ASSESSING WELLNESS IN WILD HERPTILE SPECIES IN GREATEST NEED OF CONSERVATION

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SUMMARY AND KEY FINDINGS

This project characterized the health of 3 herptile SGNC in 3 separate IWAP campaigns by developing general health profiles, identifying infectious and non-infectious causes of morbidity and mortality, and modeling the wellness of individuals and populations. The modeling framework developed in this project identifies ways to simultaneously support health and combat threats, and may represent a pathway for improving conservation outcomes in wild species of conservation concern. Specific management recommendations have been developed, and can be found later in the document.

Eastern Box Turtles

- Seven hundred health assessments were performed at five study sites
- *Abnormalities in total leukocyte count and Heterophil:Lymphocyte ratio, active shell disease, and signs of upper respiratory disease (watery eyes, nose, conjunctivitis) were the most predictive for poor health and should be used in assessing other populations.*
- Turtles were in a better plane of health and had the largest population size at the Stephen A. Forbes State Park, the site with the best habitat quality.
- Turtles at Kickapoo State Park, the site with the worst habitat quality, had a small population size and were in a poorer plane of health.
- Eastern box turtles at Kickapoo also experience a high mortality rate attributable to multiple causes, many of which are precipitated by poor health status.
- PVA indicates that the current rate of adult loss from Kickapoo is likely not sustainable.

Ornate Box Turtles

- Two hundred ninety-two health assessments were performed at the Nachusa Grasslands
- *The presence of active and inactive shell lesions and abnormalities in total leukocyte count, eosinophils, basophils, and Heterophil:Lymphocyte ratios were predictive of poor health*
- Shell lesions associated with predator trauma are the most common threat to individual health in ornate box turtles at Nachusa, and previous studies suggest that predation-associated mortality may be occurring at unsustainable rates.
- PVA revealed that the ornate box turtle population at Nachusa is small and susceptible to extinction due to stochastic events such as increased predation rates or novel disease introduction.

Silvery Salamanders

- Nine hundred and seven health assessments were performed
- *Low body condition score, injuries, and hemorrhages were the best predictors of poor health*
- Parasitic infections including *Clinostomum* sp. and *Dermotheca* sp. were observed from 2017-2019 and were occasionally associated with adult mortality. This study was the first to document the occurrence of a *Dermotheca* sp. parasite in salamanders from the Midwestern US.
- Ranavirus (FV3) mortality events resulted in the deaths of over 300 silvery salamander larvae in four ponds in 2016. Larval mortality events attributable to FV3 reoccurred in 2017 and 2019.
- FV3 is a significant threat to silvery salamander larvae, with high morbidity and > 80% mortality.
- Recurrent FV3 outbreaks, such as those observed at Kickapoo State Park, can contribute to population declines and extirpations, which is especially concerning for state-endangered species with a limited geographical distribution like silvery salamanders.

Mortality Events

- Thousands of silvery salamander and wood frog larvae died due to FV3 over multiple years.
- Head-started alligator snapping turtles intended for release into southern Illinois were diagnosed with an emerging fungal pathogen, *Emydomyces testovorans*, in 2016. Half of these turtles died.
- Radiotelemetered eastern box turtles at Kickapoo had a 61% mortality rate over 2 years due to poor health and abnormal brumation.
- Blanding's turtles were found dead due to gastrointestinal disease throughout the study.

SCIENTIFIC DELIVERABLES GENERATED

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

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- Adamovicz, L., J. Griffioen, A. Cerreta, G.A. Lewbart, M.C. Allender. 2019. Tissue enzyme activities in free-living Eastern box turtles (*Terrapene carolina carolina*). Journal of Zoo and Wildlife Medicine 50: 45-54.

- Adamovicz, L. 2019. Modeling the health of free-living Illinois herptiles: an integrated approach incorporating environmental, physiologic, spatiotemporal, and pathogen factors. PhD Dissertation, University of Illinois.

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- dePersio, S., M.C. Allender, M.J. Dreslik, L. Adamovicz, C.A. Phillips, B. Willeford, L.P. Kane, S. Joslyn, R. O'Brien. 2019. Body condition of eastern box turtles (*Terrapene carolina carolina*) evaluated by computed tomography. Journal of Zoo and Wildlife Medicine 50(2): 295-302.

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- Rayl, J.M., L. Adamovicz, A.W. Stern, M.D. Vieson, C.A. Phillips, M. Kelly, M. Beerman, M.C. Allender. 2019. Mortality investigation of monitored eastern box turtles (*Terrapene carolina carolina*) in central Illinois from 2016-2018. Journal of Wildlife Diseases 56(2):epub ahead of print.

- Adamovicz, L., D.B. Woodburn, S.V. Herrera, K. Low, C.A. Phillips, A.R. Kuhns, J.A. Crawford, M.C. Allender. Initial characterization of *Dermotheca* sp. infection in a midwestern state-endangered salamander (*Ambyostoma platineum*) and a co-occurring common species (*Ambyostoma texanum*). Parasitology, in press 10/2019.

- Adamovicz, L., S.J. Baker, E. Kessler, M. Kelly, S. Johnson, J. Winter, C.A. Phillips, M.C. Allender. Novel diagnostic techniques facilitate health assessments in free-living box turtles (*Terrapene* spp.) Ecohealth, in review 11/2019.

- Adamovicz, L., S.J. Baker, M. Merchant, M.C. Allender. Plasma antibacterial activities in ornate (*Terrapene ornate ornate*) and eastern box turtles (*Terrapene carolina carolina*). Developmental and Comparative Immunology, in review 12/2019.

- Andersson, K., L. Adamovicz, L. Mumm, J. Winter, G. Glowacki, R. Teixeira-Neto, M.J. Adkesson, M. Ivancic, M.C. Allender. Detection of a novel herpesvirus associated with squamous cell carcinoma in free-ranging Blanding's turtle (*Emydoidea blandingii*). In preparation.

- Doden, G., B. Gartlan, K. Klein, C. Maddox, L.A. Adamovicz, M.C. Allender. Prevalence and antimicrobial resistance patterns of *Salmonella* spp. in two free-ranging populations of eastern box turtles (*Terrapene carolina carolina*). In preparation.

- Doke, R., K. Hiebert, M. Repella, M. Stuart, L. Mumm, J. Winter, L. Adamovicz, G. Glowacki, E. Kessler, M.C. Allender. Prevalence of intraerythrocytic parasites in *Macrocheyls temminckii, Emydoidea blandingii, Terrapene carolina carolina,* and *Terrapene ornata ornata*. In preparation.

- Klein, K., L. Adamovicz, M.C. Allender. Measuring blood lactate concentrations in eastern box turtles (*Terrapene carolina carolina*) following capture by a canine search team. In preparation.

- Adamovicz, L., J. Webb, E. Kessler, S.J. Baker, M.C. Allender. Venous blood gas in free-living ornate box turtles (*Terrapene ornata ornata*). In preparation.

- Adamovicz, L., S.J. Baker, M. Merchant, M.C. Allender. Mechanisms of complement activation differ in the plasma of eastern (*Terrapene carolina carolina*) and ornate (*Terrapene ornata ornata*) box turtles. In preparation.

