ASSESSING WELLNESS IN WILD HERPTILE SPECIES IN GREATEST NEED OF CONSERVATION

IL SWG T-104-R-2

Report prepared by:

Matthew C. Allender and Laura Adamovicz

Wildlife Epidemiology Lab, Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL. mcallend@illinois.edu

Prepared for:
Illinois Department of Natural Resources
Annual Report
December 2021

SUMMARY & KEY FINDINGS

Health Driven Management Summary

- Kickapoo State Park habitat has led to the deterioration of Eastern Box Turtle Health and population estimates indicate extirpation in as soon as 20 years
- Kickapoo State Park has the largest pathogen associated mortality events in both eastern box turtles and silvery salamanders
- Improvement in shell lesions observed during this phase of the grant at Nachusa Grasslands, but this population remains at risk for disease-related catastrophic events
- Kennekuk Cove County Park Eastern box turtle population has decreased to nearly zero, but adjacent IDNR field property (Dynegy) has a large population and should be preserved.

Key Findings (Overall)

- Eastern box turtles remained threatened, most notably at Kickapoo State Park and Kennekuk Cove County park, and Middlefork Nature Preserve near Collison, IL
- Silvery Salamanders at Kickapoo State park have parasitic diseases and viral diseases that cause complete failure in recruitment in some years.
- Silvery salamanders with "extra" limbs were detected for the first time.
- Eastern Massasaugas have increasing prevalence of ophidiomycosis (Snake Fungal Disease) and Kirtland's snake have high prevalence of ophidiomycosis
- RNA pathogens (including coronaviruses) weer absent in herptiles from 2018-2021.
- Health in all species varies by season, sex, age class – thus a single spot check on "health" is not appropriate and multiple samples are required.
- Cancer-associated viral infection was first detected in a Blanding's turtle in Lake county

Summary of each species

Eastern Box Turtles

- Health assessments were performed in 324 eastern box turtles at five study sites.
- Physical examination findings, clinical pathology values, and pathogen presence from 2019 - 2021 were comparable to baseline data established in Phase I.
- Inflammatory leukogram changes were identified in turtles testing positive for a recently-described virus (*Terrapene* herpesvirus 2), potentially indicating negative health impacts.
- Continued health assessments and pathogen surveillance are recommended to clarify pathogen epidemiology and monitor for shifts in individual and population health.

Ornate Box Turtles

- Health assessments were performed in 202 ornate box turtles at two study sites.
- Physical examination findings, clinical pathology values, and pathogen presence from 2019 - 2021 were comparable to baseline data established in Phase I.
- Smaller body size, inflammatory leukogram changes, and increased turtle density at Ayers Sand Praire may indicate a poorer plane of health and limited resiliency in response to emerging infectious diseases compared to Nachusa Grasslands.

- Continued health assessments and pathogen surveillance are recommended at Ayers and other sites to better characterize health in Illinois ornate box turtle populations.
- Performing health assessments at additional time points during the active season is also recommended to better understand changes in health status over time.

Silvery Salamanders

- Health assessments were performed in 334 silvery salamanders at three wetlands.
- Physical examination findings and pathogen presence from 2019 - 2021 were comparable to baseline data established in Phase I.
- Hematologic assessment revealed inflammatory changes in salamanders with developmental abnormalities and *Dermotheca* sp. infection.
- We recommend incorporation of hematology and other clinical pathology testing into future amphibian health assessments to supplement existing protocols.

Eastern Massasauga Rattlesnakes

- Sixty-six health assessments were performed for eastern massasaugas at Carlyle Lake.
- *Ophidiomyces ophidiicola* was detected in 30.3% of massasauga encounters.
- Most positive snakes had "apparent ophidiomycosis" with accompanying skin lesions and hematologic changes consistent with inflammation.
- Testing body and lesion swabs in parallel provides the best diagnostic sensitivity for detecting *Ophidiomyces*. This sampling strategy is recommended for future surveys.
- Half of necropsied massasaugas had underlying health conditions which caused or contributed to death. Continued use of necropsies is recommended to support conservation objectives.

Kirtland's Snakes

- Fifty-nine Kirtland's snakes were tested for *Ophidiomyces ophidiicola* via qPCR.
- *Ophidiomyces* was detected in 48% of sampled Kirtland's snakes in 2019.
- Ophidiomycosis can cause significant morbidity and mortality in wild snakes, and additional surveillance is recommended as part of Kirtland's conservation efforts.
- We provide guidance on sample size for future *Ophidiomyces* surveillance efforts.

RNA Pathogen Surveillance

- RNA extraction protocols were developed improve RNA yield in wildlife samples.
- 308 eastern box turtle, ornate box turtle, and silvery salamander swabs were tested for seven RNA viruses (picornavirus, parvovirus, ferlavirus, reovirus, nidovirus, coronavirus, influenza). All samples were negative.
- Negative findings could be due to absence of infection, testing during time periods when infections are not detectable, or inadequate primer sensitivity.
- This provides useful baseline data that will allow rapid recognition and response to emerging RNA pathogens in our study species.
- Future testing will include additional sample types (blood) and pathogens (bunyavirus, bornavirus, flavivirus, arenavirus). De novo primer design may also be pursued.

SCIENTIFIC DELIVERABLES GENERATED

Manuscripts (supported directly or indirectly by this grant, reverse chronological order):

- Winter, J.W., N.I. Stacy, L.A. Adamovicz, M.C. Allender. 2019. Investigating the analytical variability and agreement of manual leukocyte quantification methods in Eastern box turtles (*Terrapene carolina carolina*). Frontiers in Veterinary Science. DOI:10.3389/fvets.2019.00398.
- Klein K, Gartlan B, Doden G, Fredrickson K, Adamovicz L, Allender MC. 2020. Comparing the effects of lithium heparin and dipotassium ethylenediaminetetraacetic acid on hematologic values in eastern box turtles (*Terrapene carolina carolina*). *J Zoo Wildl Med*. 2020;51(4): 999–1006.

- Adamovicz L, Baker SJ, Merchant ME, Darville L, Allender MC. 2020. Plasma complement activation mechanisms differ in ornate (*Terrapene ornata ornata*) and eastern box turtles (*Terrapene carolina carolina*). *J Exp Zool A Ecol Integr Physiol*. 2020:1–12.

- Adamovicz, L. M.C. Allender. 2020. Erythrocyte sedimentation rate in free-living eastern (*Terrapene carolina carolina*) and ornate (*Terrapene ornate*) box turtles and hemoglobin binding protein in ornate box turtles: reference intervals and demographic effects. PLoS ONE 15(6):e0234805.

- Engel AI, Adamovicz L, Wellehan JFX Jr., Allender MC. 2020. Development and validation of a quantitative PCR assay for detection of Terrapene herpesvirus 2 in eastern box turtles (*Terrapene carolina carolina*). *J Virol Methods*. 2020;286:113968.

- Edmonds, D., L. Adamovicz, M.C. Allender, M.J. Dreslik. 2020. Reproductive output of ornate box turtles (Terrapene ornate) in Illinois, USA. Herpetological Conservation and Biology 15(2):467-475.

- Sander WE, King R, Graser W, Kapfer JM, Engel A, Adamovicz L, Allender MC. 2021. Molecular detection of *Coxiella burnetii* in three different species of turtles in the Upper Midwest. *Emerg Infect Dis*. 2021:27(12):3199-3202.

- Doden G, Gartlan B, Klein K, Maddox C, Adamovicz L, Allender MC. 2021. Prevalence and antimicrobial resistance patterns of *Salmonella* spp. in two free-ranging populati9ons of eastern box turtles (*Terrapene carolina carolina*). *J Zoo Wildl Med*. 2021;52(3):863-871.

- Klein K, Adamovicz L, Phillips CA, Allender MC. 2021. Blood lactate concentrations in eastern box turtles (*Terrapene carolina carolina*) following capture by a canine search team. *J Zoo Wildl Med*. 2021;52(1),259-267.

- Andersson K, Adamovicz L, Mumm L, Winter J, Glowacki G, Teixeira-Neto R, Adkesson MJ, Hostnik ET, Haynes E, Allender MC. 2021. Detection of a novel herpesvirus associated with squamous cell carcinoma in a free-ranging Blanding's turtle. *J Vet Diagn Invest*. 2021;33(2):348-351.

- Doke, R. K. Hiebert, M. Repella, M. Stuart, L. Mumm, J. Winter, L. Adamovicz, G. Glowacki, E. Kessler, **M.C. Allender**. 2022. Prevalence of intraerythrocytic parasites in *Macrochelys temminckii*, *Emydoidea blandingii*, *Terrapene carolina carolina*, and *Terrapene ornate ornate*. Journal of Herpetological Medicine and Surgery, online ahead of print.

- Gartlan, B, L. Adamovicz, K. Klein, G. Doden, K. Fleming, M.C. Allender. Intraocular pressure and tear production of free-ranging Eastern (*Terrapene carolina carolina*) and ornate box turtles (*Terrapene ornate*). In preparation.

- Rayl, J.M., M. Kelly, M. Beerman, L. Adamovicz, C.A. Phillips, M.C. Allender. Longitudinal investigation of free-ranging Eastern box turtles (Terrapene carolina carolina) hematology and pathogen presence. In preparation.

Graduate Student Thesis/Dissertation Supported in part by samples from this project

- Low, K. Characterizing disease dynamics and temporal epidemiology of FV3 in an east-central Illinois amphibian community and investigating effects of genomic contribution on pathogenesis in tetraploid unisexual salamanders (*A. platineum*). University of Illinois, PhD Dissertation, 2019.
- Haynes, E. Characterizing the epidemiology of ophidiomycosis in North America snakes through field studies, modeling, pathogenic genomic analysis and treatment trials. PhD Dissertation, University of Illinois, 2020.
- Pohly, A. Pathological characterization of natural Ophidiomyces ophiodiicola infection in wild-caught Lake Erie watersnakes: A standardized approach to documentation of disease. University of Illinois, MS Thesis 2020.

Proceedings supported in part by this grant (and acknowledged during the presentation, reverse chronologic order):

- Adamovicz LA, Stokol T, Allender MC. Hematology and leukocyte cytochemical staining characteristics of free-living silvery salamanders (*Ambystoma platineum*) from Illinois, USA. American Association of Zoo Veterinarians Conference. Virtual, October, 2021.

- Fredrickson K, Adamovicz L, Terio K, Davidson A, Waligora A, Ladez K, Bradley S, Allender MC. *Emydomyces testavorans* surveillance in multiple free-ranging terrestrial and aquatic chelonian species in Illinois. 69th Wildlife Disease Association / 14th European Wildlife Disease Association Joint Conference. Virtual, August, 2021.

- Adamovicz LA. Health & disease in free-living box turtles (*Terrapene* spp.): The Wellness of Wildlife Project. 19th Annual Symposium on the Conservation and Biology of Tortoises and Freshwater Turtles. Virtual, August, 2021.

- **Allender, M.C**. 2021. Update on Snake Fungal Disease. Association of Zoos and Aquarium Herp Taxon Advisory Group Annual Meeting.

- Adamovicz LA, LaGrange S, Johnson S, Dreslik M, Allender MC. Comprehensive health assessment including multi-pathogen surveillance in the federally threatened eastern massasauga rattlesnake (*Sistrurus catenatus*) in Illinois, USA. American Association of Zoo Veterinarians Conference. Virtual, September, 2020.

- Engel A, Adamovicz L, Wellehan JFX, Allender MC. Development and validation of a quantitative PCR assay for detection of Terrapene herpesvirus 2 in eastern box turtles (*Terrapene carolina carolina*). American Association of Zoo Veterinarians Conference. Virtual, September, 2020.

- Klein K, Adamovicz L, Allender MC. Comparing the effects of lithium heparin and dipotassium ethylenediaminetetraacetic acid on hematologic values in eastern box turtles (*Terrapene carolina carolina*). American Association of Zoo Veterinarians Conference. Virtual, September, 2020.

- Doden G, Gartlan B, Klein K, Maddox C, Adamovicz L, Allender MC. Prevalence and antimicrobial resistance patterns of *Salmonella* spp. in two free-ranging populations of eastern box turtles (*Terrapene carolina carolina*). American Association of Zoo Veterinarians Conference. Virtual, September, 2020.

- Gartlan, B., K. Klein, G. Doden, K. Fleming, L. Adamovicz, **M.C. Allender**. 2020. Assessing ocular health of eastern and ornate box turtles (*Terrapene carolina carolina*, *Terrapene ornate ornate*) based on intraocular pressure (IOP) and tear production. Annual conference of the American Association of Zoo Veterinarians 2020.

- Adamovicz LA, Webb J, Allender MC, Kessler E, Baker S, Dreslik M. Venous blood gas in free-living ornate box turtles (*Terrapene ornata ornata*) at the Nachusa Grasslands. Nachusa Science Symposium. Dixon, IL, October, 2019.

- Winter, J., **M.C. Allender**, N.I. Stacy, L.A. Adamovicz. The reptile CBC: investigating how the method can affect results and change your clinical interpretation. Joint Meeting of the Association of Reptilian and Amphibian Veterinarians, Association of Avian Veterinarians, and Association of Exotic Mammal Veterinarians, St. Louis, MO. October 2019.

- Low, K., L. Adamovicz, C. Phillips, M.C. Allender. Genetic contribution from a sperm-host reduces susceptibility to an FV3-like ranavirus in tetraploid unisexual *A. platineum* larvae. The Wildlife Society Annual Conference, Reno, NV. October 2019.

- Adamovicz LA, Woodburn D, Virrueta Herrera S, Low K, Kuhns A, Crawford J, Phillips C, Allender MC. Characterization of a novel mesomycetozoean infection (*Dermotheca* sp.) in a state-endangered salamander (*Ambystoma platineum*) and a co-occurring common species (*Ambystoma texanum*). Joint meeting of the American Association of Zoo Veterinarians and the Association of Reptile and Amphibian Veterinarians. St. Louis, MO, September, 2019.

- Adamovicz LA, Woodburn D, Boers K, Johnson S, Willis T, Kessler E, Allender MC. Characterizing the clinical course of an emerging fungal pathogen (*Emydomyces testavorans*) in the state-endangered alligator snapping turtle (*Macrochelys temminckii*). Joint meeting of the American Association of Zoo Veterinarians and the Association of Reptile and Amphibian Veterinarians. St. Louis, MO, September, 2019.

- Adamovicz LA, Allender MC. Erythrocyte sedimentation rate and hemoglobin-binding protein in eastern and ornate box turtles (*Terrapene* spp.). Joint meeting of the American Association of Zoo Veterinarians and the Association of Reptile and Amphibian Veterinarians. St. Louis, MO, September, 2019.

- Adamovicz LA, Allender MC, Baker S, Kessler E, Kelly M, Johnson S, Dreslik M, Phillips C. Characterizing the wellness of wildlife: a comprehensive assessment of health, pathogen presence, immune function, and population viability in eastern box turtles (*Terrapene carolina carolina*). Wildlife Disease Association 68th International Conference. Tahoe City, CA, August, 2019.

- **Allender, M.C**., E. Haynes, M. Kelly, S.J. Baker. The epidemiology on snake fungal disease in eastern massasaugas over the last 10 years. Midwest Fish and Wildlife Conference, Cleveland, OH. February 2019.

- Rayl, J.M., M. Kelly, M. Beerman, L. Adamovicz, **M.C. Allender**. Spatiotemporal investigation of Eastern box turtle (*Terrapene carolina carolina*) hematology and pathogen detection through a longitudinal study in central Illinois. Midwest Fish and Wildlife Conference, Cleveland, OH. February 2019.

- **Allender, M.C**. Determining the health and disease in box turtles, integrating ecology and epidemiology. Fifth International Box Turtle Workshop, Haw River, NC. May 2019.

- **Allender, M.C**. K.M. Low, C.A. Phillips, A.R. Kuhns, J.A. Crawford, L. Adamovicz. Multiple frog virus 3-like ranaviruses at a single site causes mortality in both amphibians and reptiles. 5th International Symposium on Ranaviruses, Townsville, Australia. June 2019.

- Rayl, J.M., **M.C. Allender**. Characterizing the host hematologic and cytokine transcription response following an experimental ranavirus challenge in red-eared sliders (*Trachemys scripta elegans*) at two environmental temperatures. 5th International Symposium on Ranaviruses, Townsville, Australia. June 2019.

INTRODUCTION

Human expansion has created an unprecedented global environmental crisis characterized by habitat loss and fragmentation, modification of ecological processes, unsustainable harvesting practices, environmental toxification, climate change, and introduction of non-native plants, animals, and pathogens (Pimm et al. 1995, Wilcove et al. 1998, Tabor et al. 2001, Clavero and Garcia-Berthou 2005). Wildlife are increasingly threatened by this complex array of interacting stressors, with average population sizes declining by over 60% within the last 30 years, and current extinction rates rising 100-1,000 times above historical baselines (Pimm et al. 1995, WWF 2018). Small, fragmented wildlife populations are especially susceptible to demographic and environmental stochasticity and have an increased risk of falling into extinction vortices associated with threats such as disease (De Castro and Bolker 2005, Smith et al. 2006, Pedersen et al. 2007, Smith et al. 2009).

Infectious diseases, in conjunction with anthropogenic and environmental stressors, have contributed to an apparent increase in wildlife mortality events as well as documented extinctions and extirpations (Cunningham and Daszak 1998, Daszak et al. 1999, Van Riper et al. 2002, Lips et al. 2006, Schloegel et al. 2006, Wyatt et al. 2008, Fey et al. 2015). Disease therefore represents an important consideration for planning both in situ and ex situ conservation actions including translocations, reintroduction programs, and establishment and maintenance of healthy captive assurance colonies (Deem et al. 2008). Knowledge about pathogen epidemiology can be incorporated into formal decision-making frameworks such as Disease Risk Assessments to identify, analyze, and manage disease hazards in conservation programs, however, the utility of these analyses depends heavily on the quality of the information upon which they are based (Miller 2007, Dalziel et al. 2017, Hartley and Sainsbury 2017). Disease is understudied in many threatened and endangered species, which may result in an incomplete characterization of conservation threats and force a reactionary, rather than preventative approach to management of overall health, disease outbreaks, and mortality events – ultimately impacting the success of planned conservation interventions.

The dynamics of disease in free-living wildlife are complex, and are governed by a suite of host, pathogen, and environmental factors which can interact in ways that are difficult to predict (Pirofski and Casadevall 2012, Méthot and Alizon 2014). Furthermore, free-living wildlife are commonly co-infected with multiple pathogens which can act competitively or synergistically to influence the development of clinical signs of disease, underscoring the need for comprehensive multi-pathogen surveillance in imperiled species (Bordes and Morand 2011). Improving our understanding of disease ecology, epidemiology, and the interactions between host-pathogen communities and their surrounding environment may enable proactive strategies to prevent or manage health problems and promote more successful conservation outcomes, especially in species of special concern.

In Phase I of the "Wellness of Wildlife" project, we utilized a holistic approach to characterize health status in eastern box turtles (*Terrapene carolina carolina*), ornate box turtles (*Terrapene ornata ornata*), and silvery salamanders (*Ambystoma platineum*) in Illinois. Our research products included baseline hematologic, plasma biochemical, protein electrophoresis, and pathogen prevalence data, and models predicting individual and population health in each

study species. We sought to build upon our Phase I findings by incorporating surveillance for additional diseases in these species (RNA viruses), and by expanding pathogen surveys to two additional herptile species of conservation concern, the eastern massasauga rattlesnake (*Sistrurus catenatus*) and the Kirtland's snake (*Clonophis kirtlandii*). Cultivating the wellness of these species is integral to conserving ecosystems, assessing recovery efforts, and addressing a need identified by the Illinois Wildlife Action Plan (IWAP).

