experienced lower environmental temperatures than in 2017. These factors, or other unmeasured predictors, may help explain the difference in pathogen burden between years. Subsequent health assessments in these populations will improve our understanding of the drivers of health and disease in ornate box turtles.

The Friends of Nachusa allowed us to capitalize on our existing sampling effort in order to gather more protein electrophoresis data and evaluate a new diagnostic test in free-living ornate box turtles. The combination of hematology and protein electrophoresis appears useful for assessment of acute and chronic inflammation in ornate box turtles. Hemoglobin-binding protein may also have clinical utility, which we will be better able to assess after a second year of data collection in 2018. We have provided another year of objective evidence demonstrating that predator injuries cause significant alteration to box turtle physiology. As a result, we can make management recommendations (predator control) which may benefit box turtles at Nachusa. These funds have yielded direct results benefitting ornate box turtles, and have successfully supported the work and goals of the Nachusa Grasslands.

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 identified 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 relatively low disease burden in Nachusa's box turtle population. This could be problematic if foreign box turtles (carrying new diseases) are introduced to Nachusa later on. We therefore recommend infectious disease testing of box turtles prior to introduction. 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 22, 2016. "Ornate Box Turtle Health Assessment at Nachusa".

Nachusa Science Symposium, October 21, 2017. "Continued Ornate Box Turtle Health Assessment at Nachusa".

Have you submitted manuscripts to scientific journals? If so, which ones? If not, do you anticipate doing so? (Please keep us informed on publications.**)**

All results obtained in this study will be included in Dr. Adamovicz's thesis. As yet, this data has not been submitted for publication. However, she will be presenting the ornate box turtle health model at the Wildlife Disease Association conference in August.

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