- Adamovicz, L., E. Kessler, S.J. Baker, Y.N. Jeon, J. Webb, M. Kelly, J. Winter, C.A. Phillips, M.C. Allender. Ornate box turtle (*Terrapene ornata ornata*) hematologic and corticosterone response to transport and handling. In preparation.

- Adamovicz, L., S.A. Callahan, C.A. Phillips, A.R. Kuhns, J.A. Crawford, M.C. Allender. Hematology and cytochemical staining characteristics of free-living silvery salamander (*Ambystoma platineum*) leukocytes. In preparation.

<u>Proceedings supported in part by this grant (and acknowledged during the presentation, chronologic order)</u>

- Allender, M.C. Wildlife Epidemiology Lab: From 0 to 2770 captures in 9 years. 5th Box Turtle Conservation Workshop, Urbana, IL. May 2016.

- Allender, M.C. Chelonian sampling and infectious disease processing. 5th Box Turtle Workshop. Urbana, IL. May 2016.

- Willeford, B., M.C. Allender, B. Allan. Investigating mosquitoes as a vector for frog virus 3-like virus. 5th Box Turtle Conservation Workshop, Urbana, IL. May 2016.

- Emmel, E., M.C. Allender. Evaluation of thermal ecology and hematology of free-ranging eastern box turtles in Illinois (*Terrapene carolina carolina*). 5th Box Turtle Conservation Workshop, Urbana, IL. May 2016.

- Adamovicz, L., M.C. Allender. Box turtle mortality event investigation: Case series and recommendations. 5th Box Turtle Conservation Workshop, Urbana, IL. May 2016.

- DePersio, S., M.C. Allender, S. Joslyn, R. O'Brien. Body condition of box turtles evaluated by computed tomography. 5th Box Turtle Conservation Workshop, Urbana, IL. May 2016.

- Adamovicz, L., M.C. Allender. Investigation of multiple mortality events in eastern box turtles (*Terrapene carolina carolina*) in east-central Illinois from 2011-2015. Wildlife Disease Association Annual Conference, New York. July 2016.

- Adamovicz, L. Ornate Box Turtle (*Terrapene ornata*) Health Assessment at Nachusa. Nachusa Science Symposium, Franklin Grove, IL. October 2016.

- Adamovicz, L., C. Phillips, K. Low, K. Boers, M.C. Allender. Silvery Salamander (*Ambystoma platineum*) health assessment prior to and during a ranavirus mortality event. University of Illinois, College of Veterinary Medicine Research Symposium. Urbana, IL. April 2017.

- Low, K., M.C. Allender, C. Phillips, S. Kimble. A ranavirus-associated mass mortality event in an Illinois amphibian community. Joint Meeting of Ichthyologists and Herpetologists, Austin, TX. July 2017.

- Adamovicz, L., M.C. Allender. Drivers and impacts of single and co-pathogen occurrence in free-living eastern box turtles (*Terrapene carolina carolina*) in Illinois and Tennessee from 2013-2016. American Association of Zoo Veterinarians Annual Conference, Dallas, TX. September 2017.

- Adamovicz, L., C. Phillips, K. Low, K. Boers, M.C. Allender. Are salamanders safe? Health assessment of the silvery salamander (*Ambystoma platineum*) in Vermilion County, Illinois, USA prior to and during a ranavirus outbreak. American Association of Zoo Veterinarians Annual Conference, Dallas, TX. September 2017.

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- Rayl, J.M., M.C. Allender, A. Stern, M. Vieson, L. Adamovicz, M. Kelly, M. Beerman. Mortality investigation of eastern box turtles (*Terrapene carolina carolina*) in central Illinois from 2016-2018. Wildlife Disease Association Annual International Conference, St. Augustine, FL. August 2018.

- Adamovicz, L., M.C. Allender. Biochemical Enzyme Tissue Specificity in Eastern Box Turtles (*Terrapene carolina carolina*). ExoticsCon, Joint conference of the Association of Reptilian and Amphibian Veterinarians, The Association of Avian Veterinarians, and the Association of Exotic Mammal Veterinarians, Atlanta, GA. September 2018.

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- 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.

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- Klein, K., L. Adamovicz, M.C. Allender. Measuring blood lactate concentrations following capture by a canine search team in eastern box turtles (*Terrapene carolina carolina*). American Association of Zoo Veterinarians Annual Conference, St. Louis, MO. 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.

- Adamovicz, L., S.J. Baker, E. Kessler, M. Kelly, S. Johnson, J. Winter, C.A. Phillips, M.C. Allender. Erythrocyte sedimentation rate and hemoglobin-binding protein in eastern and ornate box turtles (*Terrapene* spp.). 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. **Plenary Talk**

- Adamovicz, L., D.B. Woodburn, S.V. Herrera, K. Low, A.R. Kuhns, J.A. Crawford, C.A. Phillips, M.C. Allender. Characterization of a novel mesomycetozoean infection (*Dermotheca* sp.) in a state-endangered salamander (*Ambystoma platineum*) and a co-occurring common species (*Ambystoma texanum*). American Association of Zoo Veterinarians Annual Conference, St. Louis, MO. October 2019. *** Winner – Graduate Award Sponsored by Animal Necessity

- 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.

INTRODUCTION

The Illinois landscape has undergone unprecedented change in the last 100 years, and many environments no longer resemble the ecosystems in which species evolved. Declines of several state species have been associated with these landscape changes, however the associated changes in pathogen presence and subsequent ability of habitats to support healthy populations remains largely unknown. Deteriorating wildlife health threatens the sustainability and successfulness of conservation efforts as has been observed in Illinois with white nose syndrome (Blehert et al., 2009), ranavirus (Johnson et al., 2008), and snake fungal disease (Allender et al., 2011). Furthermore, extinction events due to disease, while rare in wildlife, have been documented in both a species of land snail (*Partula turgida*) due to a parasite infestation (Cunningham and Daszak, 1998) and the sharp-snouted day frog (*Taudactylus acutirostris*) due to chytridiomycosis (Schloegel et al., 2006). In both cases, disease outbreaks led to rapid catastrophic declines from which populations could not demographically recover. Neither study described the wellness of individuals in the population prior to the outbreak, which may have allowed a more concerted effort to mitigate disease impact. These disease events may negate the benefits of habitat restoration executed through the Illinois Wildlife Action Plan. Thus, conserving the wellness of wildlife populations is integral to conserving ecosystems, assessing recovery efforts, and addressing a need identified by the Wildlife Action Team.

The ability to detect changes in the health of an ecosystem requires transdisciplinary cooperation utilizing multiple approaches, and wildlife sentinels have been proposed as early monitors of ecosystem health (Giulette et al., 1995; Mazet et al., 2000; Sleeman, 2008; Carson et al., 2014; Hamer et al., 2012; Childs et al., 2007). Monitoring the health of sentinel species allows early detection of ecosystem change and directly benefits species health and recovery efforts. Baseline natural history, physiological, and health-related information for sentinel species, including reptiles and amphibians, is needed and is largely absent from the current IWAP.