Eastern and ornate box turtles are biosentinel species in decline due to habitat destruction and fragmentation, road mortality, overcollection for human use, and predation (van Dijk 2011, van Dijk and Hammerson 2011). Box turtles are also increasingly threatened by infectious diseases (e.g. ranavirus, *Mycoplasmopsis* sp., herpesviruses, adenovirus) and toxicants (e.g. organochlorines, heavy metals); underscoring the need for reliable tools to characterize emerging health threats (Holcomb and Parker 1979, Beresford et al. 1981, Tangredi and Evans 1997, Feldman et al. 2006, Johnson et al. 2008, Farkas and Gál 2009, Allender et al. 2015c, Sim et al. 2015, Yonkers et al. 2015, Archer et al. 2017, Kane et al. 2017, Adamovicz et al. 2018). Over the past several years, we have been monitoring hematology, plasma biochemistries, contaminant exposure, and pathogen prevalence in Illinois box turtles. This year's work builds upon these data to identify ongoing changes that may represent deteriorating environmental conditions or specific conservation threats. Continuous health monitoring and disease investigations can provide valuable insight into ecological health as well as aid in the preservation of these species (Brown and Sleeman 2002, Sleeman 2008, Schrader et al. 2010, Stranahan et al. 2016, Sack et al. 2017).

Silvery salamanders are a triploid, all female species that co-exists with small-mouthed salamanders (*Ambystoma texanum*) in Illinois to enable their kleptogenic reproductive strategy (Uzzell and Goldblatt 1967, Morris and Brandon 1984, Spolsky et al. 1992, Phillips et al. 1997, Teltser and Greenwald 2015). They also co-occur with eastern box turtles within the Vermilion County Conservation Opportunity Area (COA). The silvery salamander is an excellent indicator of environmental change. It has a bi-phasic life cycle and as such lives in both aquatic and terrestrial habitats, transferring energy and contaminants between habitats. In addition, its permeable skin makes it sensitive to all environmental contamination. Our previous research in Phase I determined that silvery salamander larvae are highly susceptible to ranavirus infection, and recurrent mortality events have been documented in ephemeral ponds within the Vermilion County COA since 2016. Understanding the baseline health of this species prior to the emergence of additional diseases of concern (e.g. chytridiomycosis caused by *Batrachochytrium dendrobatidis* or *B. salamandrivorans*) might allow mitigation of pathogen impacts.

RNA viruses cause several important diseases in humans and animals (e.g. West Nile, Ebola, rabies, coronaviruses) and are several are associated with significant mortality in captive reptiles (Landolfi et al. 2010, Hyndman et al. 2013, Hoon-Hanks et al. 2018, Paries et al. 2019). RNA viruses have also recently been implicated in two high-profile chelonian mortality events. The endangered Bellinger river turtle (*Myuchelys georgesi*) experienced a catastrophic population decline over the course of seven weeks due to a novel nidovirus (Zhang et al. 2018). Importantly, this outbreak occurred following environmental changes suspected to disrupt the

food web and contribute to immunosuppression in the turtles, highlighting the importance of overall health status for disease development (Spencer et al. 2018). A novel bunyavirus has also been associated with an ongoing mortality event in Florida softshell turtles (*Apalone ferox*), Florida red-bellied cooters (*Pseudemys nelsoni*), and Florida cooters (*Pseudemys floridana*) (Waltzek et al. 2019). While RNA viruses are likely important for the health of captive and wild herptiles, data on pathogen presence in free-living populations is limited or non-existent. We sought determine the occurrence and clinical effects of multiple RNA viruses (ferlaviruses, nidoviruses, reoviruses, coronaviruses, parvoviruses, influenza, and picornaviruses) in wild eastern box turtles, ornate box turtles, and silvery salamanders in Illinois to supplement our understanding of health drivers in these species.

Ophidiomycosis, otherwise known as "snake fungal disease", is caused by the keratinophilic fungus *Ophidiomyces ophidiicola* (Allender et al. 2015a). This disease has been identified in over thirty snake species in the United States, Canada, and Europe, and has been associated with isolated population declines in timber rattlesnakes (*Crotalus horridus*) and eastern massasauga rattlesnakes (Clark et al. 2011, Allender et al. 2016, Lorch et al. 2016, Franklinos et al. 2017). Infection with *O. ophidiicola* results in skin lesions consisting of displaced or necrotic scales, nodules, crusts, and ulcers. While lesions are typically confined to the skin, fungal invasion into muscle or bone underlying skin lesions can occur, along with systemic dissemination (Lorch et al. 2016, Baker et al. 2019). This disease can cause significant morbidity and mortality, especially when lesions interfere with movement or food prehension. In Illinois, ophidiomycosis has been detected at annual prevalences of 14-24% in wild eastern massasaugas at Carlyle Lake, which is the last viable population of this federally threatened species in the state (Allender et al. 2011, Faust et al. 2011, Allender et al. 2015d, Allender et al. 2016, Baker et al. 2016, Szymanski et al. 2016). From 2000 – 2011, ophidiomycosis caused 7-10% of the observed mortalities in this population, highlighting the potential importance of this disease for massasauga conservation (Baker et al. 2016). In contrast, disease surveys have never been performed for the state-threatened Kirtland's snake (Illinois Endangered Species Protection Board 2015). This species is reclusive, fossorial, nocturnal, and difficult to study. However, as habitat types are similar between massasauga rattlesnakes and Kirtland's snakes (prairie wetlands), and *O. ophiodiicola* persists in soil, it is not unreasonable to expect that Kirtland's snakes may encounter and become infected by this pathogen at similar rates to massasaugas (Allender et al. 2015e). For Phase II of the "Wellness of Wildlife" project, we therefore sought characterize the epidemiology of ophidiomycosis in eastern massasauga rattlesnakes and Kirtland's snakes in Illinois.

This project addressed disease and health gaps in the IWAP for the eastern box turtle, ornate box turtle, silvery salamander, eastern massasauga, and Kirtland's snake. With the exception of the Kirtland's snake, each of these species has current or historical observations of poor health or disease susceptibility. Furthermore, the habitats of each of these species have been identified by the IWAP as locations with significant existing, or potential, wildlife habitat resources. Utilizing five species that overlap habitat, natural history, and disease threats allows integration of health results that could potentially contribute to the success of their conservation.

PURPOSE & OBJECTIVES

The purpose of this project was to assess the health of the eastern box turtle, ornate box turtle, silvery salamander, eastern massasauga rattlesnake, and Kirtland's snake through the generation of baseline hematology (EBT, OBT, SS, EMR), biochemistry (EBT, OBT), protein electrophoresis (EBT, OBT), and disease prevalence data (all species). This health monitoring established criteria that can be integrated into future conservation assessments of SGNC. Our specific objectives were:

Objective 1 – Identify at least 5 herptile species in four separate campaigns in consultation with campaign leads that fit the criteria of sample size, number of populations, and current natural history data.

Objective 2 – Establish baseline health profiles for the SGCN identified in objective 1
- Objective 2.1 – Utilize hematology, plasma biochemistry, and protein electrophoresis to characterize the general health of these species.
- Objective 2.2 – Establish baseline prevalence of common DNA pathogens in new species and expand to RNA pathogens.

Objective 3 – Assess the occurrence of emerging or ongoing infectious diseases in SGCN
- Objective 3.1 – Investigate mortality events in SGCN as they occur

Objective 4 – Provide progress reports and technical resource training
- Objective 4.1 – Prepare written quarterly and annual scientific reports
- Objective 4.2 – Provide technical resource training for IDNR staff and partners through webinars, staff presentations, or onsite training in disease detection and response

METHODS

OBJECTIVE 1

Species and Population Sampling

State agency (IDNR) staff, IWAP campaign leads, and the Wildlife Epidemiology Lab identified the eastern box turtle in the Vermillion County Conservation Opportunity Area (COA) and Stephen A. Forbes State Park, the ornate box turtle in the TNC Nachusa Grasslands Preserve, the silvery salamander in the Vermillion COA, the eastern massasauga rattlesnake at Carlyle Lake (South Shore State Park and Eldon Hazlet State Park), and the Kirtland's snake at several study sites as species of conservation concern in areas of interest.

OBJECTIVES 2.1 & 3.1

Health and Hematologic Assessments: Box Turtles

Turtles were located using a combination of human and canine search teams at five sites for eastern box turtles, and one site for ornate box turtles. Capture locations were recorded using global positioning software (GPS) via handheld devices (Garmin International Inc., Olathe, KS, USA). Turtles were returned to their exact capture location after sampling. Air temperature and substrate temperature were collected at the start and stop of each turtle search, and the results were averaged and recorded for each animal encountered (Kestrel 3000 Weather Meter, Nielsen-Kellerman, Boothwyn, PA 19061; Taylor 9878 Digital Pocket Thermometer, Taylor Precision Products, Oak Brook, IL 60523). Date, time, and categorical habitat (field, forest, edge) and microhabitat (leaves, grass, brambles, soil, road, moist area) data were also recorded at each turtle location. Deceased turtles were collected in individual sterile bags and frozen at -20^{o}C. Bone marrow samples were collected from the right bridge as previously described (Butkus et al. 2017).

Each turtle was assigned a permanent ID and mass, sex, and age status was recorded. Straight carapace length (SCL), straight carapace height (SCH), straight carapace width (SCW), and mass were determined. Physical examinations were performed, noting visual appearance of the eyes, nose, oral cavity, legs, digits, shell, and integument. Each turtle's shell was classified into one of three groups (within normal limits (WNL), active (unhealed) lesion (AL), and inactive (healed) lesion (IL)) to promote consideration of the overall condition of the shell and reduce the number of categories to include in statistical models. One combined oral and cloacal swab was collected using cotton-tipped plastic handled applicators (Fisher Scientific, Pittsburgh, PA 15275) and stored at -20^{o}C, while another was placed in RNAlater (Invitrogen, Waltham, MA, USA) and stored at -80^{o}C until RNA extraction and cDNA synthesis. A whole blood sample was taken from the subcarapacial sinus, placed in lithium heparin microtainers, and transported on wet ice until analysis later in the same day. One drop of unheparinized whole blood was also placed in RNAlater and stored at -80^{o}C.

Packed cell volume (PCV) and total solids (TS) analysis were performed by filling two sodium heparinized microhematocrit tubes (Jorgensen Laboratories, Inc., Loveland, CO 80538) from one LH microtainer tube. Each sample was centrifuged (14,500 rpm x 5 minutes) and the percent red blood cell (PCV) was recorded. Total solids were determined by refractometer (Amscope RHC-200ATC refractometer, National Industry Supply, Torrance, CA, USA) using plasma from the microhematocrit tube. Total white blood cell (WBC) counts were determined using an Avian Leukopet (Vet lab Supply, Palmetto Bay, FL, USA) on a Bright-line hemacytometer (Hausser Scientific, Horsham, PA, USA) following the manufacturer's protocol. Fresh blood smears were stained with a modified Wright's Geimsa stain and one hundred white blood cell differential counts were performed by a single observer (LA).

Plasma biochemical analysis was performed using a Beckman Coulter AU680 at University of Illinois Clinical Pathology Laboratory. Analysis includes the variables calcium, phosphorus, aspartate aminotransferase, bile acids, creatine kinase, uric acid, and glutamate dehydrogenase. Protein electrophoresis was performed using the Helena SPIFE 3000 system with split beta gels (Helena Laboratories, Inc., Beaumont, Texas 77707, USA) at the University of Miami Miller School of Medicine.

Health and Hematologic Assessments: Silvery Salamanders

Three ephemeral ponds at Kickapoo State Park in the Vermilion County COA were permanently fenced and bucket traps were installed on both the inside and outside of the fence in collaboration with another SWG (T-108-R-1). Adult silvery salamanders were captured in the bucket traps. Larval salamanders were captured at the same ponds using dipnets. Air temperature and substrate temperature were collected at the start and stop of work at each pond, and the results were averaged and recorded for each animal encountered. Adult salamanders were anesthetized using MS-222 (Sigma-Aldrich, St. Louis, MO, USA) buffered with an equal weight of sodium bicarbonate and titrated to effect. Larval salamanders were examined and sampled awake within individual Ziploc bags. Deceased salamanders were placed within individual sterile bags stored on ice, then necropsied. Samples of liver, kidney, and spleen were frozen for further diagnostics.

Each salamander was weighed and a complete physical examination was performed. Combined ventral skin and oral swabs were collected and stored in a similar manner to the box turtles. An oral swab was collected from each adult salamander and stored in RNAlater as previously described. Blood samples were collected from the ventral tail vein and non-anticoagulated blood smears were made immediately. Adult salamanders were pit-tagged for mark-recapture purposes as part of another SWG (T-108-R-1), recovered in pond water until they regained their righting reflex, and then released on the opposite side of the fence.

Health and Hematologic Assessments: Eastern Massasauga Rattlesnakes

Rattlesnakes were located using visual encounter surveys at pre-selected sites within South Shore State Park and Eldon Hazlet State Park. Air temperature and substrate temperature were collected at the start and stop of each search, and at the site of each snake capture. GPS coordinates were also taken at each site of capture, and habitat, microhabitat, degree of grass cover, and behavior were also recorded. Following capture, snakes were secured within individual pillow cases and transported to a field lab site for processing.

Each snake received a complete physical exam, during which any skin lesions consistent with ophidiomycosis were documented (see Baker et al. 2019), and snout-vent length (SVL), tail length (TL), number of rattle segments, and weight were recorded. Newly encountered snakes were pit-tagged as part of an ongoing demographic study. Blood was collected from the ventral tail vein, immediately placed into lithium heparin microtainers, and stored at 4^{o}C until processing. One swab was rubbed vigorously along the entire body of each snake (body swab), and separate swabs were collected from skin lesions, if present. Each swab was stored in a separate, sterile Eppendorf tube and frozen at -20^{o}C. Snakes were then transported back to their original sites of capture and released. Hematologic processing for massasaugas followed the box turtle protocol described above.

Health Assessments: Kirtland's Snakes

Kirtland's snakes were located under cover objects (carpet squares) placed as part of another SWG (T-118-R1). GPS, environmental, and behavioral data were collected as described above. Each snake received a complete physical examination and weight, SVL, and TL were recorded. Body swabs and lesion swabs were collected and stored as previously described.

OBJECTIVE 2.2 & 3.1

DNA and RNA Extraction:

DNA was extracted from whole blood (box turtles only), bone marrow, tissues collected during mortality events, and swab samples using a commercially available kit (QIAmp DNA Blood Mini Kit and DNAeasy kit, Qiagen, Valencia, CA, USA). For snake skin swabs, samples were incubated with 12.5 U of lyticase (Sigma-Aldrich) at 37ºC for 60 minutes immediately prior to DNA extraction. This step is designed to break down fungal cell walls to facilitate detection of *O. ophiodiicola* DNA (Allender et al. 2015b). Previously described modifications were also followed for DNA extraction from bone marrow (Butkus et al. 2017). DNA quality and purity were assessed spectrophotometrically (NanoDrop 1000, Thermo Fisher, Waltham, MA).

RNA was extracted from swab samples following an existing protocol in 2019 (Espinosa-de Aquino et al. 2017). Briefly, all tubes were vortexed and then centrifuged for 15 minutes at 16,000g. The supernatant was removed by pipette, and then tubes were centrifuged for 5 minutes at 2,000g. The supernatant was discarded, and the remaining pellet was used for RNA extraction using a commercial kit according to the manufacturer's recommendations (Qiagen RNEasy, Valencia, CA). In 2020 a new RNA extraction protocol was developed in consultation with technical advisors at Qiagen. In this protocol, tubes were first centrifuged for 3 minutes at maximum speed. Following this, all RNAlater was removed via pipetting, 600µL of buffer RLT was added, and the tubes were vortexed vigorously for two minutes. The kit protocol was then followed as-written with the exception of an additional 500µL wash step with buffer RPE. After extraction, the RNA samples were analyzed using spectrophotometry to check for quantity and purity. The RNA was stored in a -80°C freezer until reverse transcription into cDNA, which was performed following manufacturer's recommendations (Quantitect Reverse Transcription kit, Qiagen). All cDNA products were analyzed using spectrophotometry and stored at -80°C.

Disease Detection:

DNA samples from box turtles and silvery salamanders were assayed in triplicate for multiple pathogens using the Fluidigm platform at the Keck Biotechnology Center, inclusive of standard curves and negative controls. Quantitative PCR was performed in a multiplex format (Fluidigm) using published or in-house primer-probe assays as previously described (Archer et al. 2017). Box turtles were screened for the following pathogens: frog virus 3 (FV3), *Ambystoma tigrinum* virus (ATV), Bohle iridovirus (BIV), epizootic hematopoietic necrosis virus (EHNV), *Terrapene* herpesvirus 1 (TerHV1), *Terrapene* herpesvirus 2 (TerHV2), Testudinid herpesvirus 2 (TestHV2), box turtle *Mycoplasma* sp., *Mycoplasma testudineum*, *Mycoplasma agassizii*, box turtle adenovirus, *Salmonella enteritidis*, *Salmonella tymphimurium*, tortoise intranuclear coccidiosis (TINC), pathogenic leptospires, *Anaplasma phagocytophilum*, and *Borrelia burgdorferi.* Salamanders were screened for *Batrachochytrium dendrobatidis* (Bd), *Batrachochytrium salamandrivorans* (Bsal), FV3, ATV, BIV, EHNV, the Chlamydiaceae family, and Perkinsus organisms.

DNA samples from massasauga and Kirtland's snake skin swabs were assayed in triplicate for *O. ophiodiicola* using an existing Taqman qPCR assay, inclusive of a standard curve and negative controls as previously described (Allender et al. 2015b). Animals were considered qPCR positive if they had a positive result from any of their swabs (body or lesions).

Each snake was then categorized into one of four ophidiomycosis classifications (ophidiomycosis negative, *Ophidiomyces* present, possible ophidiomycosis, or apparent ophidiomycosis) based on the presence of skin lesions and qPCR results, as previously described (Baker et al. 2019).

Box turtles and silvery salamanders were screened for RNA viruses using existing consensus conventional PCR assays for picornaviruses (Marschang et al. 2016), ferlaviruses (Ahne et al. 1999, Kolesnik et al. 2017), nidoviruses (Marschang and Kolesnik 2017), reoviruses (Wellehan et al. 2009), parvoviruses (Pénzes et al. 2014), coronaviruses (Stephensen et al. 1999), and influenza (Gall et al. 2008). Products were resolved on 1% agarose gels.

Bone marrow samples and tissues collected during mortality events were tested for FV3 using an existing Taqman qPCR assay, including a standard curve and negative control, as previously described (Allender et al. 2013).

Statistical Analyses*:*

All statistical analyses were performed in R v. 3.5.1 (R Core Team 2018). The distribution of each continuous variable was assessed visually using box plots and histograms and statistically using the Shapiro-Wilk test. Descriptive statistics (mean, standard deviation, range for normally distributed variables; median, 25% and 75% percentiles, and range for non-normally distributed variables) and counts were tabulated for continuous and categorical variables, respectively.

Directed acyclic graphs (DAG) were generated separately for each species to demonstrate expected relationships among measured predictors. These diagrams were used to identify potential confounding variables and structure statistical analyses, using multivariable linear regression when necessary to control for confounders. Continuous predictor variables were assessed for multicollinearity using variance inflation factors (VIF; package car; Fox and Weisburg 2011). Strongly correlated predictor variables (VIF > 3) were not included together in statistical models. Data transformation was pursued if needed to support statistical assumptions during modeling.

Sex ratios were evaluated using binomial tests (expected ratio 0.5). Cohen's Kappa was used to evaluate agreement between *O. ophidiicola* qPCR results from body and lesion swabs. Predictors of continuous outcomes (e.g. clinical pathology parameters) were evaluated using general linear models. Predictors of categorical outcomes (e.g. pathogen presence) were evaluated using bias-reduced generalized linear models in package brglm (Kosmidis 2017). Predictors of ordinal outcomes (e.g. ophidiomycosis classification) were evaluated using multinomial logistic regression modeling (function multinom, package nnet, Venables and Ripley 2002) with a Bonferroni p-value adjustment. Post-hoc tests were performed with the contrast function (package lsmeans, Lenth 2016) using a Tukey adjustment for multiple statistical comparisons. Unless otherwise indicated, alpha was set at 0.05.