Techniques that address this data void require a health assessment approach that utilizes specific biomedical diagnostics. Hematologic, plasma biochemical, and pathogen prevalence data have been previously utilized to determine the wellness of free-ranging reptile populations (Anderson et al., 1997; Wright and Skeba, 1992; Hidalgo-Vila et al., 2011; Brown and Sleeman, 2002; Chaffin et al 2008; Sleeman et al., 2008; Schrader et al., 2010; Rose and Allender, 2011; Kimble and Williams, 2012), but have not been critically evaluated. In mammals and birds, interpretation of inflammatory responses observed on complete blood counts, elevated concentrations of kidney or liver enzymes, and/or presence of pathogens is fairly straightforward (Clarke et al., 2013; Junge and Louis, 2005; Parsons et al., 2015; Turvey et al., 2012; Williams et al., 2011). Unfortunately, assessing health in reptiles and amphibians is not well-characterized and utilizing diagnostic assays designed for mammals often leads to difficulty in interpretation. In addition, the close tie of physiological responses and temperature displayed by ectotherms (Lillywhite, 1987; Peterson et al., 1993) can complicate analysis compared to endotherms. Baseline studies that establish the same rigor and criteria for interpretation of health diagnostics are lacking in Illinois amphibian and reptile populations. The current grant aimed to address these critical needs by collecting baseline hematologic, plasma biochemical, and disease prevalence data associated with both healthy and unhealthy individuals and populations.

The Illinois WAP identifies species in greatest need of conservation (SGNC), but the wellness of the state's reptiles and amphibians is poorly investigated. To maximize the usefulness of transdisciplinary wellness assessments described above, species response to various environmental conditions need to be investigated simultaneously. Many wildlife studies utilize a single population of individuals at a specific time point. These approaches limit the ability to relate larger ecosystem changes or threats over time or between habitats. The current grant addressed this void by evaluating three species in three separate WAP

campaigns. Evaluating representative species in these campaigns allowed integration of results that have already aided in the conservation of other species within the same habitat.

The free-ranging eastern box turtle (*Terrapene carolina carolina*) is considered vulnerable by the IUCN Red List, is listed in CITES Appendix II, and has been proposed as an exemplary sentinel species (CITES Appendix II, 2012; IUCN, 2011; Sleeman, 2008; Lloyd et al., 2016). Eastern box turtles are distributed across the eastern US in a variety of habitats, have long lifespans, small home ranges, and are slow to reach reproductive maturity, all which potentiate their susceptibility to environmental stress (Sleeman, 2008) and make them excellent indicator species for environmental change (Schrader et al., 2010; Sleeman, 2008). Moreover, habitat fragmentation, infectious diseases, and toxicological exposure are of increasing concern in box turtles (Brown et al, 2004; De Voe et al., 2004; Johnson et al., 2008; Allender, 2012). Over the past six years we have been monitoring hematologic, plasma biochemical, contaminant exposure, and pathogen prevalence in eastern box turtles in Illinois. Continuous health monitoring and disease investigations such as this provide valuable insight into ecological health and aid in species preservation (Brown and Sleeman, 2002; Chaffin et al., 2008; Sleeman, 2008; Schrader et al., 2010).Our phase I project aimed to build upon these data in order to identify ongoing changes that may represent deteriorating environmental conditions or specific conservation threats to eastern box turtles.

Prior to this study, epidemiologic and systematic multiple pathogen studies on eastern box turtles were limited (Kane et al., 2017; Archer et al., 2017). This lack of understanding impacted conservation strategies for this species and its ecosystem. In a comprehensive epidemiology study investigating ranavirus in box turtles under non-outbreak conditions, prevalence of that pathogen was extremely low (Allender et al., 2013). However, the prevalence of co-pathogens *Terrapene* herpesvirus 1 (TerHV1) and *Mycoplasma* sp. has been shown to be high in wild box turtle populations (Ossiboff et al., 2015; Kane et al., 2017). These pathogens are less likely to cause mortality and may better represent individual or environmental stress. Disease ecology of each individual pathogen is largely unknown, and management strategies that target mitigation of a single disease without considering the interaction of other pathogens may be grossly over simplified. Our research filled a critical need in describing the prevalence of several eastern box turtle pathogens and providing data that aids in development of holistic conservation strategies for this species.

Evaluating the health of the box turtle can enhance other sympatric species utilizing different resources within the same environment, as has been demonstrated for other chelonian (Silbernagel et al., 2013) and wildlife (Herrera et al., 2008; Junge and Louis, 2005; Navarro-Gonzalez et al., 2014) species. The silvery salamander (*Ambystoma platineum*) co-occurs with the eastern box turtle within the Vermillion County Conservation Opportunity Area (COA). The silvery salamander is also 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. Disease threats such as ranavirus may be similar for silvery salamanders and eastern box turtles, but management responses and the role of disease in species sustainability may differ. Other threats to the wellness of the silvery salamander include *Batrachochytrium dendrobatidis* (*Bd*, chytrid), a global amphibian disease agent (Skerratt et al., 2007) that has been identified in the Vermillion COA, although not in the silvery salamander (Beyer et al., 2015). Recently in Europe, a larger threat to salamander conservation was discovered, *Batrachochytrium salamandrivorans* (Bsal), but this pathogen has yet to be detected in the US (Martel et al., 2013). The potential presence of these pathogens in the environment may represent a conservation threat for silvery salamanders, and understanding the baseline health of this species prior to disease emergence might allow efforts that mitigate the effects of novel pathogens.

While several studies exist on health and disease occurrence in eastern box turtles, fewer exist on the ornate box turtle (*Terrapene ornata ornata*), despite significant declines across its range (Cureton et

al., 2014). Historical occurrences of mortality events (Metcalf and Metcalf, 1979), disease events (Christiansen et al., 2004; Farkas and Gal, 2009), and physiological responses (heart rate, respiratory rate) to temperature and other demographic factors (Bachman, 2013; Bethea, 1971) have occurred in this species. Despite these reports, there is a paucity of information on disease threats or wellness in this species anywhere across the range, including Illinois (Harden et al., 2018). Utilizing similar techniques applied to the eastern box turtle enables integration of data sets in species with similar physiology and helps elucidate different approaches to maintaining wellness.

There are several threats to the success of conservation programs for the eastern box turtle, ornate box turtle, and silvery salamander. 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 WAP as locations with significant existing, or potential wildlife habitat resources. Utilizing three species that overlap habitat, natural history, and disease threats allows integration of health results that can contribute to the success of their conservation.

PURPOSE AND OBJECTIVES

The purpose of this project was to assess the health of the eastern box turtle, ornate box turtle, and silvery salamander through the generation of baseline hematology, biochemistry, protein electrophoresis, and disease prevalence data. This health monitoring established criteria that can be integrated into future conservation assessments of SGNC. Our specific objectives were:

1. Identify three herptile SGNC in three separate campaigns (Forests and Woodlands; Prairie and Farmland; Wetlands) in consultation with campaign leads that fit the criteria of sample size, number of populations, and current natural history data.

2. Establish baseline health profiles for the SGNC identified in objective 1
    a. Utilize hematology, plasma biochemistry, and protein electrophoresis to characterize the general health of herptile SGNC in three campaigns.
    b. Establish baseline prevalence of common pathogens
    c. Provide technical resources training for IDNR staff to characterize baseline health and its impacts

3. Assess the occurrence of emerging or ongoing infectious diseases in SGNC
    a. Investigate mortality events in SGNC as they occur
    b. 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 previously 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, and the silvery salamander in the Vermillion COA as species of conservation concern in areas of interest.

OBJECTIVE 2A/3A

***Health and Hematologic Assessments**:* 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 (Boers et al., 2017). 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 -20oC. 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 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. Combined oral and cloacal swabs were collected using cotton-tipped plastic handled applicators (Fisher Scientific, Pittsburgh, PA 15275) and stored at -20ºC. 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.

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) 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 (2018 & 2019). 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.

Three - thirteen ephemeral ponds per year 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 other SWGs (T-108-R-1, T-113-R-1, T-118-R-1). Adult silvery salamanders were captured in the bucket traps. Larval salamanders were captured at the same ponds using dipnets later in the season. Salamanders were 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. Adult salamanders were toe-clipped (2016 – 2018) or pit-tagged (2019) for mark-recapture purposes and then released on the opposite side of the fence.

OBJECTIVE 2B/3A

Disease Detection: 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). Samples were subsequently 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. 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), Testudinid herpesvirus 2 (TestHV2), box turtle *Mycoplasma* sp., *Mycoplasma testudineum*, *Mycoplasma agassizii*, box turtle adenovirus, *Salmonella enteritidis*, *Salmonella tymphimurium*, tortoise intranuclear coccidiosis (TINC), *Anaplasma phagocytophilum*, and *Borrelia burgdorferi.* Salamanders were screened for *Batrachochytrium dendrobatidis* (Bd), *Batrachochytrium salamandrivorans* (Bsal), FV3, ATV, BIV, EHNV, and the Chlamydiaceae family.

Statistics & Modeling: 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, 10% and 90% 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 to control for confounders (Joffé et al., 2012). Continuous predictor variables were assessed for multicollinearity using Pearson's correlation coefficient and variance inflation factors (VIF; package car; Fox and Weisburg 2011), with $r > 0.5$ and $VIF > 5$ considered "strongly" correlated. Strongly correlated predictor variables were not included together in statistical models. Data transformation was pursued if needed to support statistical assumptions during modeling.

Univariable analyses were conducted using data from 2016 and 2017 to select predictors for inclusion in the final health models for eastern and ornate box turtles. A liberal p-value of 0.15 was utilized to include all variables which may explain biologically important variation in health parameters. Sex ratios were evaluated using binomial tests (expected ratio 0.5). Predictors of continuous outcomes (clinical pathology parameters) were evaluated using general linear models. Post-hoc between group differences were evaluated using the contrasts function in the lsmeans package with a Tukey adjustment for multiple statistical comparisons. (Lenth, 2016). Predictors of categorical outcomes (pathogen status, presence/absence of physical exam abnormalities) were assessed using bias-reduced generalized linear models in package brglm (Kosmidis, 2017).

Reference intervals were constructed for each clinical pathology parameter in each year using the nonparametric method in the referenceIntervals package according to American Society for Veterinary Clinical Pathology guidelines (Friedrichs, 2012). Turtles with abnormal physical examination findings were excluded from the reference interval dataset, and intervals were further partitioned based on year, season, age class, and/or sex as appropriate based on univariate analyses. Outliers were visually identified using box plots and excluded using Horn's method (Horn et al., 2001).

All turtles were then assigned to a health category ("apparently healthy" vs. "unhealthy"). Apparently healthy turtles met the following three criteria (Figure 1):

1. **No clinically significant physical examination abnormalities.** A clinically significant abnormality is one expected to affect the animal's ability to navigate, prehend food, mate, and protect itself from predation. Examples include open wounds, active fractures, or respiratory distress. Physical examination abnormalities that are not considered clinically significant include healed injuries and congenital abnormalities like fused digits or supranumerary scutes.
2. **No more than three clinical pathology parameters outside of reference intervals.** By definition, reference intervals comprise 95% of the values of a "healthy" population, so 5% of "healthy" animals can be expected to have a value outside of the reference interval (Freidrichs 2012). Increasing the number of abnormal values required to classify an animal as "abnormal" decreases the risk of misclassifying an otherwise "healthy" animal.
3. **No clinically apparent qPCR detection of infectious disease.** The presence of a pathogen does not necessarily equate to disease (Thompson et al., 2010; Pirofski, 2012; Méthot, 2014). Furthermore, some pathogens are host-adapted and have limited associated clinical signs in an otherwise healthy animal (Ryser-Degiorgis, 2013). The present study only considers pathogen-positive animals "unhealthy" if they have concurrent clinical signs of illness or if they have greater than three abnormal clinical pathology values, indicating a negative physiologic effect of the pathogen.

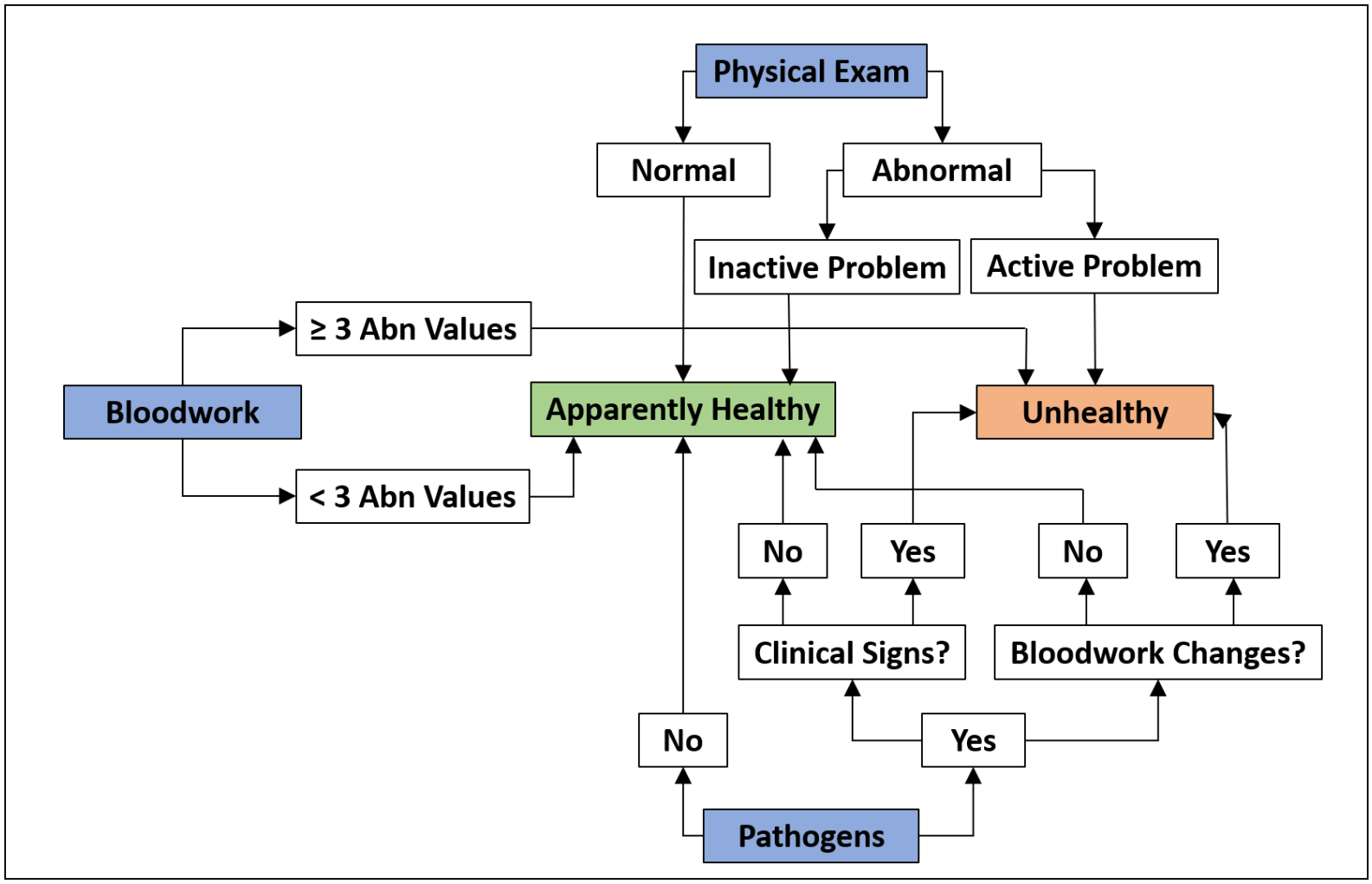

Figure 1. Health classification decision tree for eastern and ornate box turtles.