Sample size calculations were performed to determine the number of Kirtland's snake swabs needed to detect varying levels of *Ophidiomyces ophidiicola* prevalence (1%, 2%, 3%, 5%, 10%, 20%), at different snake population sizes (50, 100, 200, 300, 500, and 1000). The

specificity for qPCR detection of *Ophidiomyces* DNA was assumed to be one, while a value of 0.8 was chosen for sensitivity, based on a previous study estimating the probability of false negative qPCR results when multiple swabs are collected and tested (Hileman et al., 2018). Herd sensitivity, or the probability of infected snakes being detected at a site where ophidiomycosis is actually present, was set at 0.95. These calculations were performed using an online tool provided at epitools.ausvet.com.au, based on an approximation of the hypergeometric distribution modified to account for imperfect diagnostic test sensitivity (MacDiarmid 1988).

OBJECTIVE 4.2

Technical Resource Training:

Onsite technical training was provided on search days at each site for IDNR staff and partners present. This occurred yearly at each site when possible. Formal technical training and summary information from this project have also been performed in 2018 for AFWA Midwest Fish and Wildlife Forum, in 2019 for the Midwest Fish and Wildlife Conference, Nachusa Grasslands Science Symposium, Illinois Association of Conservation Districts, Illinois Naturalist meeting, in 2020 at the Midwest Fish and Wildlife Conference, and in 2021 for the Chicago Zoological Society Science Series, and the Fish and Wildlife Service Aquatic Chelonian Research Group. Formal presentations were done remotely in 2020 as COVID-19 impacted many activities. Scientific presentations given based on data from this project are listed above.

RESULTS

Pandemic-Associated Limitations

We experienced a global pandemic that impacted both field and laboratory work starting in April, 2020. Many aspects of this project were impacted or delayed due to restricted access to field sites and safety protocols put in place by the university. Unfortunately, massasauga sampling was not possible in 2020 due to state-wide stay-at-home orders during the month of April. Sample sizes were reduced in 2020 compared to a more typical year, and access to laboratory spaces was limited for safety reasons. Sampling occurred on a more normal schedule in 2021 and overall sample sizes for this project were met or exceeded.

Eastern Box Turtles

A total of 324 eastern box turtles were evaluated at five study sites from 2019 – 2021. Demographic data, physical examination abnormalities, and pathogen presence is summarized by study site in Table 1 and Figures 1 & 2. Co-pathogens were detected less frequently than single pathogens. The most common co-detected pathogens were *Mycoplasmopsis* sp. and TerHV2 (N = 4) followed by TerHV1 and adenovirus (N = 3), TerHV2 and adenovirus (N = 3), *Mycoplasmopsis* sp. and adenovirus (N = 3), and TerHV1 and *Mycoplasmopsis* sp. (N = 3). Least common were TerHV1, TerHV2, and adenovirus (N = 1), and TerHV1 and TerHV2 (N = 1). Nine deceased turtles were collected over the last three years, all tested negative for FV3. Clinical pathology data are presented in Table 4.

Habitat use differed by study site, age class, and sex. Turtles at Kennekuk were sampled in edge habitats more frequently ($p < 0.0001$) and forest habitats less frequently ($p < 0.0001$) than turtles at other study sites. Juvenile turtles were sampled more frequently in field habitats vs. forests ($p = 0.01$), and adults were sampled more frequently in forests vs. fields ($p = 0.01$). Female turtles were sampled more frequently in edge habitats ($p = 0.007$) and males were sampled more frequently in forests ($p = 0.007$).

The occurrence of physical examination abnormalities differed by age class. Specifically, the odds of upper respiratory disease were 3.85 times higher (95% CI = 1.15 – 12.9, $p = 0.01$) and the odds of shell abnormalities were 4.28 times higher (95% CI = 1.34 – 13.7, $p = 0.01$) in adults than juveniles. Similarly, the odds of any physical examination abnormality were 2.62 times higher in adults than juveniles (95% CI = 1.17 – 5.89, $p = 0.02$).

Pathogen prevalence differed by season, age class, sex, and the presence of physical exam abnormalities. The odds of adenovirus detection were 3.32 times higher in juveniles vs. adults (95% CI = 1.5 – 7.35, $p = 0.003$) and 2.57 times higher in spring vs. summer (95% CI = 1.13 – 5.85, $p = 0.02$). The odds of TerHV1 detection were 2.9 times higher in males vs. females (95% CI = 1.15 – 7.3, $p = 0.02$). The odds of *Mycoplasmopsis* sp. detection were 2.63 times higher in turtles with any physical examination abnormality (95% CI = 1.24 – 5.59, $p = 0.01$).

Clinical pathology parameters differed by year, season, study site, sex, age class, the presence of physical exam abnormalities, and pathogen status. All parameters except PCV, total leukocyte count (WBC), eosinophil count, basophil count, erythrocyte sedimentation rate (ESR), bile acids, uric acid, calcium, creatine kinase (CK), and albumin significantly varied by year ($p < 0.05$). Study site differences largely occurred between Forbes, Kennekuk, and Collison. Specifically, turtles from Forbes had higher total solids (TS) (effect size = 0.55 g/dL, 95% CI = 0.05 – 1.06 g/dL, $p = 0.02$), total protein (TP) (effect size = 0.62 g/dL, 95% CI = 0.09 – 1.16 g/dL, $p = 0.01$), albumin (effect size = 0.2 g/dL, 95% CI = 0.04 – 0.36 g/dL, $p = 0.006$), and bile acids (effect size = 2.78 μmol/L, 95% CI = 0.32 – 5.24 μmol/L, $p = 0.02$) and lower heterophil counts (effect size = 1,537 cells/μL, 95% CI = 305 – 2,768 cells/μL, $p = 0.006$) compared to turtles from Kennekuk. Forbes turtles also had higher TP (effect size = 0.87 g/dL, 95% CI = 0.07 – 1.68 g/dL, $p = 0.03$) and albumin (effect size = 0.33 g/dL, 95% CI = 0.09 – 0.57 g/dL, $p = 0.002$) compared to Collison turtles. Alpha 1 globulins were also higher at Forbes compared to all other sites ($p < 0.0001$). Turtles from Forest Glen had lower bile acids compared to Collison (effect size = 5.3 μmol/L, 95% CI = 1.0 – 9.56 μmol/L, $p = 0.007$) and Forbes (effect size = 3.5 μmol/L, 95% CI = 0.34 – 6.61 μmol/L, $p = 0.02$). Finally, turtles from Collison had higher bile acids compared to those sampled at Kennekuk (effect size = 4.6 μmol/L, 95% CI = 0.8 – 8.4 μmol/L, $p = 0.009$).

Significant seasonal variability was noted in several clinical pathology parameters. The heterophil to lymphocyte ratio (H:L) was significantly higher in spring vs. summer (effect size = 0.277, 95% CI = 0.195 – 0.358, $p < 0.0001$) and fall (effect size = 0.230, 95% CI = 0.063 – 0.397, $p = 0.001$). The same trend was observed for alpha 1 globulins, which were higher in spring vs. summer (effect size = 0.1 g/dL, 95% CI = 0.06 – 0.14 g/dL, $p < 0.0001$) and fall (effect size = 0.11 g/dL, 95% CI = 0.02 – 0.2 g/dL, $p = 0.003$). GLDH concentrations were significantly higher in summer vs. spring (effect size = 11.64 U/L, 95% CI = 3.83 – 19.4 U/L, p

= 0.0005) and fall (effect size = 17.88 U/L, 95% CI = 4.28 – 31.5 U/L, p = 0.002). Similarly, aspartate aminotransferase (AST) concentrations were higher in summer vs. spring (effect size = 18.75 U/L, 95% CI = 3.1 – 34.4 U/L, p = 0.005) and fall (effect size = 26.42 U/L, 95% CI = 1.44 – 54.3 U/L, p = 0.03). TP was significantly higher in spring vs. summer (effect size = 0.43 g/dL, 95% CI = 0.05 – 0.81 g/dL, p = 0.009), as were alpha 2 globulins (effect size = 0.15 g/dL, 95% CI = 0.02 – 0.27 g/dL, p = 0.007), heterophils, (effect size = 2,708 cells/μL, 95% CI = 1,893 – 3,523 cells/μL, p < 0.0001) and monocytes (effect size = 298 cells/μL, 95% CI = 136 – 460 cells/μL, p = 0.0001). Bile acids (effect size = 2.4 μmol/L, 95% CI = 0.54 – 4.26 μmol/L, p = 0.003) and the albumin to globulin ratio (AGR) (effect size = 0.044, 95% CI = 0.02 – 0.68, p = 0.0001) were significantly higher in summer vs. spring. Prealbumin concentrations were significantly lower in fall compared to spring (effect size = 0.12 g/dL, 95% CI = 0.05 – 0.19 g/dL, p < 0.0001) and summer (effect size = 0.14 g/dL, 95% CI = 0.07 – 0.21 g/dL, p < 0.0001). Gamma globulin concentrations were significantly higher in fall compared to both spring (effect size = 0.16 g/dL, 95% CI = 0.02 – 0.3 g/dL, p = 0.007) and summer (effect size = 0.2 g/dL, 95% CI = 0.06 – 0.34 g/dL, p = 0.0009).

Many expected age and sex-related clinical pathology associations were identified. Adult turtles had higher TS (effect size = 0.76 g/dL, 95% CI = 0.32 – 1.21 g/dL, p = 0.02), calcium (effect size = 2.2 mg/dL, 95% CI = 0.35 – 4.06 mg/dL, p = 0.02), calcium to phosphorous ratios (effect size = 0.56 mg/dL, 95% CI = 0.184 – 0.935 mg/dL, p = 0.004), and beta globulins (effect size = 0.63 g/dL, 95% CI = 0.34 – 0.92 g/dL, p < 0.0001) than juveniles. Juvenile turtles had higher lymphocyte counts (effect size = 5,559 cells/μL, 95% CI = 2,420 – 8,698 cells/μL, p = 0.0006), basophil counts (effect size = 1,867 cells/μL, 95% CI = 1,198 – 2,537 cells/μL, p < 0.0001), creatine kinase (CK) concentrations (effect size = 306 U/L, 95% CI = 154 – 457 U/L, p = 0.0001), albumin (effect size = 0.22 g/dL, 95% CI = 0.05 – 0.4 g/dL, p = 0.01), and AGR (effect size = 0.1, 95% CI = 0.062 – 0.131, p < 0.0001) compared to adults. Female turtles had higher TS (effect size = 0.37 g/dL, 95% CI = 0.07 – 0.68 g/dL, p = 0.02), calcium (effect size = 5.52 mg/dL, 95% CI = 4.61 – 6.44 mg/dL, p < 0.0001), phosphorous (effect size = 1.31 mg/dL, 95% CI = 1.01 – 1.62 mg/dL, p < 0.0001), prealbumin (effect size = 0.03 g/dL, 95% CI = 0.006 – 0.059 g/dL, p = 0.02), and beta globulins (effect size = 0.47 g/dL, 95% CI = 0.31 – 0.63 g/dL, p < 0.0001) than males. Males had higher CK (effect size = 143 U/L, 95% CI = 53 – 233 U/L, p = 0.002), albumin (effect size = 0.18 g/dL, 95% CI = 0.08 – 0.27 g/dL, p = 0.0005), alpha 1 globulins (effect size = 0.04 g/dL, 95% CI = 0.003 – 0.07 g/dL, p = 0.03), and AGR (effect size = 0.054, 95% CI = 0.035 – 0.073, p < 0.0001).

Interestingly, several clinical pathology parameters were associated with clinical signs of illness and pathogen detection. Turtles with upper respiratory disease had lower lymphocyte counts (effect size = 2,770 cells/μL, 95% CI = 330 – 5,211 cells/μL, p = 0.03) and AGR (effect size = 0.027, 95% CI = 0.001 – 0.053, p = 0.04) and higher prealbumin concentrations (effect size = 0.05 g/dL, 95% CI = 0.019 – 0.082 g/dL, p = 0.002). Turtles with any physical exam abnormality had higher prealbumin concentrations (effect size = 0.038 g/dL, 95% CI = 0.012 – 0.064 g/dL, p = 0.004) and lower albumin concentrations (effect size = 0.12 g/dL, 95% CI = 0.01 – 0.22 g/dL, p = 0.03) and AGR (effect size = 0.023, 95% CI = 0.001 – 0.044, p = 0.04) than turtles without apparent physical abnormalities. Turtles testing positive for TerHV2 had higher WBC counts (effect size = 5,738 cells/μL, 95% CI = 484 – 10,992 cells/μL, p = 0.03), heterophil counts (effect size = 1,963 cells/μL, 95% CI = 637 – 3,288 cells/μL, p = 0.004), and basophil

counts (effect size = 1,012 cells/μL, 95% CI = 180 – 1,843 cells/μL, p = 0.02). Turtles testing positive for *Mycoplasmopsis* sp. had higher heterophil counts (effect size = 1,340 cells/μL, 95% CI = 135 – 2,545 cells/μL, p = 0.03), monocyte counts (effect size = 240 cells/μL, 95% CI = 18 – 462 cells/μL, p = 0.03), and H:L ratios (effect size = 0.142, 95% CI = 0.029 – 0.255, p = 0.01). Turtles testing positive for Terrapene adenovirus 1 had lower albumin concentrations (effect size = 0.16 g/dL, 95% CI = 0.02 – 0.31 g/dL, p = 0.03) and higher alpha 1 globulins (effect size = 0.07 g/dL, 95% CI = 0.02 – 0.12 g/dL, p = 0.005) and alpha 2 globulins (effect size = 0.25 g/dL, 95% CI = 0.09 – 0.4 g/dL, p = 0.002).

Table 1. Demographics, physical exam abnormalities, and pathogen presence in eastern box turtles by study site 2019 – 2021.

		Collison	Kennekuk	Forest Glen	Kickapoo	Forbes
Year	**2019**	2	31	16	10	51
	2020	15	40	9	11	29
	2021	18	46	23	10	13
Season	**Spring**	9	59	14	12	51
	Summer	26	47	24	19	42
	Fall	0	11	10	0	0
Age Class	**Adult**	32	95	45	27	84
	Juvenile	3	22	3	4	9
Sex	**Female**	17	63	17	17	39
	Male	15	32	28	12	45
	Unknown	3	22	3	2	9
Physical Exam	**Asymmetrical Nares**	11 (31%)	15 (13%)	7 (15%)	7 (33%)	10 (11%)
	Upper Respiratory Disease	2 (6%)	9 (8%)	4 (8%)	3 (14%)	7 (8%)
	Aural Abscess	0	1 (0.9%)	1 (2%)	0	4 (4%)
	Head, Limb, or Tail Trauma	5 (14%)	10 (9%)	6 (13%)	1 (5%)	8 (9%)
	Burn Injury	0	1 (0.9%)	1 (2%)	1 (5%)	3 (3%)
	Developmental Abnormality	3 (9%)	5 (4%)	2 (4%)	3 (14%)	6 (6%)
	Active Shell Lesion	7 (20%)	15 (13%)	4 (8%)	2 (10%)	6 (6%)
	Inactive Shell Lesion	17 (49%)	35 (30%)	14 (29%)	14 (67%)	22 (24%)
	# Turtles Tested	35	114	46	31	92
Pathogens	***Terrapene* adenovirus**	1 (3%)	17 (15%)	6 (13%)	1 (3%)	19 (21%)
	***Terrapene* herpesvirus 1**	4 (11%)	5 (4%)	5 (11%)	0	10 (11%)
	***Terrapene* herpesvirus 2**	3 (9%)	9 (8%)	6 (13%)	0	6 (7%)
	***Mycoplasmopsis* sp.**	2 (6%)	14 (12%)	3 (7%)	1 (3%)	11 (12%)
Total		35	117	48	21	93

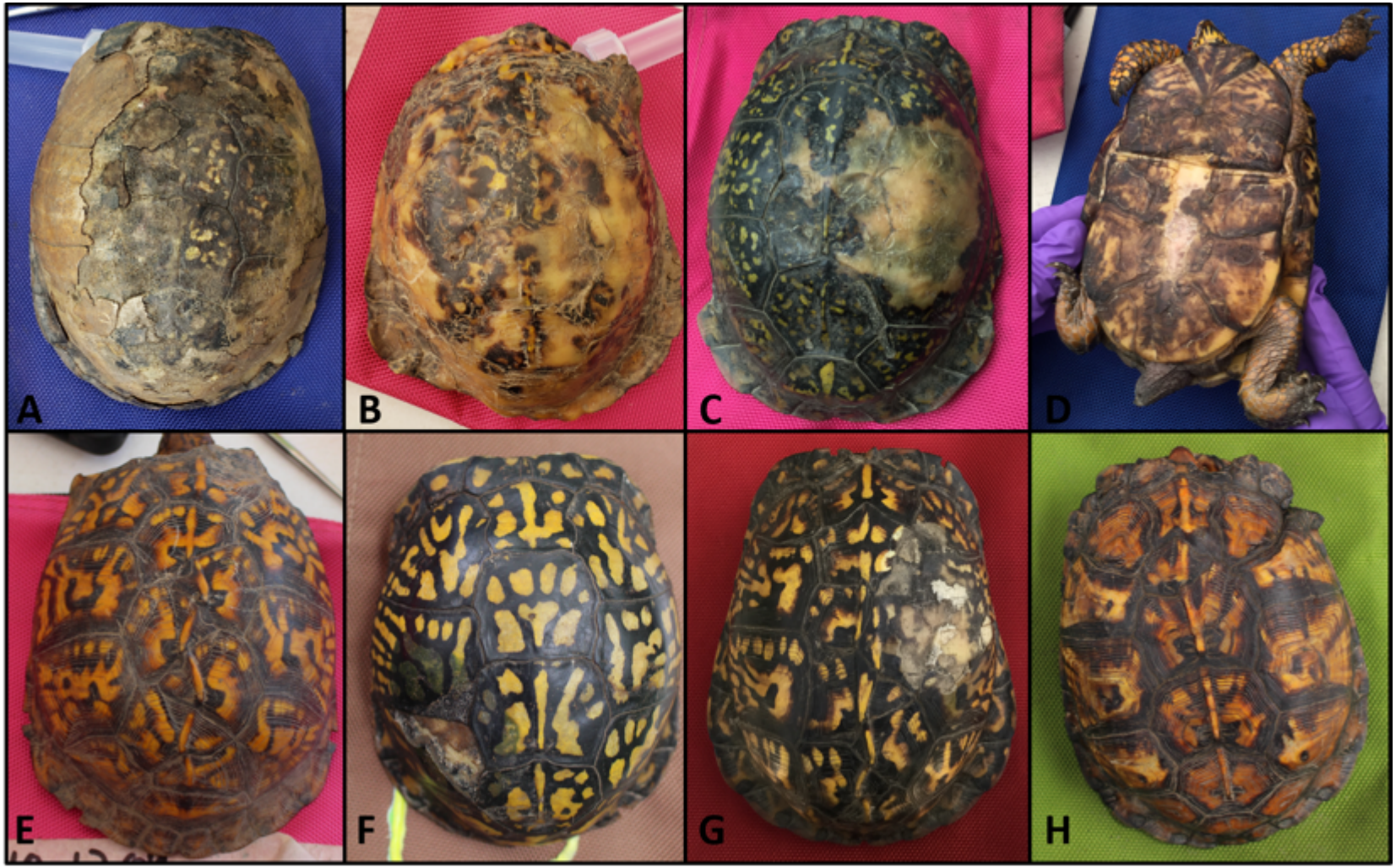

Figure 1. Common eastern box turtle shell abnormalities. A – D) Burn injuries in various stages of healing. E) Developmental abnormality – supranumerary vertebral scutes. F & G) Active shell lesions characterized by moist, soft foci. Turtle G also has supranumerary vertebral scutes. H) Inactive shell lesion – healed fracture of the 1st right costal scute. This individual also has supranumerary vertebral scutes.