Individual health status was evaluated using sets of candidate generalized linear models ranked using an information-theoretic approach with the MuMIn package (Barton, 2018). To generate predictive health models with a minimum number of variables, each significant clinical pathology parameter was centered based on age, sex, and year-specific median values and health models were reconstructed. Internal model validation was performed via bootstrapping with 500 replicates using the 2016 and 2017 datasets and the rms package. External model validation was performed with the 2018 & 2019 data using the rms, caret, and pROC packages (Robin et al., 2011; Harrell, 2018; Kuhn et al., 2018).

Silvery salamander health modeling was performed in a manner similar to the box turtles, but model construction was performed using all available data due to the relatively small number of animals classified as "unhealthy". External validation will be pursued using data collected in Phase 2. The definition of health for salamanders was also slightly different than the box turtle definition because clinical pathology testing was not pursued. Apparently healthy silvery salamanders had to meet the following two criteria:

1. **No clinically significant physical examination abnormalities.**
2. **No clinically apparent qPCR detection of infectious disease.**

Estimating Population Size & Population Viability Analysis in Box Turtles: Population size estimates with 95% confidence intervals were produced using the Schumacher-Eschmeyer method in the fishmethods package for both eastern and ornate box turtles (Schumacher and Eschmeyer, 1943).

The stability of each population and its resiliency to perturbance was evaluated as a proxy for population health using population viability analysis (PVA). Individual-based PVA were performed using VORTEX v10 (Lacy, 2018). This program utilizes deterministic and stochastic modeling to produce estimates of population growth rate, extinction rate, and extinction time, enabling the user to examine factors that affect population stability (health).

PVA scenarios were simulated over 100 years with 1000 iterations for three different starting population sizes: 1) expected, 2) optimistic, and 3) pessimistic based on the Schumacher-Eschmeyer estimate and its confidence intervals. Parameter and rate estimates were obtained from the literature (Tables 1 & 2).

Sensitivity testing was performed to examine the effect of removing turtles of different age classes and sexes on each PVA scenario, representing increased losses due to predation, disease, or human collection. The specific scenarios tested included annual removal of 1-5 adult females, annual removal of 1-5 adult males, and annual removal of 1-5 female hatchlings.

Table 1. Parameter estimates and sources for population viability analysis in free-living eastern box turtles.

Parameter		Value	Source
Breeding System		Polygamy	Dodd 2001
Age of 1st Offspring: Female		8	Dodd 1997
Age of 1st Offspring: Male		6	Dodd 1997
Maximum Lifespan		50	Stickel 1978
Maximum # Broods/Year		2	Burke 2011b, Willey 2012, Wilson 2005, Wilson 2008
Maximum # Progeny/Brood		6	Burke 2011b, Willey 2012, Wilson 2005
Sex Ratio at Birth		0.5	
Maximum Age of Reproduction		50	Stickel 1978
% Adult Females Breeding		0.33	Willey 2012, Wilson 2005
Mean # Offspring/Female/Brood (SD)		2 (2)	Burke 2011b, Willey 2012, Wilson 2005
Mortality Rates (SD):	0-1	42% (10)	Burke 2011b, Congello 1978, Dodge 1978, Ewing 1933, Flitz 2006,
	1-2	33% (10)	Willey 2012, Wilson 2005, Forsythe 2004, Heppell 1998,
	2-3	28% (10)	Currylow 2011, Nazdrowicz 2008
	3-4	24% (10)	
	4-5	19% (3)	
	5-6	15% (3)	
	6-7	6% (3)	
	7-8	6% (3)	
	8 and up	6% (3)	
Initial Population Size		Pop-based	Present study
Carrying Capacity		1000 - 5000	Present study

Table 2. Parameter estimates and sources for population viability analysis in free-living ornate box turtles.

Parameter		Value	Source
Breeding System		Polygamy	Dodd 2001
Age of 1st Offspring: Female		10	Legler 1960
Age of 1st Offspring: Male		8	Legler 1960
Maximum Lifespan		37	Christiansen 2004
Maximum # Broods/Year		2	Legler 1960, Vogt 1981, Temple 1987, Doroff 1990, Blair 1976
Maximum # Progeny/Brood		5	Vogt 1981, Temple 1987, Doroff 1990, Blair 1976, Caldwell 1981
Sex Ratio at Birth		0.5	
Maximum Age of Reproduction		37	Henry 2003
% Adult Females Breeding		0.57	Doroff 1990, Redder 2006
Mean # Offspring/Female/Brood (SD)		2 (2)	Doroff 1990
Mortality Rates (SD):	0-1	42% (10)	Burke 2011b, Congello 1978, Dodge 1978, Ewing 1933, Flitz 2006,
	1-2	33% (10)	Willey 2012, Wilson 2005, Forsythe 2004, Heppell 1998,
	2-3	28% (10)	Currylow 2011, Nazdrowicz 2008
	3-4	24% (10)	
	4-5	19% (3)	
	5-6	15% (3)	
	6-7	6% (3)	
	7-8	6% (3)	
	8-9	6% (3)	
	9-10	3% (2)	
	10 and up	3% (2)	Bowen 2004
Initial Population Size		184, 268, 493	Present study
Carrying Capacity		1000	

***Geospatial Analysis**:* Turtle GPS locations were mapped using ArcGIS on the World Imagery background layer and visually examined for demographic clustering (ArcMap 10.4.1, Esri, Redlands, CA 92373). Spatial clustering of poor BCS and extreme clinical pathology values was evaluated using Gettis G Ord hotspot analysis. The Bernoulli model was used to test for statistically significant spatial clusters of physical examination abnormalities and pathogens using SaTScan v9.4.4 (Kulldorff 1997). Output files from SaTScan were uploaded into ArcGIS to visualize the extent of significant clusters.

Mortality Events: Specimens and environmental variables were collected during herptile mortality events reported to the IDNR. Necropsy was performed on suitable carcasses and samples were collected for disease diagnostics. Existing conventional PCR assays were employed to facilitate comparison of ranavirus sequences between species, sites, and years. These included consensus assays targeting the major capsid protein (MCP, primers 1+2, 3+4, 5+6), the viral homolog of the alpha subunit of eukaryotic initiation factor 2 (vIF-2α), and the DNA polymerase gene (DNApol) (Hyatt et al., 2000, Holopainen et al., 2009, Stöhr et al., 2015). Products were resolved on a 1% agarose gel and samples with appropriately-sized bands (DNApol = 560bp, vIF-2 α = 250 or 1050bp, and MCP = approximately 500bp) were treated with ExoSAP-IT (USB Corporation, 26111 Miles Road, Cleveland, OH, USA), and commercially sequenced in both directions. Sequences were trimmed of primers and low-quality base-pair calls in Geneious v.11.1.3 (Kearse et al., 2012), the full MCP sequence was constructed from the three different MCP primer-pairs, and all products were compared to known sequences in the NCBI GenBank database using BLASTN (Benson et al., 2008).

Samples of liver, spleen, and kidney from frozen silvery salamander larvae were pooled for virus isolation, finely diced with a scalpel blade, immersed in 10mL Minimum Essential Medium (MEM, Thermo Fisher Scientific, Waltham, MA, USA) with 5μg/mL amphotericin B, 200U/mL penicillin, 200μg/mL streptomycin, and 100μg/mL gentamycin (Sigma Chemical Co. St. Louis, MO 61378), and homogenized using a Polytron homogenizer (Type P10/35 with power control unit (PCU-2-110), Brinkmann Instruments, Westbury, NY 11590, USA) with a sterilized saw-toothed rotor/stator at 6,000rpm for 60 seconds on ice. Each homogenized sample was centrifuged at 2000rpm for 20 minutes at 4ºC. The supernatant was passed through a 0.45μM filter and inoculated onto Terrapene heart cells (TH-1), fathead minnow epithelial cells (FHM), and viper heart cells (VH) grown to 80% confluence in 75 cm^3 flasks. Following a 10-minute incubation at 27ºC, 20mL of Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 100U/mL penicillin, 100μg/mL streptomycin, and 2.5μg/mL amphotericin B was added to each flask, and cells were maintained at 27ºC with 5% CO2. Blind passaging was performed after inoculated cells reached 100% confluency up to three times. Flasks were frozen at -80ºC, thawed, and vortexed three times. One milliliter of the flask contents was used to inoculate a new flask containing TH-1 cells grown to 80% confluency. Infected flasks were monitored daily for signs of cytopathic effects (CPE).

OBJECTIVE 2C/3B

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 from this project have also been performed for Vermilion County Conservation District staff in April 2017, Illinois DNR Heritage meeting in May 2017, Nachusa Grasslands in October 2017, Chicago Wilderness Meeting in March 2018, Nachusa Grasslands in October 2018, PIJAC health forum in November 2018, Illinois Association of Conservation Districts in February 2019, and Champaign county Naturalist program in November 2019. Scientific presentations given based on data from this project are listed above.

RESULTS

Eastern Box Turtles

Seven hundred eastern box turtle health assessments were performed at five study sites between 2016 and 2019 (Figure 2). Demographic data, physical examination abnormalities, and pathogen presence is summarized by study site in Table 3 and Figures 3 & 4. Co-pathogens were detected less frequently than single pathogens. The most common co-detected pathogens were TerHV1 and adenovirus (N = 15) closely followed by *Mycoplasma* sp. and adenovirus (N = 14), then TerHV1 and *Mycoplasma* sp. (N = 2), *S. typhimurium* and adenovirus (N = 1) and *S. typhimurium* and *Mycoplasma* sp. (N = 1). Twenty-one deceased turtles were collected over the last four years, all tested negative for FV3. Clinical pathology data are presented in Table 4.

Habitat use differed by study site, sex, and age class. Turtles at Forbes were sampled more frequently in forests than fields and edges compared to Collison ($p = 0.0005$, $p = 0.004$), Kennekuk ($p = 0.0001$, $p = 0.0001$), and Forest Glen ($p = 0.0064$, $p = 0.0003$). Turtles at Kennekuk were sampled more frequently in fields and edges than forests compared to Collison ($p = 0.008$, $p = 0.003$), Forest Glen ($p = 0.0004$, $p = 0.0017$), and Kickapoo ($p = 0.0001$, $p = 0.0003$). Adult turtles were sampled more frequently in forest habitat ($p = 0.004$) compared to juveniles, and juveniles occupied field habitats more frequently than adults ($p = 0.0005$). Male turtles were sampled more frequently in forest habitats than females ($p < 0.0001$) and females were sampled more frequently in fields than males ($p < 0.0001$).

The occurrence of physical examination abnormalities differed by study site and age class. Specifically, the odds of asymmetrical nares were 2.9 times higher at Collison compared to Forbes (95% CI = 1.45 – 5.82, $p = 0.02$) and 3.3 times higher in adult turtles than juveniles (95% CI = 1.3 – 8.9, $p = 0.02$). The odds of active shell lesions were 6.8 times higher in turtles from Collison compared to Forest Glen (95% CI = 1.8 – 26.3, $p = 0.003$). The odds of any physical exam abnormality were 3.3 times higher in adult turtles compared to juveniles (95% CI = 2.1 – 5.3, $p < 0.0001$).

Pathogen prevalence differed by study site, season, age class, and the presence of physical exam abnormalities. The odds of TerHV1 detection were 4.4 times higher at Forbes compared to Kennekuk (95% CI = 1.7 – 11.5, $p = 0.03$). The odds of adenovirus detection were 3.1 times higher at Forbes (95% CI = 1.2 – 8, $p = 0.02$) and 3.3 times higher at Kennekuk (95% CI = 1.2 – 9.2, $p = 0.02$) compared to Collison. Finally, the odds of *Mycoplasma* sp. were 5.5 and 4.7 times higher at Kennekuk compared to Collison (95% CI = 1.2 – 24.5, $p = 0.02$) and Kickapoo (95% CI = 1.7 – 12.3, $p = 0.007$), respectively. TerHV1 detection was significantly higher in the summer compared to spring (OR = 13, 95% CI = 3 – 57, $p = 0.002$) and fall (OR = 36, 95% CI = 6 – 217, $p = 0.0003$). Adenovirus detection was more common in spring (OR = 4.6, 95% CI = 1.5 – 12, $p = 0.01$) and summer (OR = 4.2, 95% CI = 1.1 – 21.1, $p = 0.04$) compared to fall. Adenovirus was also significantly more prevalent in juveniles than adults (OR = 2.8, 95% CI = 1.6 – 4.8, $p = 0.0002$). *Mycoplasma* sp. detection was significantly associated with nasal discharge (OR = 5.8, 95% CI = 1.8 – 18.7, $p = 0.003$), blepharoedema (OR = 4, 95% CI = 1.1 – 14.3, $p = 0.03$), oral plaques (OR = 5.1, 95% CI = 1.7 – 15, $p = 0.009$), and the presence of any clinical sign of upper respiratory disease (OR = 3.3, 95% CI = 1.2 – 9, $p = 0.02$).