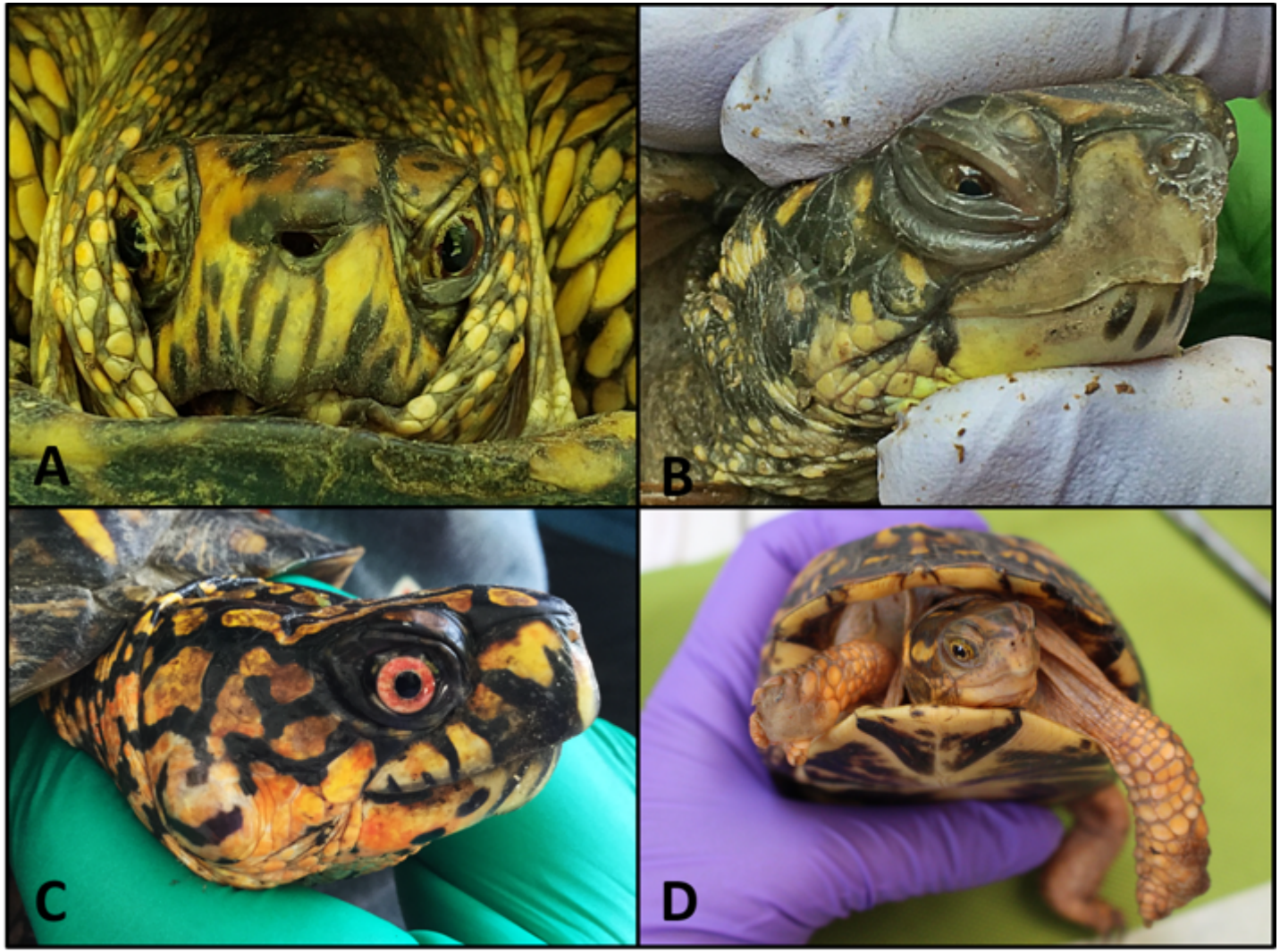

Figure 2. Common physical examination abnormalities in eastern box turtles. A & B) Clinical signs of upper respiratory disease including nasal asymmetry or erosion (A), blepharoedema (B), and nasal discharge (B). C) Aural abscess. D) Trauma – this individual previously lost both front feet.

Table 2. Descriptive statistics for eastern box turtle hematology, plasma biochemistries, and protein electrophoresis from 2019 – 2021. Central tendency = mean or median, Dispersion = standard deviation or 10^{th} – 90^{th} percentiles depending upon data distribution.

Analyte	N	Distribution	Central Tendency	Dispersion	Min	Max
Packed Cell Volume (%)	318	Non-normal	24.5	20 – 28	7	48.5
Total Solids (g/dL)	318	Non-normal	6.3	5.4 – 7.2	1.6	11.0
Total Leukocyte Count (/µL)	317	Non-normal	21,120	15,644 – 28,160	3,367	127,424
Heterophils (/µL)	317	Non-normal	2,673	1,564 – 4,572	253	24,334
Lymphocytes (/µL)	317	Non-normal	11,092	7,523 – 17,389	1,993	104,488
Monocytes (/µL)	317	Non-normal	375	162 – 714	0	4,259
Eosinophils (/µL)	317	Non-normal	2,984	1,827 – 4,267	209	18,204
Basophils (/µL)	317	Non-normal	1,545	908 – 2,620	0	15,291
Heterophil / Lymphocyte	317	Non-normal	0.250	0.133 – 0.447	0.011	2.526
Erythrocyte Sedimentation Rate (mm)	203	Non-normal	4.89	4.00 – 6.36	2.10	46.00
Glutamate Dehydrogenase (U/L)	221	Non-normal	11.4	7 – 20.5	1.5	191.3
Bile Acids (µmol/L)	221	Non-normal	6.0	3.6 – 7.98	1.1	40.5
Uric Acid (mg/dL)	221	Non-normal	1.4	1.1 – 1.5	0.8	5.0
Calcium (mg/dL)	221	Non-normal	11.75	10.3 – 15.83	8.0	30.7
Phosphorous (mg/dL)	221	Non-normal	3.8	3.1 – 4.7	1.9	9.9
Calcium / Phosphorous	221	Non-normal	3.36	2.83 – 3.94	1.57	6.43
Aspartate Aminotransferase (U/L)	221	Non-normal	70	50.8 – 98.8	19	293
Creatine Kinase (U/L)	221	Non-normal	285	165 – 494	17	2,346
Total Protein (g/dL)	238	Normal	6.4	1.2	2.8	10.1
Prealbumin (g/dL)	238	Non-normal	0.19	0.14 – 0.26	0.01	0.89
Albumin (g/dL)	238	Non-normal	1.44	1.23 – 1.7	0.57	2.61
Alpha 1 Globulins (g/dL)	238	Non-normal	0.38	0.29 – 0.46	0.17	0.83
Alpha 2 Globulins (g/dL)	238	Non-normal	1.64	1.37 – 1.9	0.63	3.27
Beta Globulins (g/dL)	238	Non-normal	1.98	1.64 – 2.4	0.81	4.78
Gamma Globulins (g/dL)	238	Non-normal	0.55	0.46 – 0.67	0.09	1.46
Albumin / Globulin	238	Normal	0.361	0.08	0.18	0.59

Ornate Box Turtles

A total of 202 ornate box turtles were evaluated from 2019 – 2021. Turtles were assessed at the Orland Track and South Bison Unit of the Nachusa Grasslands (N = 138) in all three years, while turtles at the INPC Ayers Sand Prairie Nature Preserve (N = 64) were only sampled in 2019. Two shells were recovered from Nachusa and both tested negative for FV3. Demographic data, physical examination abnormalities, and pathogen presence is summarized in Table 3 and Figure 3. Clinical pathology results are summarized in Table 4.

Turtles sampled at Nachusa were larger in both weight (effect size = 71g, 95% CI = 51 – 91g, $p < 0.0001$) and multiple standardized shell measurements (SCL, SCW, CW, etc.; $p < 0.05$) than those at Ayers. Nachusa turtles also had higher body condition scores (effect size = 1.36, 95% CI = 0.92 – 1.8, $p < 0.0001$). Distribution of age classes, sexes, and physical examination abnormalities was similar between sites ($p > 0.05$), however, turtles at Ayers had more evidence of shell trauma secondary to vehicular or mower strike than turtles at Nachusa.

Clinical pathology values differed by year, site, month, sex, age class, and condition of the shell. All parameters except WBC count, monocyte count, basophil count, bile acids, uric acid, calcium, calcium to phosphorous ratio, AST, CK, and beta globulins varied between years ($p < 0.05$). Turtles at Ayers had higher PCV (effect size = 2.3%, 95% CI = 0.8 – 3.8%, $p = 0.003$), WBC count (effect size = 2,748 cells/μL, 95% CI = 281 – 5,215 cells/μL, $p = 0.03$), heterophil count (effect size = 4,757 cells/μL, 95% CI = 3,672 – 5,841 cells/μL, $p < 0.0001$), and H:L (effect size = 1.278, 95% CI = 0.875 – 1.680, $p < 0.0001$) compared to turtles at Nachusa. In contrast, Nachusa turtles had higher TS (effect size = 2.05 g/dL, 95% CI = 1.79 – 2.31 g/dL, $p < 0.0001$) and basophils (effect size = 213 cells/μL, 95% CI = 15 – 411 cells/μL, $p = 0.03$).

Turtles sampled in May had higher PCV (effect size = 2.9%, 95% CI = 1.2 – 4.6%, $p = 0.0009$), heterophil count (effect size = 4,203 cells/μL, 95% CI = 2,882 – 5,523 cells/μL, $p < 0.0001$), H:L (effect size = 0.998, 95% CI = 0.517 – 1.478, $p < 0.0001$), and alpha 1 globulins (effect size = 0.072 g/dL, 95% CI = 0.025 – 0.119 g/dL, $p = 0.003$) than those sampled in June. June turtles had higher TS (effect size = 0.83 g/dL, 95% CI = 0.4 – 1.25 g/dL, $p = 0.0002$), lymphocyte count (effect size = 5,268 cells/μL, 95% CI = 3,388 – 7,148 cells/μL, $p < 0.0001$), eosinophil count (effect size = 1,333 cells/μL, 95% CI = 803 – 1,862 cells/μL, $p < 0.0001$), ESR (effect size = 2.38 mm, 95% CI = 1.61 – 3.15 mm, $p < 0.0001$), phosphorous (effect size = 0.7 mg/dL, 95% CI = 0.23 – 1.17 mg/dL, $p = 0.004$), prealbumin (effect size = 0.08 g/dL, 95% CI = 0.05 – 0.11 g/dL, $p < 0.0001$), alpha 2 globulins (effect size = 0.164 g/dL, 95% CI = 0.033 – 0.294 g/dL, $p = 0.01$), and AGR (effect size = 0.043, 95% CI = 0.02 – 0.066, $p = 0.0004$).

Juvenile turtles had higher heterophil counts (effect size = 2,374 cells/μL, 95% CI = 324 – 4,423 cells/μL, $p = 0.02$), monocyte counts (effect size = 427 cells/μL, 95% CI = 102 – 751 cells/μL, $p = 0.01$), basophil counts (effect size = 347 cells/μL, 95% CI = 24 – 670 cells/μL, $p = 0.04$), H:L (effect size = 1.01, 95% CI = 0.3 – 1.71, $p = 0.005$), and AGR (effect size = 0.095, 95% CI = 0.033 – 0.157, $p = 0.003$) than adults.

Female turtles had higher calcium (effect size = 4.41 mg/dL, 95% CI = 3.28 – 5.54 mg/dL, $p < 0.0001$) and phosphorous (effect size = 1.02 mg/dL, 95% CI = 0.6 – 1.45 mg/dL, $p <$

0.0001) than males. Male turtles had higher PCV (effect size = 3%, 95% CI = 1.5 – 4.4%, p < 0.0001), AST (effect size = 57 U/L, 95% CI = 27 – 87 U/L, p = 0.0004), and alpha 1 globulins (effect size = 0.079 g/dL, 95% CI = 0.035 – 0.123 g/dL, p = 0.0006) than females.

Turtles with normal shells had lower TS than those with inactive shell injuries (effect size = 0.5 g/dL, 95% CI = 0.0004 – 1.0 g/dL, p = 0.04) and active shell injuries (effect size = 0.61 g/dL, 95% CI = 0.07 – 1.15 g/dL, p = 0.0002). Turtles with active shell injuries had higher GLDH than turtles with normal shells (effect size = 18.66 U/L, 95% CI = 5.33 – 32 U/L, p = 0.004). Sample size was too low to evaluate for relationships between pathogen presence and clinical pathology parameters.

Table 3. Demographics, physical exam abnormalities, and pathogen presence in ornate box turtles by capture site 2019 – 2021: Nachusa's Orland Track and South Bison Unit (SBU) vs. Ayers Sand Prairie.

		Orland	Ayers
Year	**2019**	66	64
	2020	28	0
	2021	44	0
Month	**May**	95	64
	June	43	0
Age Class	**Adult**	127	57
	Juvenile	11	7
Sex	**Female**	62	19
	Male	64	37
	Unknown	12	8
Physical Exam	**Asymmetrical Nares**	6 (4%)	0
	Head, Limb, or Tail Trauma	5 (4%)	6 (9%)
	Burn Injury	2 (1%)	0
	Developmental Abnormality	1 (0.7%)	2 (3%)
	Active Shell Lesion	37 (27%)	8 (13%)
	Inactive Shell Lesion	44 (32%)	14 (22%)
Pathogens	***Terrapene* adenovirus**	3 (2%)	NA
	Leptospirosis	1 (0.7%)	NA
Total		138	64

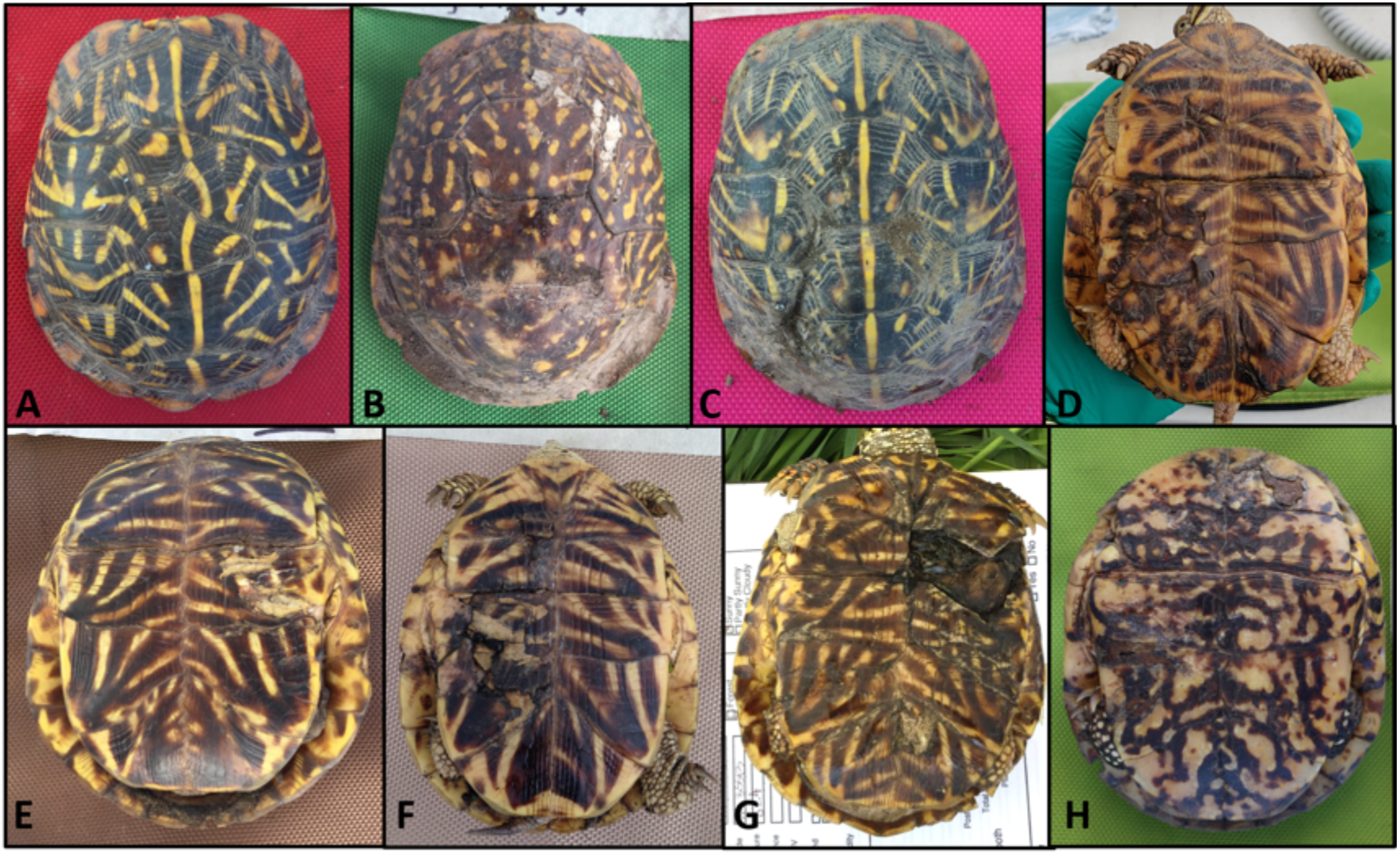

Figure 3. Common ornate box turtle shell abnormalities. A) Developmental abnormality – supranumerary vertebral scutes. B) Healed burn injury. C & D) Inactive shell lesions including a healed fracture (C) and healed shell trauma (D). E – H) Active shell lesions characterized by moist, soft foci and/or bleeding upon manipulation.

Table 4. Descriptive statistics for ornate box turtle hematology, plasma biochemistries, and protein electrophoresis 2019 - 2021. Central tendency = mean or median, Dispersion = standard deviation or 10^{th} – 90^{th} percentiles depending upon data distribution.

Analyte	N	Distribution	Central Tendency	Dispersion	Min	Max
Packed Cell Volume (%)	202	Non-normal	25	22.6 – 28.9	12.5	45
Total Solids (g/dL)	202	Non-normal	6.0	5 – 6.8	3.0	9.55
Total Leukocyte Count (/µL)	202	Non-normal	19,603	14,879 – 25,674	5,927	52,467
Heterophils (µL)	202	Non-normal	5,626	3,824 – 8,652	1,119	27,779
Lymphocytes (µL)	202	Non-normal	8,599	5,570 – 13,998	1,067	32,326
Monocytes (µL)	202	Non-normal	716	378 – 1,090	0	4,956
Eosinophils (µL)	202	Non-normal	2,374	1,375 – 3,518	118	7,715
Basophils (µL)	202	Non-normal	475	205 - 835	0	5,697
Heterophil / Lymphocyte	202	Non-normal	0.700	0.401 – 1.19	0.048	12.43
Erythrocyte Sedimentation Rate (mm)	71	Non-normal	4.25	3.02 – 5.56	1.83	11.8
Glutamate Dehydrogenase (U/L)	68	Non-normal	18.95	8.4 – 33.83	3.1	83.2
Bile Acids (µmol/L)	68	Non-normal	4.6	3.6 – 6.9	2.4	21.7
Uric Acid (mg/dL)	68	Non-normal	1.4	1.2 – 1.6	0.8	2.9
Calcium (mg/dL)	68	Non-normal	10.6	9.08 – 13.4	7.7	22.5
Phosphorous (mg/dL)	68	Non-normal	3.1	2.8 – 3.9	1.9	6.0
Calcium / Phosphorous	68	Normal	3.59	0.8	1.56	5.21
Aspartate Aminotransferase (U/L)	68	Non-normal	94	66 – 137	29	420
Creatine Kinase (U/L)	68	Non-normal	314	195 - 547	59	12,930
Total Protein (g/dL)	125	Normal	6.5	1.06	3.0	9.6
Prealbumin (g/dL)	125	Non-normal	0.15	0.1 – 0.2	0.01	0.39
Albumin (g/dL)	125	Normal	1.81	0.34	0.82	2.75
Alpha 1 Globulins (g/dL)	125	Non-normal	0.4	0.32 – 0.5	0.15	0.76
Alpha 2 Globulins (g/dL)	125	Normal	1.69	0.35	0.77	2.45
Beta Globulins (g/dL)	125	Non-normal	1.73	1.53 – 2.05	0.84	3.29
Gamma Globulins (g/dL)	125	Normal	0.58	0.13	0.29	0.93
Albumin / Globulin	125	Normal	0.443	0.064	0.28	0.59

Silvery Salamanders

Three hundred thirty-four silvery salamanders were evaluated from three wetlands at Kickapoo State Park from 2019 – 2021. Fluidigm qPCR was performed for 254 individuals; all samples were pathogen-negative. Demographics and physical exam findings are summarized by wetland in Table 5 and Figure 4. Hematology was performed in 25 salamanders and summary statistics are presented in Table 6. Weights and body condition scores did not vary between ponds and were not significantly associated with the presence of physical examination abnormalities ($p > 0.05$). The occurrence of physical examination abnormalities was not significantly different between ponds ($p > 0.05$).

Salamanders with developmental abnormalities had higher WBC counts (effect size = 7,321 cells/μL, 95% CI = 1,317 – 13,326 cells/μL, $p = 0.01$), heterophil counts (effect size = 3,777 cells/μL, 95% CI = 861 – 6,694 cells/μL, $p = 0.01$), and monocyte counts (effect size = 1,114 cells/μL, 95% CI = 400 – 1,828 cells/μL, $p = 0.004$) than animals without. Salamanders with *Dermotheca* sp. infection had higher monocyte counts (effect size = 1,169 cells/μL, 95% CI = 472 – 1,867 cells/μL, $p = 0.002$) than those without.

Table 5. Demographics and physical exam abnormalities in silvery salamanders by wetland, 2019 – 2021.

		67	**282**	**283**
Year	**2019**	48	29	60
	2020	22	16	81
	2021	26	23	29
Age Class	**Adult**	90	60	157
	Larva	6	8	13
Physical Exam	***Dermotheca* sp.**	8 (8%)	3 (4%)	20 (12%)
	***Clinostomum* sp.**	1 (1%)	0	0
	Developmental Abnormality	8 (8%)	8 (12%)	18 (11%)
	Fresh Injuries	7 (7%)	6 (9%)	9 (5%)
	Healed Injuries	21 (21%)	8 (12%)	13 (8%)
	Cutaneous Ulcer	0	1 (1%)	4 (2%)
	Hemorrhage	2 (2%)	0	1 (0.6%)
Total		96	68	170

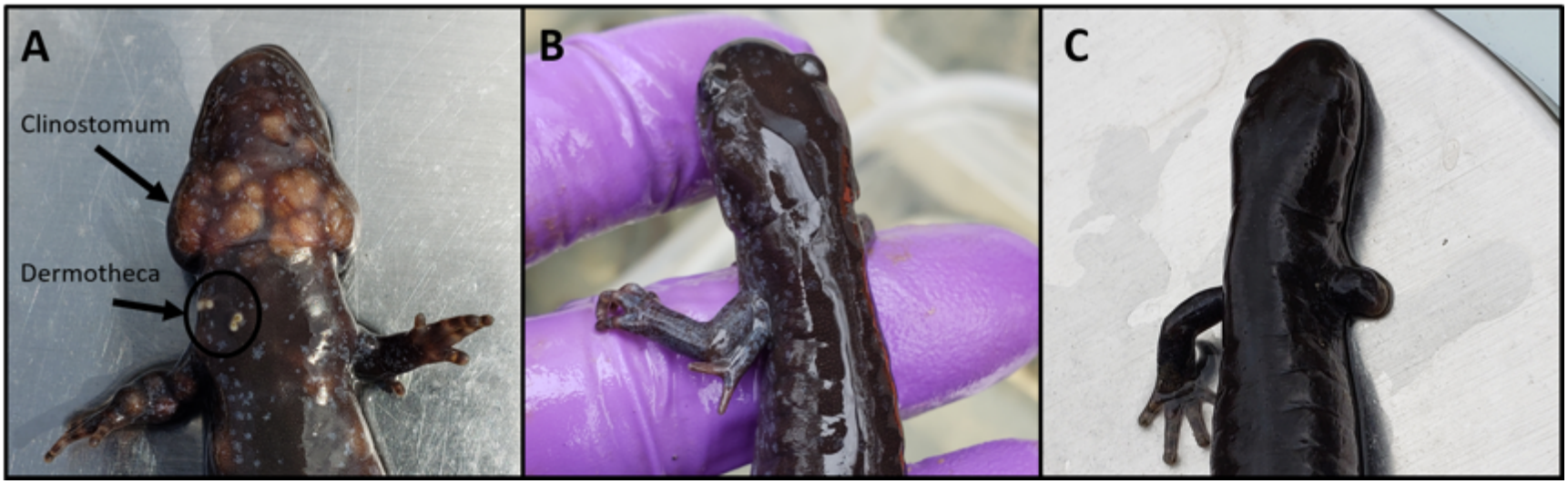

Figure 4. Common silvery salamander physical examination abnormalities. A) *Clinostomum* sp. and *Dermotheca* sp. infection. B) Developmental abnormality – extra forelimb. C) Trauma.

Table 6. Descriptive statistics for silvery salamander hematology 2019 – 2021. Central tendency = mean or median, Dispersion = standard deviation or 10^{th} – 90^{th} percentiles depending upon data distribution.

Analyte	N	Distribution	Central Tendency	Dispersion	Min	Max
Total Leukocyte Count (/µL)	25	Non-normal	10,400	8,200 - 12,800	4,800	28,000
Neutrophils (/µL)	25	Non-normal	1,908	924 – 2,720	520	12,320
Lymphocytes (/µL)	25	Non-normal	588	270 – 896	0	4,940
Monocytes (/µL)	25	Non-normal	86	0 – 168	0	3,360
Eosinophils (/µL)	25	Normal	7,059	3,494	1,480	16,590
Basophils (/µL)	25	Normal	854	369	304	1,482
Neutrophil / Lymphocyte	25	Non-normal	2.81	1.64 – 5.75	0.31	17

RNA Extraction Protocol Refinement

RNA virus surveillance is rarely attempted in wildlife due to logistical constraints about RNA stability and the ubiquitous nature of RNAses. Snap freezing samples at -80ºC or in liquid nitrogen is one of the best ways to preserve RNA in biological samples. However, this is highly impractical in a field setting. RNA preservatives, such as RNAlater, provide an alternative means of stabilizing RNA when instant freezing is impossible. Unfortunately, few commercially available RNA extraction kits are designed to work with RNA stabilizing buffers like RNAlater. To deal with this issue, we initially relied upon a published protocol for extracting RNA from mucosal swabs preserved in RNAlater (Espinosa-de Aquino et al. 2017). This protocol assumes that many mucosal cells detach from the swab head and/or lyse into the RNAlater solution. The initial centrifugation step therefore concentrates cellular material and cellular contents at the bottom of the tube. The swab is removed, the RNAlater is decanted, and RNA extraction proceeds from that point using a commercial kit and the cellular pellet. After using this protocol in 2019, we found that herptile cellular material tends to remain associated with the swab head. By removing the swab from the extraction, our RNA yields were lower than anticipated. We modified this protocol by performing the initial centrifugation as recommended, decanting the RNAlater, and aspirating RNAlater from the swab head with a sterile pipette tip. This protocol allows the swab head to be retained in the tube and subjected to RNA extraction (Qiagen RNEasy, Valencia, CA). Following this protocol adjustment, our RNA yields improved 10-fold.

We have also developed a protocol for extracting RNA from whole blood preserved in RNAlater, which is challenging for species with nucleated erythrocytes. This protocol involves an initial centrifugation to pellet the erythrocytes. The RNAlater is removed via pipetting, then the erythrocyte pellet is homogenized using a bead beater. RNA extraction is performed on the homogenate using a commercial kit with a tissue extraction protocol (Qiagen RNEasy, Valencia, CA). These protocol alterations have enhanced the quality and quantity of RNA retrieved from field-collected herptile samples, and may improve the diagnostic sensitivity of downstream pathogen PCR. We hope that our protocols can be used by other wildlife disease researchers to expand pathogen surveillance to RNA viruses.

RNA Virus Surveillance

Thirty-five samples from eastern box turtles, ornate box turtles, and silvery salamanders were targeted each year for RNA extraction and PCR. Samples were selected to balance study sites, sexes, age classes, seasons, and the presence or absence of clinical signs. A total of 308 cloacal/oral swab (box turtles) or oral swab (salamanders) samples were tested (only 28 ornate box turtle samples were collected in 2020) for seven RNA viruses. All samples were negative.

Eastern Massasauga Rattlesnakes

A total of sixty-six health assessments were performed for Carlyle Lake massasaugas in 2019 (N = 37 assessments of 35 individuals), 2020 (N = 1 assessment of 1 individual), and 2021 (N = 28 assessments of 27 individuals). Field surveys were not performed in 2020 due to COVID-related shelter in place orders, the single individual sampled that year was observed basking multiple times in December and was transported to UIUC for health assessment and ophidiomycosis treatment. Three snakes were sampled in both 2019 and 2021, including an adult non-gravid female which was in the "ophidiomycosis negative" category in 2019, then in the "possible ophidiomycosis" category in 2021. A juvenile female was in the "possible ophidiomycosis" category in 2019, then in the "apparent ophidiomycosis" category as an adult in 2021. Finally, an adult female that was non-gravid in 2019 and gravid in 2021 was in the "apparent ophidiomycosis" category at both assessments. Demographic, site, lesion, and ophidiomycosis data are summarized in Table 7 and Figure 5. Hematology was performed in 46 individuals and results are presented in Table 8.

Overall, 30.3% of massasauga encounters tested positive for *O. ophiodiicola* DNA. Fifty-six percent were classified as ophidiomycosis negative, 13.6% were possible ophidiomycosis, 1.5% were *Ophidiomyces* present, and 28.9% were in the apparent ophidiomycosis category. There was no significant difference in apparent ophidiomycosis prevalence between 2019 (22.9%, 95% CI = 12.1 – 39.0%) and 2021 (28.6%, 95% CI = 15.2 – 47.1%) and the proportion of snakes in each ophidiomycosis category did not significantly differ between South Shore and Eldon Hazlet ($p > 0.05$). The probability of a lesion testing qPCR positive for *Ophidiomyces* was not significantly associated with lesion type or anatomic location ($p > 0.05$). Lesion type and anatomic location were also not significantly associated with *Ophidiomyces* copy number ($p > 0.05$).

Agreement in *Ophidiomyces* testing between body and lesion swabs was moderate (kappa = 0.53). The sensitivity of body swabs for determining whether the animal would test positive for *O. ophiodiicola* DNA was 45% (95% CI = 24 – 68%) while the specificity was 100% (95% CI = 90 – 100%). Body swab positive predictive value was 100% (95% CI = 63 – 100%) while negative predictive value was 81% (95% CI = 68 – 90%). In contrast, lesion swab sensitivity was 76% (95% CI = 61 – 87%), specificity was 100% (95% CI = 68 – 100%), positive predictive value was 100% (95% CI = 88 – 100%), and negative predictive value was 50% (29 – 71%).

Packed cell volume (effect size = 8.1%, 95% CI = 4.1 – 12.1%, $p = 0.0002$) and heterophil:lymphocyte ratio (effect size = 0.504, 95% CI = 0.052 – 0.955, $p = 0.03$) were significantly higher in 2019 than 2021. WBC counts (effect size = 6,935 cells/μL, 95% CI = 2,298 – 11,572 cells/μL, $p = 0.004$) and lymphocyte counts (effect size = 7,287 cells/μL, 95% CI = 4,056 – 10,518 cells/μL, $p < 0.0001$) were significantly higher in 2021 vs. 2019. Monocyte counts were significantly higher at South Shore vs. Eldon Hazlet (effect size = 299 cells/μL, 95% CI = 37 – 562 cells/μL, $p = 0.03$). Snakes with skin lesions (effect size = 1 g/dL, 95% CI = 0.3 – 1.7 g/dL, $p = 0.007$) and those that tested positive for *Ophidiomyces* (effect size = 0.8 g/dL, 95% CI = 0.06 – 1.6 g/dL, $p = 0.04$) had significantly higher TS concentrations than snakes without lesions or with negative qPCR results, respectively. Similarly, snakes with apparent ophidiomycosis had higher TS than snakes in the ophidiomycosis negative category (effect size = 1 g/dL, 95% CI = 0.08 – 2.0 g/dL, $p = 0.03$). Snakes testing qPCR positive for *Ophidiomyces*

had higher heterophil counts (effect size = 455 cells/μL, 95% CI = 77 – 833 cells/μL, p = 0.02) than negative snakes. Heterophil counts also tended to be higher in snakes with skin lesions and in those with apparent ophidiomycosis, though these associations were not statistically significant (p = 0.06).

Table 7. Summary demographic and ophidiomycosis data by study site for eastern massasauga rattlesnakes sampled from 2019 – 2021.

		South Shore	Eldon Hazlet
Age Class	**Adult**	31	21
	Juvenile	4	2
	Neonate	7	1
Sex	**Female**	26	11
	Male	15	12
	Unknown	1	1
Ophidiomycosis	**Negative**	23 (56%)	14 (58%)
	Possible	9 (21%)	0
	Present	1 (2%)	0
	Apparent	9 (21%)	10 (42%)
Total		42	24

Table 8. Descriptive statistics for eastern massasauga rattlesnake hematology, 2019 – 2021. Central tendency = mean or median, Dispersion = standard deviation or 10^{th} – 90^{th} percentiles depending upon normality of data distribution.

Analyte	N	Distribution	Central Tendency	Dispersion	Min	Max
Packed Cell Volume (%)	46	Normal	27	7.6	8	40
Total Solids (g/dL)	46	Normal	7.5	1.2	4.8	10.2
Total Leukocyte Count (/μL)	46	Non-normal	10,230	5773 - 15,569	668	44,880
Heterophils (/μL)	46	Normal	1,068	625	167	2,534
Lymphocytes (/μL)	46	Non-normal	5,003	2,300 - 11,440	220	28,723
Azurophils (/μL)	46	Non-normal	2,631	1,001 - 4,732	0	13,015
Monocytes (/μL)	46	Non-normal	229	0 - 475	0	1,877
Eosinophils (/μL)	46	Non-normal	0	0 - 0	0	601
Basophils (/μL)	46	Non-normal	273	96 - 452	0	1,056
Heterophil / Lymphocyte	46	Non-normal	0.218	0.087 - 0.444	0.017	4.3

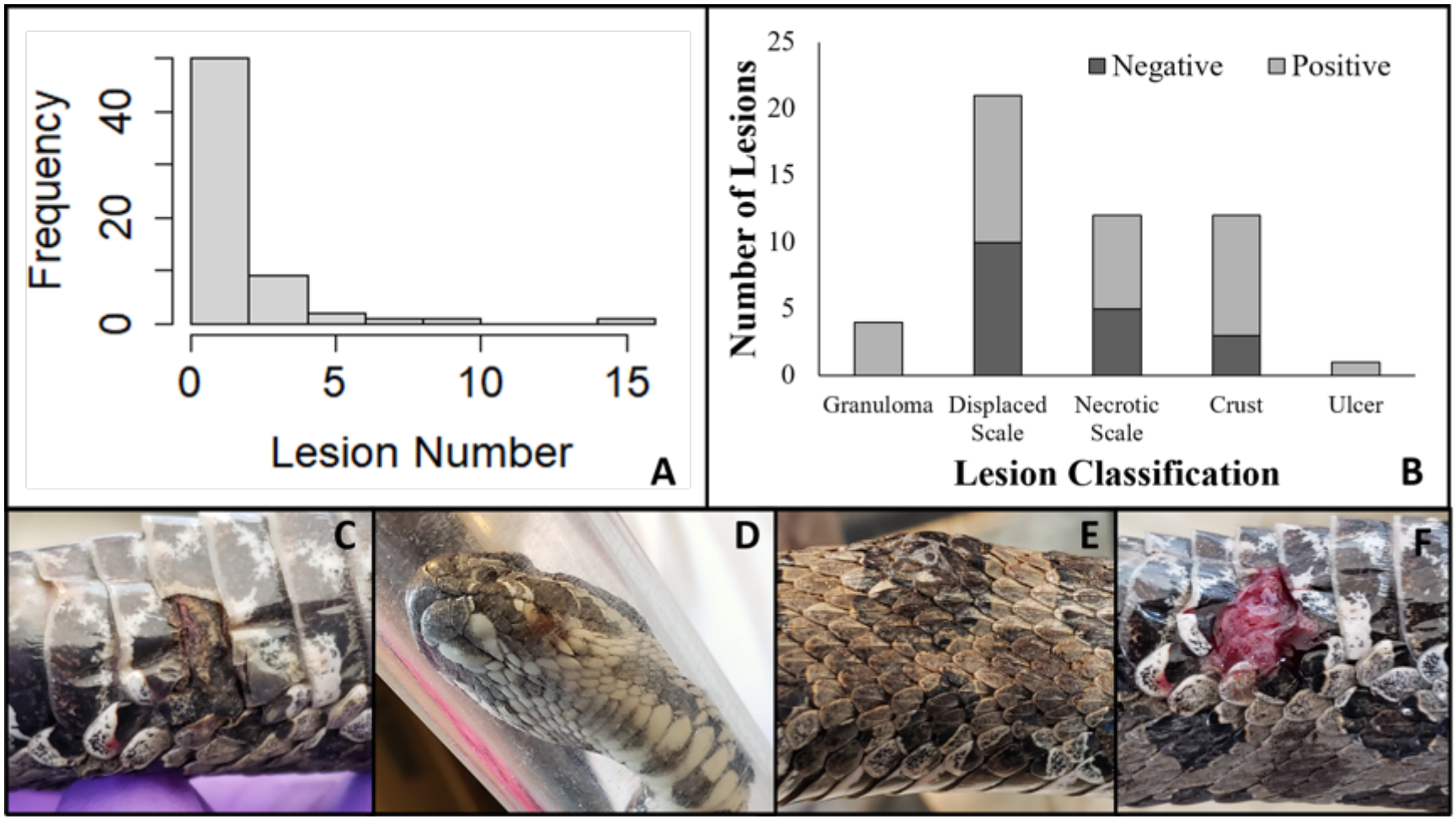

Figure 5. Skin lesion characteristics in eastern massasauga rattlesnakes. A) Histogram of the number of skin lesions per snake. B) Number of skin lesions by lesion type and *Ophidiomyces* qPCR result. C) Skin crust. D) Necrotic scales. E) Granuloma. F) Ulcer.