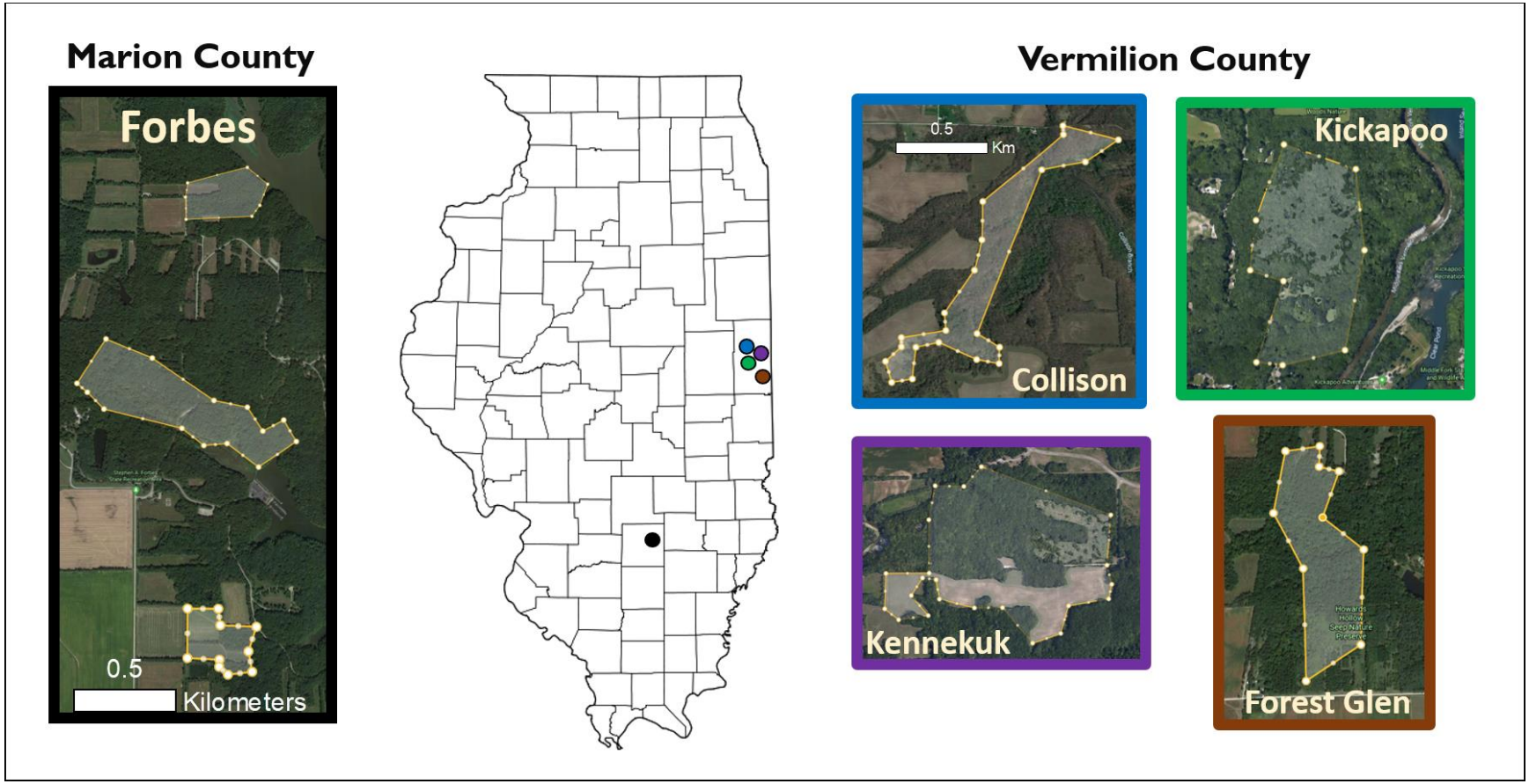

Figure 2. Eastern box turtle study sites in Illinois. Specific search areas are outlined in yellow.

Table 3. 2019 Demographics, physical exam abnormalities, and pathogen presence in eastern box turtles by study site.

		Collison	Kennekuk	Forest Glen	Kickapoo	Forbes
Year	**2016**	29	25	28	20	137
	2017	11	35	24	7	61
	2018	31	45	58	23	54
	2019	2	32	16	10	52
Season	**Spring**	32	82	70	28	249
	Summer	31	50	48	25	0
	Fall	10	5	8	7	55
Age Class	**Adult**	64	107	109	50	258
	Juvenile	9	30	17	10	46
Sex	**Female**	42	66	51	23	122
	Male	22	46	62	28	151
	Unknown	9	25	13	9	31
Physical Exam	**Asymmetrical Nares**	15 (21%)	19 (7%)	9 (7%)	7 (12%)	25 (8%)
	Upper Respiratory Disease	0	9 (7%)	6 (4%)	5 (8%)	10 (3%)
	Aural Abscess	3 (4%)	5 (4%)	1 (5%)	1 (2%)	11 (4%)
	Head, Limb, or Tail Trauma	4 (5%)	9 (7%)	12 (10%)	7 (12%)	20 (7%)
	Burn Injury	0	6 (4%)	1 (5%)	0	11 (4%)
	Developmental Abnormality	5 (7%)	9 (7%)	10 (8%)	5 (8%)	16 (5%)
	Active Shell Lesion	10 (14%)	13 (9%)	3 (2%)	2 (4%)	20 (7%)
	Inactive Shell Lesion	25 (34%)	45 (33%)	45 (36%)	18 (30%)	80 (26%)
	# Turtles Tested	67	118	103	58	243
Pathogens	***Terrapene* adenovirus**	5 (8%)	25 (21%)	18 (17%)	7 (12%)	48 (20%)
	***Terrapene* herpesvirus 1**	5 (8%)	4 (3%)	11 (11%)	0	33 (14%)
	***Mycoplasma* sp.**	2 (3%)	17 (14%)	4 (4%)	2 (3%)	15 (6%)
	Salmonella typhimurium	0	1 (0.8%)	1 (1%)	1 (2%)	0
Total		73	137	126	60	304