Kirtland's Snakes

A total of 59 Kirtland's snakes were tested for *Ophidiomyces* in 2019 (N = 25), 2020 (N = 9), and 2021 (N = 25) at four study sites. Demographic, site, and ophidiomycosis data are summarized in Table 9. In 2019 48% (95% CI = 30 – 66.5%) of sampled Kirtland's snakes tested qPCR positive for *O. ophidiicola* with pathogen detection occurring at all study sites. All snakes tested negative in 2020 (95% CI = 0 – 30%) and 2021 (95% CI = 0 – 13.3%). Most snakes had normal physical examinations, and qPCR positive individuals were classified as "*Ophidiomyces* present". However, one adult male from Sangchris in 2019 had what appeared to be an injured ventral scale. This individual tested positive for *Ophidiomyces ophidiicola* and was placed in the "apparent ophidiomycosis" category. Sample size calculations were performed to direct future *Ophidiomyces* surveillance efforts in Kirtland's snakes, results are displayed in Table 10.

Table 9. Summary demographic and ophidiomycosis data by study site for Kirtland's snakes tested for *Ophidiomyces* from 2019 – 2021.

		Study Site			
		Clinton Lake	**Goodenow**	**Sangchris**	**Theodore Stone**
Sex	**Female**	0	19	9	1
	Male	2	7	15	3
	Unknown	0	2	1	0
Age Class	**Adult**	1	13	15	4
	Subadult	0	2	2	0
	Juvenile	1	13	8	0
Ophidiomycosis	**Negative**	0	24 (86%)	20 (80%)	3 (75%)
	Possible	0	0	0	0
	Present	2 (100%)	4 (14%)	4 (16%)	1 (25%)
	Apparent	0	0	1 (4%)	0
Total		2	28	25	4

Table 10. Number of Kirtland's snake skin swab qPCR tests needed to detect *Ophidiomyces ophidiicola* at different levels of prevalence and different snake population sizes.

	***Ophidiomyces ophidiicola* Prevalence**					
Population Size	**1%**	**2%**	**3%**	**5%**	**10%**	**20%**
50	50	50	49	40	29	17
100	100	98	79	57	33	18
200	195	132	99	65	35	19
300	237	148	107	68	36	19
500	282	162	114	71	37	19
1000	324	174	119	73	37	19

Mortality Investigation

Lake County Ranavirus Outbreak

In early June 2019, the Lake County Forest Preserve District was notified of a freshwater turtle mortality event in a pond adjacent to Lakewood Forest Preserve. On June 10th, the shells of 22 deceased turtles were recovered from the pond, including painted turtles (*Chrysemys picta*), red-eared sliders (*Trachemys scripta elegans*), and common snapping turtles (*Chelydra serpentina*). Bone marrow and tissue samples were collected from the retrieved shells and pooled by species. The painted turtle bone marrow and tissue pools tested positive for ranavirus via qPCR. Hoop traps were placed in the pond on June 17th to enable sampling of live turtles and amphibians. On June 18th, one live common snapping turtle was captured and two painted turtle plastrons were incidentally recovered. The snapping turtle had deep ulcerations on the head, but no other physical examination abnormalities (Figure 6). This animal tested qPCR positive for ranavirus in whole blood and oral/cloacal swab samples.

Figure 6. Deep cutaneous ulceration in a common snapping turtle with ranavirus from Lake County, IL. A stick was placed inside the mouth as a bite guard during sampling.

Blanding's Turtle Mortalities & Novel Disease Investigation

In May, 2018 an adult male Blanding's turtle from Lake County was incidentally encountered. Physical examination revealed an intra-oral 1.5cm broad-based right mandibular mass. The turtle was transported to the University of Illinois for mass removal and further diagnostics. Histopathology revealed squamous cell carcinoma, and PCR on tissues from the mass revealed a novel herpesvirus (*Emydoidea* herpesvirus 2). The turtle recovered uneventfully

and was released at its site of capture. In 2019, the mass had regrown. The turtle was transported to the University of Illinois and the mass was aggressively removed. *Emydoidea* herpesvirus 2 DNA was again detected within the mass via PCR. The turtle recovered and was re-released in Lake County with a radio transmitter to facilitate continued monitoring. This is the first detection of a cancer-associated herpesvirus in a Blanding's turtle, and the case report was recently published (Andersson et al., 2021). Aside from decreases in weight associated with initial growth and regrowth of the mass, likely attributable to difficulty in prehending food, the turtle appeared otherwise healthy.

Figure 7. Photographs of an adult male Blanding's turtle with oral squamous cell carcinoma-associated herpesvirus (Emydoidea herpesvirus 2) in Lake County, Illinois in 2019. A) May 14th, 2019; oral squamous cell carcinoma with caseous debris. B, C, & D) June 21st, 2019; oral cavity immediately before (B & C) and after (D) surgical debridement. E & F) August 30th, 2019; oral cavity and right beak commissure 10 weeks post-op.

In late July, 2019 an adult male Blanding's turtle (2019-1) from Lake County was incidentally discovered with labored breathing and tracheal discharge. The animal was transported to the Wildlife Epidemiology Lab for diagnostic testing and clinical management. The turtle tested negative for ranavirus, *Emydoidea* herpesvirus 1, and *Mycoplasmopsis* sp. Treatment was initiated for suspected pneumonia with antibiotics and heat support. The turtle's attitude, appetite, and respiratory signs improved over the course of several weeks, however, intermittent respiratory noise was still appreciated. The turtle unexpectedly and rapidly declined at the end of October and was euthanized. Gross necropsy revealed severe and extensive fibrinonecrotic and hemorrhagic enterocolitis, along with residual pneumonia. Histopathology revealed fungal sepsis caused by a *Mucorales* organism. This fungus caused systemic vasculitis

and thrombosis, which ultimately led to necrosis of the gastrointestinal tract and the rapid clinical deterioration.

On May 20th, 2021 an adult female Blanding's turtle (2021-1) was found after being hit by a car. The turtle sustained multiple fractures to the left cranial carapace, left bridge, and skull. She was transported to the University of Illinois for additional diagnostics and treatment. Radiographs revealed additional fractures to the left scapula and left distal humerus. The turtle was stabilized with fluids, pain medication, broad-spectrum antibiotics, and heat support for several days. Fracture repair and feeding tube placement were pursued without incident. The turtle's mentation and movement improved over the course of several weeks, but at the end of June she developed intermittent bloody oral discharge and clear nasal discharge. Testing for *Mycoplasmopsis* sp. was positive, and additional antibiotics were added to combat this infection. Unfortunately, the turtle's condition rapidly deteriorated over the course of 36 hours and she was found dead on July 2nd. Gross necropsy and histopathology revealed chronic bacterial pneumonia, which may have caused the turtle to be basking on the road prior to vehicular strike.

An adult radiotelemetered female Blanding's turtle from Kane County (2021-2) was discovered dead on June 8th, 2021. The remains were in poor condition, the head and limbs had been scavenged and most of the viscera was autolyzed. Several shelled eggs were present within the coelomic cavity. Histopathology was attempted, but was uninformative due to the state of the carcass. There was no evidence of antemortem hemorrhage or tissue damage to indicate predation and the cause of death is unknown, though the presence of eggs may implicate egg binding.

An adult radiotelemetered female Blanding's turtle from Lake County (2021-3) was discovered dead on July 7th, 2021. She had last been seen alive on June 29th. On external exam, the turtle was moderately autolyzed with significant insect activity. The left femur was exposed and the distal left hind limb was missing. A 3cm laceration through the skin and coelomic membrane of the left prefemoral fossa was also present. Histopathology revealed bacterial ceolomitis, likely extending from the aforementioned laceration. Ulceration of the skin around the remnants of the left hind leg was also present, indicating pre-mortem injury. Death likely occurred secondary to traumatic amputation of the left hind leg with concurrent penetrating trauma to the coelomic cavity. Attempted predation is considered highly likely. A summary of Blanding's turtle necropsy findings is provided in Table 11.

Table 11. Summary findings of Blanding's turtle necropsies from 2019 – 2021.

Turtle ID	Cause of Death	Underlying Health Issues
2019-1	Fungal Sepsis	Yes
2021-1	Pneumonia	Yes
2021-2	Unknown	Unknown
2021-3	Predation	No

Eastern Massasauga Mortalities

An adult female massasauga (20-EMR-01) from Clinton County was visualized aboveground and basking multiple times in December, 2020 and was captured and transported to the University of Illinois for evaluation on December 29th. Physical examination revealed a quiet attitude, 15 skin lesions consistent with ophidiomycosis (multifocal crusts, necrotic scales, and displaced/thickened scales, worst around the cloaca – Figure 8), and thickened caudal coelomic contents. Blood film review revealed toxic changes in the heterophils (cytoplasmic basophilia, vacuolization, and partial degranulation) and azurophils (cytoplasmic vacuolation) indicating a significant inflammatory process such as active bacterial or fungal infection. Skin swabs were qPCR positive for *Ophidiomyces*, and this snake was classified in the "apparent ophidiomycosis" category. Treatment was initiated with terbinafine nebulization and broad-spectrum systemic antibiotics, but the snake was found dead on January 10, 2021. Necropsy revealed changes in the skin consistent with ophidiomycosis (severe granulomatous and ulcerative dermatitis and panniculitis with intralesional fungal hyphae), extension of *Ophidiomyces* infection into the liver with a concurrent inflammatory response (regionally extensive, severe granulomatous and necrotizing hepatitis with intralesional fungal hyphae), and culture-confirmed *Salmonella* sp. infection of the oviduct, resulting in the thickened coelomic contents palpable on physical examination (severe granulomatous and ulcerative salpingitis with intralesional bacterial organisms). Cause of death was suspected to be sepsis secondary to salmonellosis, though *Ophidiomyces* infection also caused significant pathology in this animal.

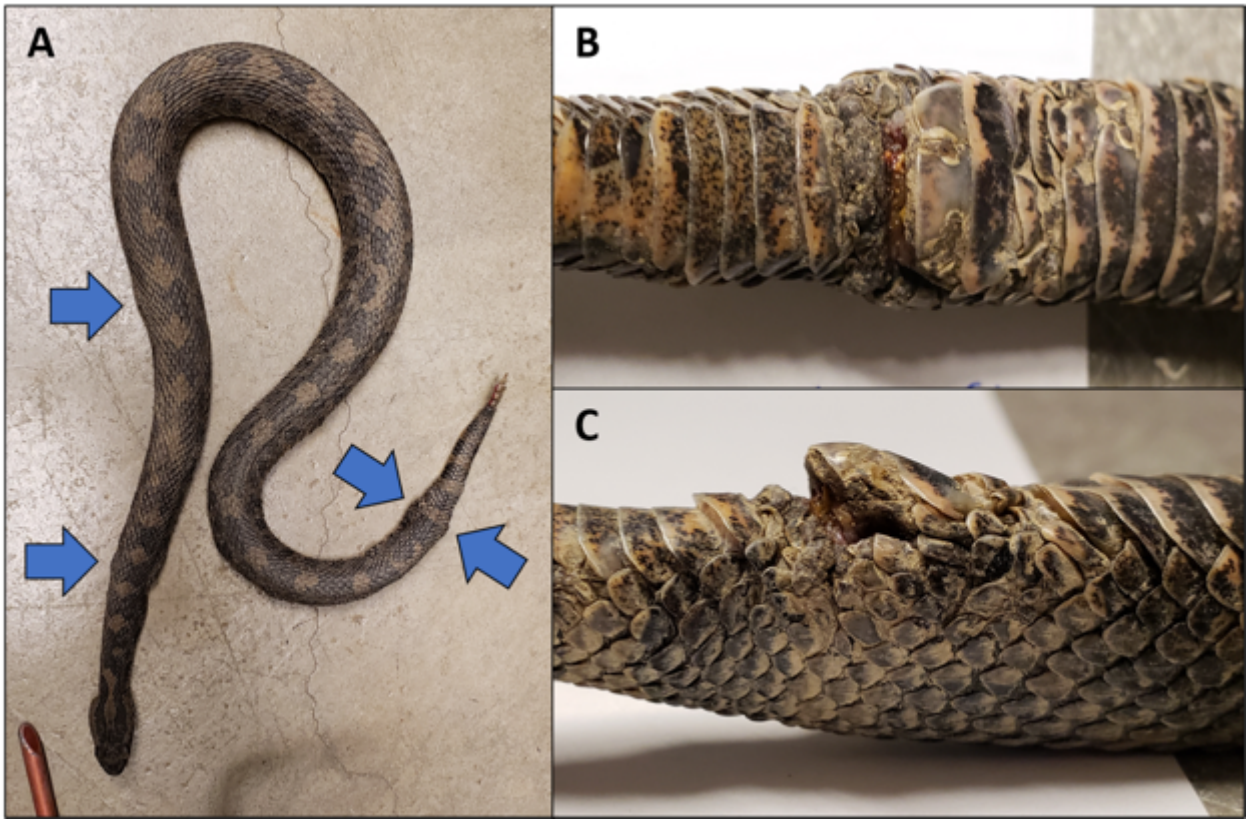

Figure 8. External appearance of an adult female eastern massasauga rattlesnake presented for assessment in December 2020. A) Top-down view of the entire snake with multiple areas of displaced scales (arrows). B & C) Appearance of the cloaca, significant swelling and crusting lesions are present.

Seven additional deceased wild snakes from Clinton County were presented for necropsy and *Ophidiomyces* testing from 2019 - 2021. Snakes 2019-1 and 2019-2 were presented in late December 2019, snakes 2020-1 and 2020-2 were presented in early January 2020, snake 2020-3 was presented in November 2020, and snakes 2021-1 and 2021-2 were frozen and presented together in October 2021. Snake 2019-1 (Site number: 11231-EHSP; Field # CP Area #12, per history) was a well-conditioned adult male with the head, heart, and lungs missing. No gross or histologic lesions were noted, and the snake tested negative for *Ophidiomyces* via qPCR. Snake 2019-2 (Site number: 11231-1 EHSP; Field # CP Areas #6, per history) was a moderately-conditioned adult male with significant bacterial and fungal infection in the skin on the head (chronic, segmental hyperkeratosis with intralesional bacteria and fungi). The fungal morphology was not consistent with *Ophidiomyces*, and qPCR for this organism was negative. Snake 2020-1 was recovered as a partial body on 1/2/20. While this animal had been either predated or scavenged, necropsy revealed a lack of coelomic fat stores and moderate granulomatous hepatitis, indicating compromised health status which may have contributed to death. This snake tested negative for *Ophidiomyces*. Snake 2020-2 was a well-conditioned adult female found dead on 1/4/20. Grossly, this snake had multiple skin lesions consisting of thickened, displaced scales and ulcers which are consistent with ophidiomycosis. Histology confirmed the presence of severe, locally extensive granulomatous dermatitis with intralesional fungi, and *Ophidiomyces* qPCR was positive. Snake 2020-3 was a well-conditioned adult found dead north of Apache Road at EHSP on 8/17/20. This snake had significant evidence of internal and external trauma, consistent with a vehicle strike. *Ophidiomyces* qPCR was negative. Snake 2021-1 (EHSP_09302021_1_DEAD, per history) was an adult female found dead on September 30th in the ditch along field 3 at Eldon Hazlet. Mowing had occurred in this area the week prior. External examination revealed multiple skull, spinal, and rib fractures, a dorso-ventrally flattened section in the middle of the snake, and a 3cm full-thickness skin laceration in the cranial third of the body. Mild insect activity was present. Internal examination confirmed multiple fractures and significant soft tissue trauma. The remains were severely autolyzed and unsuitable for histopathology, so we were unable to evaluate for the presence of underlying illness. *Ophidiomyces* qPCR was negative. Cause of death was trauma, likely mower strike. Snake 2021-2 (EHSP_10012021_1_DEAD, per history) was an adult female found dead on a road at Eldon Hazlet on October 1st. External exam revealed a single 4cm x 2cm full-thickness laceration approximately 10cm caudal to the head on the left side. No fractures were palpable and the snake was in good body condition. Internal exam revealed significant crush injury and hemorrhage in the cranial half of the body and normal appearing organs in the caudal half of the body. Histopathology confirmed crush injury in the cranial half of the snake, with no significant findings in organs from the caudal half of the snake. *Ophidiomyces* qPCR was negative. Cause of death was trauma, likely vehicular strike. A summary of massasauga necropsy findings is provided in Table 12.

Table 12. Summary findings of eastern massasauga rattlesnake necropsies from 2019 – 2021.

Snake ID	*Ophidiomyces* qPCR	Cause of Death	Underlying Health Issues
2019-1	Negative	Unknown	Unknown
2019-2	Negative	Unknown	Yes
2020-1	Negative	Unknown	Yes
2020-2	Positive	Unknown	Yes
2020-3	Negative	Trauma	No
20-EMR-01	Positive	Sepsis	Yes
2021-1	Negative	Trauma	Unknown
2021-2	Negative	Trauma	No

DISCUSSION

The Wellness of Wildlife project was impacted by the global pandemic in 2020 and 2021. Ironically, SARS-CoV-2, the virus which causes COVID-19, likely has a wildlife origin, highlighting the importance of projects such as this one. Despite the sampling and laboratory limitations we met or exceeded sampling goals for all species and refined laboratory methodology to better meet the needs of the unique animals with which we work. Specific findings will now be reviewed for each of our study species individually.

RNA Virus Surveillance

RNA virus testing in eastern and ornate box turtle cloacal/oral swab samples and silvery salamander oral swabs has been negative for seven viruses (picornaviruses, ferlaviruses, nidoviruses, reoviruses, parvoviruses, coronaviruses, and influenza) so far. This may indicate lack of infection, inadequate primer sensitivity, testing of inappropriate samples (though this seems unlikely based on recommendations in the literature), testing during time periods when infections are not detectable (i.e., early in the disease course, following disease resolution, or during periods of latency), or issues with sample quality. To rule out sample quality issues, we developed revised RNA extraction protocols for herptile samples that have dramatically increased RNA yield. We have also optimized our existing consensus PCR protocols and incorporated new assays for arenaviruses, bornaviruses, bunyaviruses, and flaviviruses, dramatically expanding our capacity to survey for RNA viruses. We plan to employ these updated protocols to test for additional RNA viruses during the next phase of the Wellness of Wildlife project. Animals will be tested from all study sites, sexes, age classes, and health statuses in order to increase our potential for detecting RNA virus positive animals. If no viruses are detected, we will align all available sequence data for herptile RNA viruses and design *de novo* primers during the next phase of this project.

Eastern Box Turtle Health Assessment & Recommendations

Physical examination findings, clinical pathology values, and pathogen presence from 2019 - 2021 were comparable to baseline data established in Phase I of the Wellness of Wildlife project. Differences in clinical pathology parameters between sexes were largely consistent with vitellogenesis in females (elevated TS, calcium, phosphorous, and beta globulins) and male territorial and mating behaviors (elevated CK and alpha 1 globulins) (Wilkinson 2004). Age-related changes in clinical pathology parameters (e.g., higher TS, calcium, calcium to phosphorous ratio, and beta globulins in adults, higher lymphocytes and basophils in juveniles) are consistent with those reported in the literature (Wilkinson 2004). Clinicopathologic alterations in association with physical examination abnormalities also followed expected patterns. Specifically, decreases in albumin and the AGR are consistent with an acute phase inflammatory response in turtles with upper respiratory disease and general physical examination abnormalities. Several clinical pathology parameters differed between study sites and seasons, likely reflecting differences in health status and/or general physiology. However, since not all sites were sampled in all seasons, it is difficult to differentiate the effects of season and study site. This highlights the difficulty inherent in reptile health assessment and supports large-scale studies such as this one that collate data from multiple years, locations, and seasons.

Adenovirus was more commonly detected in juvenile turtles and the odds of detection were higher in spring vs. summer, which is consistent with our findings during Phase I. Eastern box turtles with adenovirus detection typically lack clinical signs of illness, and this pathogen is suspected to be host-adapted in box turtles (Franzen-Klein et al., 2020). Interestingly, changes in protein electrophoresis were noted during this study that indicate the presence of acute inflammation in adenovirus positive turtles (decreased albumin, elevated alpha 1 and 2 globulins). Host-adapted viruses can cause clinical disease in young or immunocompromised hosts, and it is possible that we identified some individuals with negative clinical impacts secondary to adenovirus infection during this study. Challenge studies will ultimately be necessary to fully elucidate the effects of adenovirus infection in box turtles, but until the virus has been successfully cultured, continued study of free-living populations provides the most useful data on this host-pathogen system.

Mycoplasmopsis sp. detection was more likely in turtles with physical examination abnormalities and was associated with inflammatory leukogram changes including elevations in heterophils, monocytes, and H:L. These findings are also generally consistent with what we documented during Phase I, with *Mycoplasmopsis* sp. being the only pathogen besides frog virus 3 (ranavirus) associated with clinical illness in box turtles. Similarly, *Mycoplasmopsis* is the pathogen associated with significant management implications for desert and gopher tortoises. It is yet to be seen if *Mycoplasmopsis* in emydid turtles will require such intense intervention, but declining populations are beginning concern in Illinois. While *Mycoplasmopsis* sp. prevalence is generally low, significant morbidity and mortality have been documented in infected box turtles from Vermilion County (Adamovicz et al., 2018), and continuing to monitor the occurrence and clinical effects of this pathogen is recommended.

Terrapene herpesvirus 1 is suspected to be host-adapted in box turtles, and limited clinical effects are anticipated secondary to infection (Kane et al., 2017). This is consistent with

our findings in Phase II, however, continued TerHV1 surveillance is recommended to assess for changes in prevalence and development of clinical disease, both of which may indicate shifts in underlying health status of individuals and populations. The pathogenicity of *Terrapene* herpesvirus 2 is less well understood, as it was originally reported in association with fibropapilloma development in a single turtle (Yonkers et al., 2015). Our findings demonstrate an association between TerHV2 detection and elevations in WBC count, heterophil count, and basophil count. Taken together, these changes are consistent with inflammation and chronic antigenic stimulation. This may indicate that TerHV2 causes more pathology than TerHV1, potentially enough to impact host wellness. Additional research will be necessary to clarify the relationship between TerHV2 detection and box turtle health.

Ranavirus was not detected in box turtles during this study period, which is consistent with previous cross-sectional studies and is likely due to the acute onset, rapid progression, and high mortality nature of this pathogen (Allender et al., 2013). However, ranavirus has been previously associated with mortality events at Kennekuk and Kickapoo, and this pathogen should be considered a significant threat to eastern box turtles (Adamovicz et al., 2018). Continued pathogen surveillance, mortality investigations, and comprehensive health assessments will be necessary to clarify the epidemiology and contextualize the clinical importance of pathogens in Illinois box turtle populations.

Ornate Box Turtle Health Assessment & Recommendations

Physical examination findings, clinical pathology values, and pathogen presence in 2019 – 2021 were generally comparable to baseline data established in Phase I. During Phase II, we were able to expand to an additional study site at Ayers Sand Prairie. Turtles at this site are physically smaller and subjectively more densely packed than those at Nachusa. While shell damage is identified at similar rates between sites, more turtles at Ayers had healed fractures secondary to vehicular strike or encounters with mowing equipment. This is likely due to the close proximity of Ayers to agricultural fields and roads, whereas the Orland Track and South Bison Unit at Nachusa are fenced off from these hazards. Several hematologic differences were identified between these populations and many of these (e.g. elevated WBC count, heterophils, H:L) may indicate higher levels of stress and inflammation in turtles at Ayers compared to Nachusa. This could potentially be explained by differences in habitat size and quality between the sites. It could also possibly be attributable to differences in infectious disease burden, however, pathogen testing was not performed for turtles from Ayers and this should be a goal for future studies. Disease surveillance could be particularly interesting at Ayers due to the subjective high density of turtles at this site, which may facilitate pathogen transmission. We recommend that future health assessment studies incorporate sampling at Ayers to better understand health and disease status of Illinois ornate box turtle populations.

Compared to eastern box turtles, pathogen burden is low in ornate box turtles. Two pathogens were detected from 2019 – 2021: *Terrapene* adenovirus 1 and a human-pathogenic leptospire. While adenovirus was also detected in Phase I and does not appear to be associated with clinical signs of illness in this species, identification of *Leptospira* sp. DNA in ornate box turtles is interesting and somewhat unexpected. Leptospirosis is an important zoonotic disease

with abundant wildlife reservoirs, however, its presence in chelonians is poorly described. So far, *Leptospira* spp. shedding has only been documented in the Blanding's turtle (*Emydoidea blandingii*), which appears to be a competent reservoir species (Rockwell et al., 2019). *Leptospira* spp. primers were only incorporated into our Fluidigm qPCR pathogen surveillance program in 2020, but this detection in an ornate box turtle demonstrates that continued testing is indicated. Additional surveillance will help determine whether *Leptospira* spp. are associated with clinical disease in ornate box turtles, and may support a role for this species as a reservoir host for this zoonotic pathogen. While limited pathogen detection in ornate box turtles may appear to be a positive finding for their overall health status, it is important to keep in mind that the turtles sampled at Nachusa represent a fairly isolated population. Introduction of novel diseases to these turtles could pose a significant threat, and continued pathogen surveillance at this and other sites is recommended to allow for rapid detection of new pathogens and monitor for changes in known pathogen prevalence which could indicate deteriorating health status.

Ornate box turtle sampling in Phase I was all conducted in May. In Phase II, we sampled turtles in both May and June in 2019 and in June, 2020. Expanding our sampling to two time points during the active season uncovered several clinical pathology changes which have not been previously described for this species. We recommend continuing to sample at multiple time points during the year to better characterize ornate box turtle health status.

Silvery Salamander Health Assessments & Recommendations

Silvery salamander physical exam findings were similar to those identified during Phase 1. *Dermotheca* sp. was documented each year and appears to be established within the silvery salamander populations at Kickapoo State Park. Other pathogens were not detected in silvery salamanders from 2019 – 2021. Given previous ranavirus mortality events during Phase I, the lack of ranavirus detection during Phase II is likely a good sign for silvery salamander health at this site.

Phase II of the Wellness of Wildlife project included hematologic assessments in a small number of silvery salamanders. Hematology is rarely employed in amphibians, especially wild individuals due to sample volume constraints and a lack of reference values for comparison. Despite this, we identified associations between several hematologic parameters and both developmental abnormalities and *Dermotheca* sp. infection. These changes complimented physical examination findings and provided a more thorough understanding of health in sampled individuals. We recommend incorporating hematology and other clinical pathology testing into future amphibian health assessments to supplement physical examination and pathogen surveillance findings and provide a more comprehensive understanding of wellness.

Eastern Massasauga Rattlesnake *Ophidiomyces* Surveillance & Recommendations

Eastern massasauga rattlesnakes from Carlyle Lake were found to have detectable *Ophidiomyces ophiodiicola* DNA at prevalences similar and higher to previous surveys (Allender et al. 2011, Allender et al. 2015d, Allender et al. 2016, Baker et al. 2016), however, this study represents the first application of the recently-proposed ophidiomycosis categorization system in this species (Baker et al. 2019). We found that most snakes with a positive qPCR result

also had skin lesions, and were placed within the apparent ophidiomycosis category. These findings are consistent with previous reports of high susceptibility to the development of clinical ophidiomycosis in pit vipers (Allender et al. 2011, Allender et al. 2016, Baker et al. 2019). Perhaps unsurprisingly, the three snakes evaluated in both 2019 and 2021 developed progressive disease and were placed in worsening disease categories over time. While all of these snakes survived with ophidiomycosis for two years, it is likely that others do not and additional research is needed to better characterize the impacts of ophidiomycosis on individual survival/reproduction and population stability.

Hematologic assessment demonstrated higher TS in "apparent ophidiomycosis" snakes, likely consistent with an acute-phase response and resulting hyperproteinemia (Campbell 2015). Leukocyte counts (including heterophils) tended to shift towards an inflammatory leukogram in snakes with apparent ophidiomycosis, which has been previously documented in massasaugas (Allender et al. 2016); however, these associations were not statistically significant. Hematologic changes were also observed between years and sites, indicating the need for additional research to better characterize how clinical pathology parameters change based on spatiotemporal factors in this species.

Lesion swabs were more sensitive than body swabs for detection of *O. ophiodiicola* DNA, however, body swabs had a higher negative predictive value for the *Ophidiomyces* status of the snake. This demonstrates that a combination of lesion and body swabs (e.g. testing "in parallel") yields a higher overall diagnostic sensitivity for *Ophidiomyces* detection and future disease surveys should incorporate both sampling techniques.

Necropsies of deceased snakes provided valuable information about cause of death and the presence of underlying health conditions. While scavenging and autolysis were complicating factors in several cases, vehicular/mower trauma was confirmed as the cause of death in three out of eight cases, and sepsis was identified as the cause in a single case. Underlying health problems (salpingitis, hepatitis, dermatitis, ophidiomycosis) were identified in half of necropsied snakes, some of which may have contributed to debilitation and death. Necropsy data are very difficult to obtain in wildlife, but can provide critical information for understanding the overall health of individuals and identifying potential mitigation strategies to improve survival. Continued utilization of necropsies in conjunction with comprehensive health assessments and ophidiomycosis surveillance is recommended to support conservation of eastern massasauga rattlesnakes at Carlyle Lake.

Kirtland's Snake *Ophidiomyces* Surveillance & Recommendations

Kirtland's snakes are threatened in Illinois, but have not yet been granted federal protection due to a lack of population and health data. Ophidiomycosis is recognized as a potential threat to this cryptic species (U.S. Fish and Wildlife Service, 2017), and this study is the first to perform *Ophidiomyces* surveillance in Illinois Kirtland's snakes. *Ophidiomyces* was detected at all sampling sites, but most positive snakes lacked skin lesions. This could be due to detection of *Ophidiomyces* DNA without concurrent infection; qPCR is highly sensitive but does not distinguish between viable and non-viable pathogen presence. Alternatively, sampling may

have occurred when clinical disease was not present, i.e. prior to the development of skin lesions, following a shed cycle, or immediately after disease resolution. Identification of a single *Ophidiomyces* positive snake with compatible skin lesions indicates that Kirtland's snakes can be clinically affected by ophidiomycosis. Additional research pairing pathogen surveillance with more comprehensive health assessments within the context of population demography will be necessary to determine the importance of ophidiomycosis for Kirtland's conservation.

We detected *Ophidiomyces* in 48% of Kirtland's snakes sampled in 2019, but not in snakes sampled in 2020 or 2021. Sample size calculations indicate that sampling effort in 2020 and 2021 was inadequate to detect *Ophidiomyces* at a prevalence of less than 20%. Detecting this pathogen is key to characterizing its epidemiology and understanding threats to Kirtland's snakes. Ongoing disease surveillance efforts should therefore include a larger sample size. We have produced a table of minimum sample sizes to detect *Ophidiomyces* in snake populations of varying size (Table 10). This should provide guidance for future pathogen detection in Kirtland's snakes. Ophidiomycosis can cause significant morbidity and mortality in wild snakes (Clark et al 2011, Allender et al. 2016, Lorch et al 2016) and the high prevalence detected in Kirtland's snakes in 2019 necessitates further investigation through additional disease surveillance and health assessment.

Objectives for Future Research

1. Expand disease surveillance to include emerging pathogens which may threaten SGNC in Illinois.
    a. Additional RNA viruses including arenaviruses, bunyaviruses, bornaviruses, and flaviviruses.
    b. Parasitic and bacterial infections including *Cryptosporidium* spp. and *Helicobacter* spp.
    c. Recently-described box turtle pathogens such as adintovirus.
2. Incorporate toxicological testing and determine the importance of contaminants for herptile health
    a. Heavy metals, endocrine disrupting chemicals, organochlorine compounds
3. Characterize herptile immune status using functional assays and immunogenetics.
    a. Plasma antibacterial activity, sheep erythrocyte hemolysis, major histocompatibility complex and toll-like receptor allelic diversity, etc.
4. Perform longitudinal health assessments on monitored individuals to determine relationships between health status, reproductive capacity, and survival, ultimately contextualizing the importance of health for conservation in Illinois herptiles.
5. Apply health assessment protocols and the health modeling framework to additional SGNC in Illinois to support stable populations and guide practical conservation actions.
    a. Certain species are of particular interest due to the availability of baseline health and mortality data:
        i. Eastern and ornate box turtles from other populations
        ii. Eastern massasauga rattlesnakes
        iii. Blanding's turtles (*Emydoidea blandingii*)

iv. Alligator snapping turtles (*Macrochelys temminckii*)

Conclusions

The information gathered during this three-year study provides documentation of deteriorating health of some SGCN (Eastern box turtles at Kickapoo and Kennekuk), emergence of disease events (EBT, Blanding's, painted turtles, common snapping turtle), baseline health data for several populations which to measure the future impacts of several diseases. We have provided specific guidance on sample sizes and sampling strategies to detect *Ophidiomyces* in two snake species of conservation concern. We have also developed baseline data for seven RNA viruses in three herptile species of conservation concern. Continued surveillance for these viruses will allow us to rapidly detect and proactively respond to emerging infectious diseases. Future efforts will expand upon this foundation by testing for additional RNA pathogens in these and new herptile species. The RNA extraction protocols that we have developed can be used by wildlife health researchers and practitioners to advance understanding of disease ecology and promote successful conservation outcomes in multiple species. We have addressed important knowledge gaps within the current Wildlife Action Plan (appendix II) for species in greatest need of conservation, and plan to continue this work in the next phase of the Wellness of Wildlife project.

REFERENCES

Ahne W, Batts WN, Kurath G, Winton JR. Comparative sequence analyses of sixteen reptilian paramyxoviruses. Virus Res. 1999 Sep;63(1-2):65-74.

Adamovicz L, Allender MC, Archer G, Rzadkowska M, Boers K, Phillips C, Driskell E, Kinsel MJ, Chu C (2018). Investigation of multiple mortality events in eastern box turtles (Terrapene carolina carolina). PLoS One 13(4):e0195617.

Adamovicz L, Baker SJ, Kessler E, Kelly M, Johnson S, Winter J, Phillips CA, Allender MC. Erythrocyte sedimentation rate and hemoglobin-binding protein in free-living box turtles (Terrapene spp.). PLoS One. 2020;15(6):e0234805.

Adamovicz L, Griffioen J, Cerreta A, Allender MC, Lewbart GA. Tissue enzyme activities in free-living eastern box turtles (Terrapene carolina carolina). J Zoo Wildl Med. 2019;50(1):45-54.

Allender MC, Dreslik M, Wylie S, Phillips C, Wylie DB, Maddox C, Delaney MA, Kinsel MJ. Chrysosporium sp. infection in eastern massasauga rattlesnakes. Emerg Infect Dis. 2011 Dec;17(12):2383-4.

Allender MC, Bunick D, Mitchell MA. Development and validation of TaqMan quantitative PCR for detection of frog virus 3-like virus in eastern box turtles (Terrapene carolina carolina). J Virol Methods. 2013 Mar;188(1-2):121–125.

Allender MC, Mitchell MA, Torres T, Sekowska J, Driskell EA Pathogenicity of frog virus 3-like virus in red-eared slider turtles (Trachemys scripta elegans) at two environmental temperatures. Journal of Comparative Pathology 2013;149:356-67 DOI 10.1016/j.jcpa.2013.01.007.

Allender M, Baker S, Wylie D, Loper D, Dreslik MJ, Phillips CA, et al. (2015a). Development of snake fungal disease after experimental challenge with Ophidiomyces ophiodiicola in cottonmouths (Agkistrodon piscivorous). PloS one, 10(10), e0140193

Allender MC, Bunick D, Dzhaman E, Burrus L, Maddox C. Development and use of a real-time polymerase chain reaction assay for the detection of *Ophidiomyces ophiodiicola* in snakes. J Vet Diagn Invest. 2015b Mar;27(2):217-20.

Allender MC, Dreslik MJ, Patel B, Luber EL, Byrd J, Phillips CA, Scott JW (2015c) Select metal and metalloid surveillance of free-ranging eastern box turtles from Illinois and Tennessee (Terrapene carolina carolina). Ecotoxicology 24(6):1269-1278; DOI: 10.1007/s10646-015-1498-5.

Allender MC, Junge RE, Baker-Wylie S, Hileman ET, Faust LJ, Cray C. Plasma electrophoretic profiles in the eastern massasauga (Sistrurus catenatus) and influences of age, sex, year, location, and snake fungal disease. J Zoo Wildl Med. 2015d;46(4):767-773.

Allender M, Raudabaugh D, Gleason F, and Miller A (2015e). The natural history, ecology, and epidemiology of Ophidiomyces ophiodiicola and its potential impact on free-ranging snake populations. Fungal Ecology, 17:187-196.

Allender MC, Phillips CA, Baker SJ, Wylie DB, Narotsky A, Dreslik MJ. Hematology in an eastern massasauga (*Sistrurus catenatus*) population and the emergence of *Ophidiomyces* in Illinois, USA. J Wildl Dis. 2016 Apr 28;52(2):258-69.

Archer G, Phillips CA, Adamovicz L, Band M, Allender MC (2017) Detection of co-pathogens in free-ranging eastern box turtles (Terrapene carolina carolina) in Illinois and Tennessee. Journal of Zoo and Wildlife Medicine 48(4):1127–1134; DOI: 10.1638/2017-0148R.1.

Baker SJ, Dreslik MJ, Wylie DB, Phillips CA. Sources of mortality in the endangered Eastern Massasauga (Sistrurus catenatus) in Illinois. Herpetol Conserv Biol. 2016;11:335–343.

Baker SJ, Haynes E, Gramhofer M, Stanford K, Bailey S, Christman M, Conley K, Frasca A Jr, Ossiboff RJ, Lobato D, Allender MC. Case definition and diagnostic testing for snake fungal disease. Herp Rev. 2019;50(2):279-285.

Barton K (2018). MuMIn: Multi-Model Inference. R package version 1.40.4. https://CRAN.R-project.org/package=MuMIn.

Beresford WA, Donovan MP, Henninger JM, Waalkes MP (1981). Lead in the bone and soft tissues of box turtles caught near smelters. Bulletin of Environmental Contamination and Toxicology 27(3):349-352; DOI: 10.1007/bf01611031.

Bordes F, Morand S. The impact of multiple infections on wild animal hosts: a review. Infect Ecol Epidemiol. 2011;1.

Brenes R, Gray MJ, Waltzek TB, Wilkes RP, Miller DL. Transmission of ranavirus between ectothermic vertebrate hosts. PLoS One. 2014a;9:e92476.

Brown JD, Sleeman JM. 2002. Morbidity and mortality of reptiles admitted to the wildlife center of Virginia, 1991 to 2000. Journal of Wildlife Diseases 38: 699–705.

Butkus CE, Allender MC, Phillips CA, Adamovicz LA. Diagnosis of ranavirus using bone marrow harvested from mortality events in eastern box turtles (Terrapene carolina carolina). J Zoo Wildl Med. 2017;48(4):1210–1214.

Campbell TW. Clinical pathology. In: Mader D, Divers S (eds.). Current therapy in reptile medicine and surgery 1st edition. Ames (IA): John Wiley & Sons; 2015. p. 70-93.

Clark RW, Marchand MN, Clifford BJ, Stechert R, Stephens S. Decline of an isolated timber rattlesnake (Crotalus horridus) population: Interactions between climate change, disease, and loss of genetic diversity. Biol Conserv. 2011;144:886–891.

Clavero M and Garcia-Berthou E. Invasive species are a leading cause of animal extinctions. Trends Ecol Evol. 2005;20:110.

Converse KA, Green DE. Diseases of salamanders. In: Majumdar SK, Huffman JE, Brenner FJ, Panah AI (eds.). Wildlife diseases: Landscape epidemiology, spatial distribution and utilization of remote sensing technology. Easton (PA): The Pennsylvania Academy of Science; 2005. p. 118-130.

Cunningham AA and Daszak P. Extinction of a species of land snail due to infection with a microsporidian parasite. Conserv Biol. 1998;12:1139–1141.

Dalziel AE, Sainsbury AW, McInnes K, Jakob-Hoff R, Ewen JG. A comparison of disease risk analysis tools for conservation translocations. Ecohealth. 2017 Mar;14(Suppl 1):30-41.

Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R. Emerging infectious diseases and amphibian population declines. Emerg Infect Dis. 1999;5:735–748.

De Castro F and Bolker B. Mechanisms of disease-induced extinction. Ecol Lett. 2005;8:117–126.

Deem S, Parker P, Miller E. Building bridges: Connecting the health and conservation professions. Biotopica. 2008;40:662–665.

Espinosa-de Aquino W, Olvera-Ramirez A, Arellano-Carbajal F, Lanz-Mendoza H, Villagran-Herrera E, Acevedo-Whitehouse K. 2017. Protein and RNA extraction from mucosal swabs: a minimally invasive source of ecological data for studies of natural populations. Methods in Ecol and Evol 8:370-378.

Farkas SL, Gál J (2009) Adenovirus and mycoplasma infection in an ornate box turtle (Terrapene ornata ornata) in Hungary. Veterinary Microbiology 138:169–173; DOI: 10.1016/j.vetmic.2009.03.016.

Faust L, Szymanski J, Redmer M. Range wide extinction risk modeling for the Eastern Massasauga Rattlesnake (Sistrurus catenatus catenatus). Chicago, IL: Lincoln Park Zoo and U.S. Fish and Wildlife Service. 2011. 66p. Available: https://www.fws.gov/midwest/endangered/Reptiles/eama/pdf/Faust2011EMRModelingFinalReport.pdf.

Feldman SH, Wimsatt J, Marchang RE, Johnson AJ, Brown W, Mitchell JC, Sleeman JM (2006) A novel mycoplasma detected in association with upper respiratory disease syndrome in free-ranging eastern box turtles (Terrapene carolina carolina) in Virginia. Journal of Wildlife Diseases 42:279–289; DOI: 10.7589/0090-3558-42.2.279.

Fey SB, Siepielski AM, Nusslé S, Cervantes-Yoshida K, Hwan JL, Huber ER, Fey MJ, Catenazzi A, Carlson SM. Recent shifts in the occurrence, cause, and magnitude of animal mass mortality events. Proc Natl Acad Sci USA. 2015 Jan 27;112(4):1083-8.

Fox J, Weisberg S (2011). An {R} Companion to Applied Regression, Second Edition. Thousand Oaks CA: Sage. URL: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.

Franklinos LHV, Lorch JM, Bohuski E, Rodriguez-Ramos Fernandez J, Wright ON, Fitzpatrick L, Petrovan S, Durrant C, Linton C, Baláž V, Cunningham AA, Lawson B. Emerging fungal pathogen Ophidiomyces ophiodiicola in wild European snakes. Sci Rep. 2017 Jun 19;7(1):3844.

Gall A, Hoffmann B, Harder T, Grund C, Beer M. Universal primer set for amplification and sequencing of HA0 cleavage sites of all influenza A viruses. J Clin Microbiol. 2008 Aug;46(8):2561-7.

Goodman RM, Miller DL, Ararso YT. Prevalence of ranavirus in Virginia turtles as detected by tail-clip sampling versus oral-cloacal swabbing. Northeastern Naturalist 2013;20:325-32 DOI 10.1656/045.020.0208.

Hartley M, Sainsbury A. Methods of disease risk analysis in wildlife translocations for conservation purposes. Ecohealth. 2017 Mar;14(Suppl 1):16-29.

Hileman ET, Allender MC, Bradke DR, Faust LJ, Moore JA, Ravesi MJ, Tetzlaff SJ. Estimation of *Ophidiomyces* prevalence to evaluate snake fungal disease risk. Jour Wild Mgmt. 2018;82:173-181.

Holcomb CM, Parker WS (1979) Mirex residues in eggs and livers of two long-lived reptiles (*Chrysemys scripta* and *Terrapene carolina*) in Mississippi, 1970-1977. Bulletin of Environmental Contamination and Toxicology 23(3):369-371; DOI: 10.1007/bf01769972.

Holopainen R, Ohlemeyer S, Schütze H, Bergmann SM, Tapiovaara H. Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. Dis Aquat Organ. 2009 Jun 10;85(2):81-91.

Hoon-Hanks LL, Layton ML, Ossiboff RJ, Parker JSL, Dubovi EJ, Stenglein MD. Respiratory disease in ball pythons (Python regius) experimentally infected with ball python nidovirus. Virology. 2018 Apr;517:77-87.

Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J, Coupar BE. Comparative studies of piscine and amphibian iridoviruses. Arch Virol. 2000;145(2):301-331.

Hyndman TH, Shilton CM, Marschang RE. Paramyxoviruses in reptiles: a review. Vet Microbiol. 2013 Aug 30;165(3-4):200-13.

Illinois Endangered Species Protection Board (2015) Checklist of Endangered and Threatened Animals and Plants of Illinois. Retrieved from the Illinois Department of Natural Resources website: https://www.dnr.illinois.gov/ESPB/Documents/2015_ChecklistFINAL_for_webpage_051915.pdf. (accessed 15 September 2019).

Johnson AJ, Pessier AP, Jacobson ER. Experimental transmission and induction of ranaviral disease in western ornate box turtles (Terrapene ornata ornata) and red-eared sliders (Trachemys scripta elegans). Veterinary Pathology 2007;44:285-97 DOI 10.1354/vp.44-3-285.

Johnson AJ, Pessier AP, Wellehan JFX, Childress A, Norton TM, Stedman NL, Bloom DC, Belzer W, Titus VR, Wagner R, Brooks JW, Spratt J, Jacobson ER (2008) Ranavirus infection of free-ranging and captive box turtles and tortoises in the United States. Journal of Wildlife Diseases 44:851–863; DOI: 10.7589/0090-3558-44.4.851.

Kane LP, Allender MC, Archer G, Dzhaman E, Pauley J, Moore AR, Ruiz MO, Smith RL, Byrd J, Phillips CA (2017) Prevalence of Terrapene herpesvirus 1 in free-ranging eastern box turtles (Terrapene carolina carolina) in Tennesee and Illinois, USA. Journal of Wildlife Diseases 53(2):285-295; DOI: 10.7589/2016-06-138.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28(12):1647-1649.

Kolesnik E, Obiegala A, Marschang RE. Detection of Mycoplasma spp., herpesviruses, topiviruses, and ferlaviruses in samples from chelonians in Europe. J Vet Diagn Invest. 2017;29(6):820-832.

Kosmidis I (2017). brglm: Bias Reduction in Binary-Response Generalized Linear Models. R package version 0.6.1, http://www.ucl.ac.uk/~ucakiko/software.html.

Landolfi JA, Terio KA, Kinsel MJ, Langan J, Zachariah TT, Childress AL, Wellehan JF Jr. Orthoreovirus infection and concurrent cryptosporidiosis in rough green snakes (Opheodrys aestivus): pathology and identification of a novel orthoreovirus strain via polymerase chain reaction and sequencing. J Vet Diagn Invest. 2010 Jan;22(1):37-43.

Lenth RV (2016). Least-Squares Means: The R Package lsmeans. Journal of Statistical Software, 69(1), 1-33. doi:10.18637/jss.v069.i01

Lips KR, Bren F, Brenes R, Reeve JD, Alford RA, Voyles J, Careys C, Livo A, Pessier AP, Collins JP. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA. 2006;103:3165–3170.

Lorch JM, Knowles S, Lankton JS, Michell K, Edwards JL, Kapfer JM, Staffen RA, Wild ER, Schmidt KZ, Ballmann AE, Blodgett D, Farrell TM, Glorioso BM, Last LA, Price SJ, Schuler KL, Smith CE, Wellehan JF Jr, Blehert DS. Snake fungal disease: an emerging threat to wild snakes. Philos Trans R Soc Lond B Biol Sci. 2016 Dec 5;371(1709).

MacDiarmid SC Future options for brucellosis surveillance in New Zealand beef herds. N Z Vet J. 1988;36:39–42.

Marschang RE, Ihász K, Kugler R, Lengyel G, Fehér E, Marton S, Bányai K, Aqrawi T, Farkas SL. Development of a consensus reverse transcription PCR assay for the specific detection of tortoise picornaviruses. J Vet Diagn Invest. 2016 May;28(3):309-14.

Marschang RE, Kolesnik E. Detection of nidoviruses in live pythons and boas. Tierarztl Prax Ausg K Kleintiere Heimtiere. 2017 Feb 9;45(1):22-26.

McAllister CT, Bursey CR, Niemiller ML, Miller BT. A noteworthy infection of Clinostomum complanatum (Digenea: Clinostomidae) in a cave salamander, Eurycea lucifuga (Caudata: Plethodontidae), from northcentral Tennessee. Tex J Sci. 2007;59:321–326.

McAllister CT, Bursey CR, Crawford JA, Kuhns AR, Shaffer C, Trauth SE. Metacercariae of Clinostomum (Trematoda: Digenea) from three species of Ambystoma (Caudata: Ambystomatidae) from Arkansas and Illinois, U.S.A. Comp Parasitol. 2010;77(1):25–30.

McKenzie CM, Piczak ML, Snyman HN, Joseph T, Theijin C, Chow-Fraser P, Jardine CM. First report of ranavirus mortality in a common snapping turtle Chelydra serpentina. Dis Aquat Organ 2019;132:221–7.

Méthot PO, Alizon S. What is a pathogen? Toward a process view of host-parasite interactions. Virulence. 2014;5:775–785.

Miller PS. Tools and techniques for disease risk assessment in threatened wildlife conservation programmes. Int Zoo Yb. 2007;41: 38–51.

Morris, MA and Brandon, RA (1984) Gynogenesis and hybridization between Ambystoma platineum and Ambystoma texanum in Illinois. Copeia 1984, 324–337.

Paries S, Funcke S, Kershaw O, Failing K, Lierz M. The role of Virus "X" (Tortoise Picornavirus) in kidney disease and shell weakness syndrome in European tortoise species determined by experimental infection. PLoS One. 2019 Feb 19;14(2):e0210790.

Pedersen AB, Jones KE, Nunn CL, Altizer S. Infectious diseases and extinction risk in wild mammals. Conserv Biol. 2007;21:1269–1279.

Pénzes JJ, Benkö M. (2014) Novel parvovirus from the worm lizard Trogonophis wiegmanni – first virus ever detected in amphisbaenian hosts. Acta Vet Hung 62: 284–292.

Perpiñán D, Garner MM, Trupkiewicz JG, Malarchik J, Armstrong DL, Lucio-Forster A, Bowman DD. Scoliosis in a tiger salamander (Ambystoma tigrinum) associated with encysted digenetic trematodes of the genus *Clinostomum*. J Wild Dis. 2010;46(2):579–584.

Petrikkos, G, Tsioutis, C. Recent Advances in the Pathogenesis of Mucormycoses. Clinical Therapeutics. Vol 40(6). 2018. 894-902.

Phillips, CA, Uzzell, T, Spolsky, CM, Serb, JM, Szafoni, RE and Pollowy, TR (1997) Persistent high levels of tetraploidy in salamanders of the Ambystoma jeffersonianum complex. Journal of Herpetology 31, 530-535. doi: 10.2307/1565605.

Pimm SL, Russell GJ, Gittleman JL, Brooks TM. The future of biodiversity. Science. 1995;269:347–350.

Pirofski LA, Casadevall A. Q and A: What is a pathogen? A question that begs the point. BMC Biol. 2012;10:6.

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rivera S, Wellehan JFX, McManamon R, Innis CJ, Garner MM, Raphael BL, Gregory CR, Latimer KS, Rodriguez CE, Diaz-Figueroa O, Marlar AB, Nyaoke A, Gates AE, Gilbert K, Childress AL, Risatti GR, Frasca S. Systemic adenovirus infection in Sulawesi tortoises (Indotestudo forsteni) caused by a novel siadenovirus. J Vet Diagnostic Investig. 2009;21:415–426.

Rockwell KE, Thompson D, Maddox C, Mitchell MA. Blanding's turtles (Emydoidea blandingii) as a reservoir for Leptospira spp. PLoS One. 2019;14(6):e0210688.

Sack A, Butler E, Cowen P, Lewbart GA. Morbidity and mortality of wild turtles at a North Carolina wildlife clinic: a 10-year retrospective. J Zoo Wildl Med. 2017;48(3):716-724.

Schloegel LM, Hero JM, Berger L, Speare R, McDonald K, Daszak P. The decline of the sharp-snouted day frog (Taudactylus acutirostris): the first documented case of extinction by infection in a free-ranging wildlife species? EcoHealth. 2006;3:35–40.

Schrader GM, Allender MC, Odoi A. Diagnosis, treatment, and outcome of eastern box turtles (Terrapene carolina carolina) presented to a wildlife clinic in Tennessee, USA, 1995-2007. J Wildl Dis. 2010;46(4):1079-1085.

Schumacher VL, Innis CJ, Garner MM, Risatti GR, Nordhausen RW, Gilbert-Marcheterre K, Wellehan JFX, Childress AL, Frasca S. Sulawesi tortoise adenovirus-1 in two impressed tortoises (Manouria impressa) and a Burmese star tortoise (Geochelone platynota). J Zoo Wildl Med. 2012;43(3):501–510.

Sim RR, Norton TM, Bronson E, Allender MC, Stedman N, Childress AL, Wellehan JF Jr (2015) Identification of a novel herpesvirus in captive eastern box turtles (Terrapene carolina carolina). Veterinary Microbiology 175:218–223; DOI: 10.1016/j.vetmic.2014.11.029.

Sleeman J. 2008. Use of wildlife rehabilitation centers as monitors of ecosystem health. In Zoo and wild animal medicine, current therapy 6, Fowler M. E., Miller R. E. (editors.). St. Louis, Missouri: Saunders Elsevier,,pp. 97–104.

Smith KF, Sax DF, Lafferty KD. Evidence for the role of infectious disease in species extinction and endangerment. Conserv Biol. 2006;20:1349–1357.

Smith KF, Acevedo-Whitehouse K, Pedersen AB. The role of infectious diseases in biological conservation. Animal Conserv. 2009;12:1–12.

Spencer R-J, Van Dyke J, Petrov K, Ferronato B, McDougall F, Austin M, et al. Profiling a possible rapid extinction event in a long-lived species. Biological Conservation. 2018; 221: 190–7.

Spolsky, C, Phillips, CA and Uzzell, T (1992) Gynogenetic reproduction in hybrid mole salamanders (Genus Ambystoma). Evolution 46, 1935-1944.

Stranahan L, Alpi KM, Passingham RK, Kosmerick TJ, Lewbart GA. 2016. Descriptive epidemiology for turtles admitted to the North Carolina State University College of Veterinary

Medicine Turtle Rescue Team. Journal of Fish and Wildlife Management 7(2):520–525; e1944-687X. doi: 10.3996/072015-JFWM-056

Stephensen CB, Casebolt DB, Gangopadhyay NN. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res. 1999 Apr;60(2):181-9. doi: 10.1016/s0168-1702(99)00017-9. PMID: 10392726; PMCID: PMC7126282.

Stöhr AC, López-Bueno A, Blahak S, Caeiro MF, Rosa GM, Alves de Matos AP, Martel A, Alejo A, Marschang RE. Phylogeny and differentiation of reptilian and amphibian ranaviruses detected in Europe. PLoS One. 2015;10(2):e0118633.

Szymanski J, Pollack C, Ragan L, Redmer M, Clemency L, Voorhies K, JaKa J. Species status assessment for the eastern massasauga rattlesnake (Sistrurus catenatus) report version 2. U.S. Fish and Wildlife Service. 2016. 103p. Available: https://www.fws.gov/midwest/endangered/Reptiles/eama/pdf/SSAFinalV2July2016EMR.pdf.

Tabor GM, Ostfeld R, Poss M, Dobson A, Aguirre AA. Conservation biology and the health sciences. Defining the research priorities of conservation medicine. In: Soulé ME and Orians GH (eds.). Conservation biology. Research priorities for the next decade. Washington, DC: Island Press; 2001. p. 155-174.

Tangredi BP, Evans RH (1997) Organochlorine pesticides associated with ocular, nasal, or otic infection in the eastern box turtle (Terrapene carolina carolina). Journal of Zoo and Wildlife Medicine 28:97–100.

Teltser, C and Greenwald, KR (2015) Survivorship of ploidy-variable unisexual Ambystoma salamanders across developmental stages. Herpetologica 71, 81-87. doi: 10.1655/HERPETOLOGICA-D-14-00007.

U.S. Fish and Wildlife Service. 2017. Species status assessment report for Kirtland's snake (*Clonophis kirtlandii*). 43 pp.

Uzzell, TM Jr. and Goldblatt, SM (1967) Serum proteins of salamanders of the Ambystoma jeffersonianum complex, and the origin of the triploid species of this group. Evolution 21, 345-354.

van Dijk PP (2011) Terrapene carolina (errata version published in 2016). The IUCN Red List of Threatened Species. Available: http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T21641A9303747.en. [accessed September 17 2018]

van Dijk PP, Hammerson GA (2011) Terrapene ornata. The IUCN Red List of Threatened Species. Available: http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T21644A9304752.en. [accessed September 17 2018]

Van Riper C, Van Riper SG, Hansen WR. Epizootiology and effect of avian pox on Hawaiian forest birds. The Auk. 2002;119:929–942.

Venables WN, Ripley BD (2002). Modern Applied Statistics with S. Fourth Edition. Springer, New York. ISBN 0-387-95457-0

Waltzek TB, Stacy BA, Ossiboff R, Stacy N, Fraser W, Yan A, Mohan S, Rodrigues T, Subramaniam K, Guzman-Vargas V, Shender L. Characterization of a novel bunyavirus isolated from moribund farmed and free-ranging freshwater turtles in Florida. In: Proceedings of the 68th Annual Wildlife Disease Association Conference. August 4 – 9th, Tahoe City, CA.

Wellehan JF Jr, Childress AL, Marschang RE, Johnson AJ, Lamirande EW, Roberts JF, Vickers ML, Gaskin JM, Jacobson ER. Consensus nested PCR amplification and sequencing of diverse reptilian, avian, and mammalian orthoreoviruses. Vet Microbiol. 2009 Jan 1;133(1-2):34-42.

Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E. Quantifying threats to imperiled species in the United States. Bio-Science. 1998;48:607–615.

Wilkinson R. Clinical Pathology. In: McArthur S, Wilkinson R, Meyer J (eds.). Medicine and surgery of tortoises and turtles. Oxford (UK): Blackwell Publishing Ltd; 2004. p. 141-186.

WWF. Living Planet Report 2018 Aiming Higher. Grooten M, Almond REA (Eds). WWF, Gland, Switzerland. 2018. 75p.

Wyatt KB, Campos PF, Gilbert MTP, Kolokotronis S-O, Hynes WH, DeSalle R, Daszak P, MacPhee RDE, Greenwood AD. Historical mammal extinction on Christmas Island (Indian Ocean) correlates with introduced infectious disease. PLoS One. 2008;3:1–9.

Yonkers SB, Schneider R, Reavill DR, Archer LL, Childress AL, Wellehan JF Jr (2015) Coinfection with a novel fibropapilloma-associated herpesvirus and a novel Spirorchis sp. in an eastern box turtle (Terrapene carolina) in Florida. Journal of Veterinary Diagnostic Investigation 27(4):408-413; DOI: 10.1177/1040638715589612.

Zhang J, Finlaison DS, Frost MJ, Gestier S, Gu X, Hall J, et al. (2018) Identification of a novel nidovirus as a potential cause of large scale mortalities in the endangered Bellinger River snapping turtle (Myuchelys georgesi). PLoS ONE 13(10): e0205209. https://doi.org/10.1371/journal.pone.0205209