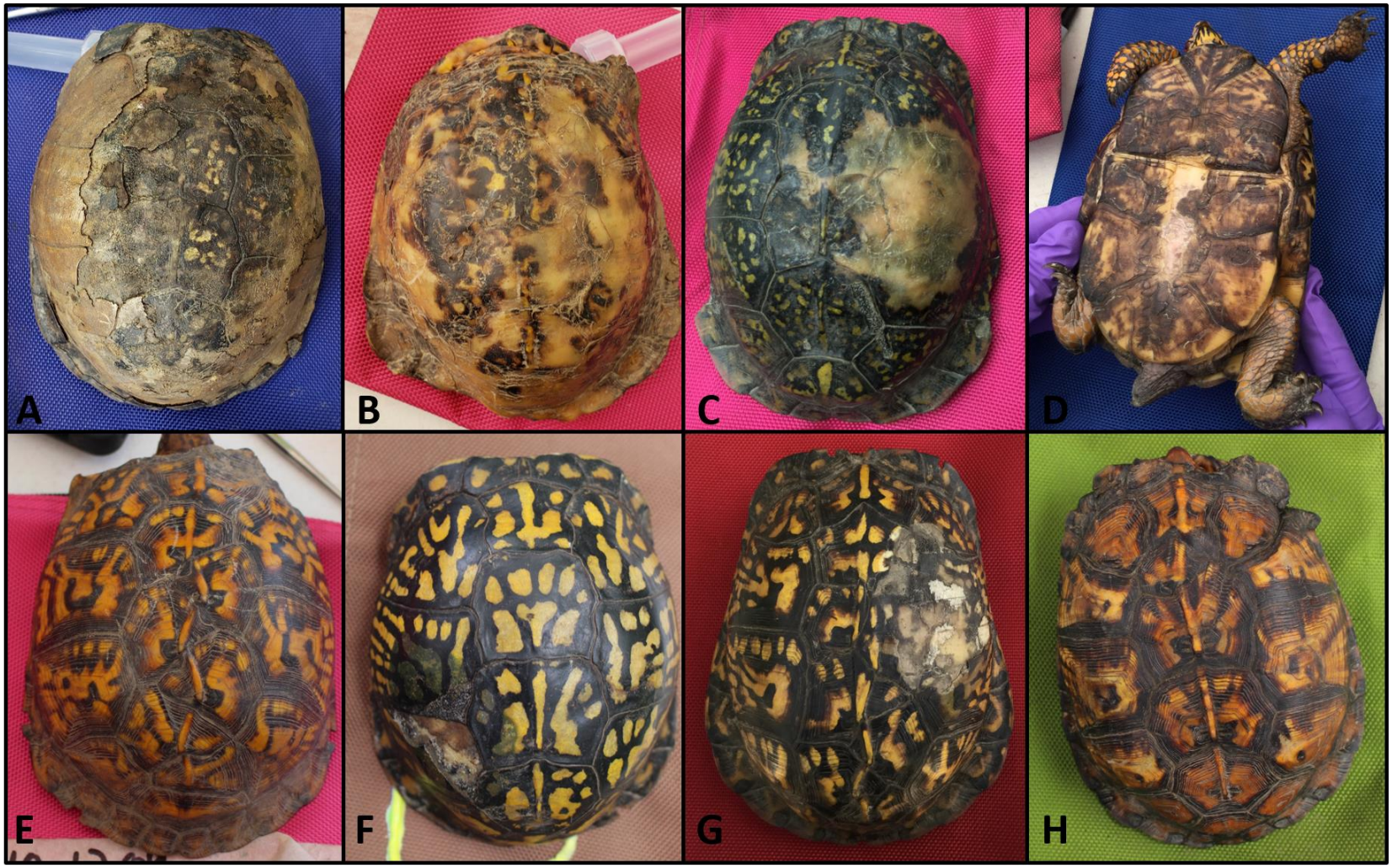

Figure 3. Common eastern box turtle shell abnormalities. A – D) Burn injuries in various stages of healing. E) Developmental abnormality – supranumerary vertebral scutes. F & G) Active shell lesions characterized by moist, soft foci. Turtle G also has supranumerary vertebral scutes. H) Inactive shell lesion – healed fracture of the 1st right costal scute. This individual also has supranumerary vertebral scutes.

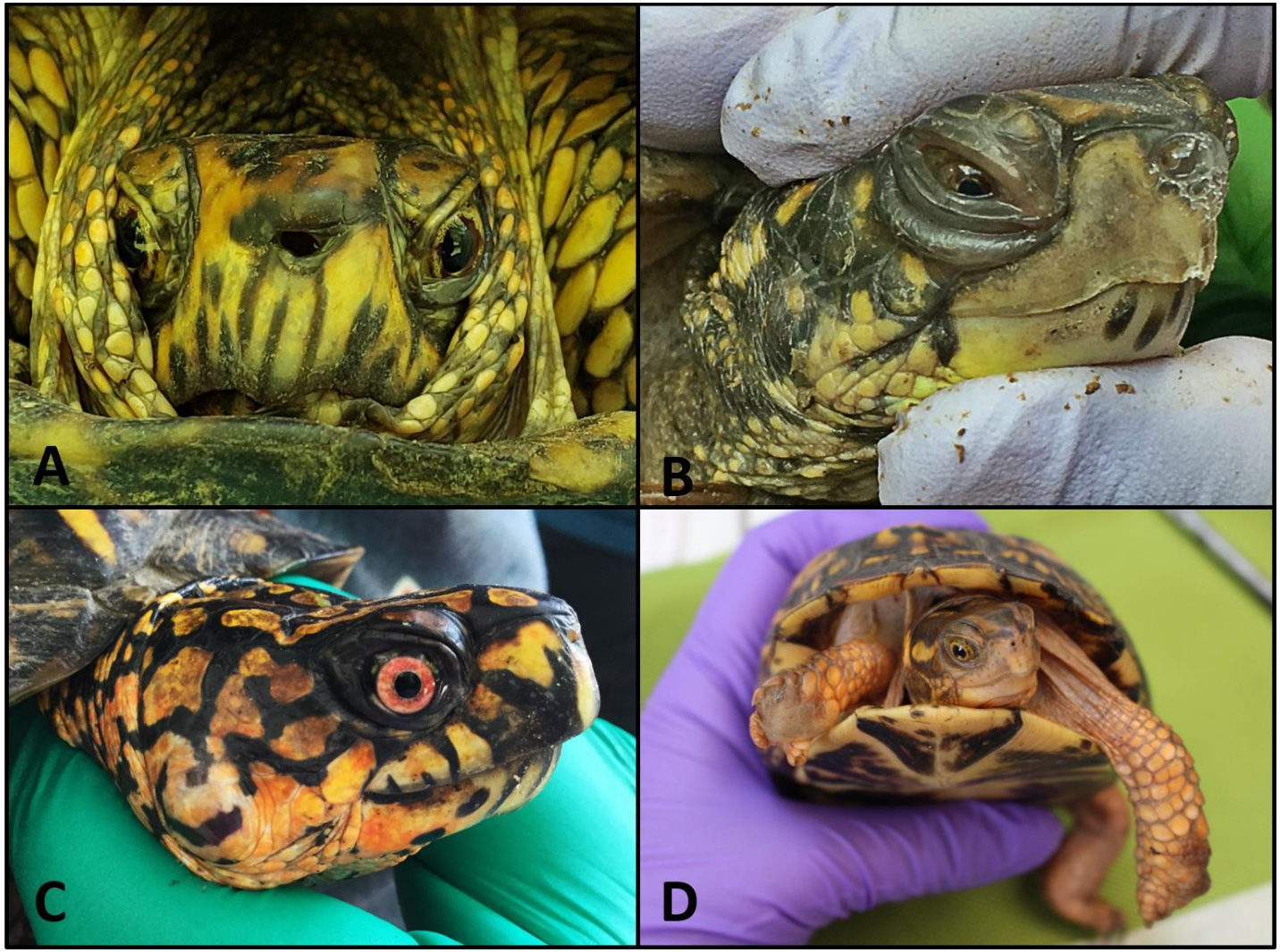

Figure 4. Common physical examination abnormalities in eastern box turtles. A & B) Clinical signs of upper respiratory disease including nasal asymmetry or erosion (A), blepharoedema (B), and nasal discharge (B). C) Aural abscess. D) Trauma – this individual previously lost both front feet.

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

Analyte	N	Distribution	Central Tendency	Dispersion	Min	Max
Packed Cell Volume (%)	660	Non-normal	24	15 - 32	5	48.5
Total Solids (g/dL)	660	Non-normal	6.65	5 - 8.5	2.2	12
Total Leukocyte Count (/μL)	660	Non-normal	19,515	10,213 - 34,366	920	168,080
Heterophils (μL)	660	Non-normal	2,799	1,056 - 5,906	184	24,334
Lymphocytes (μL)	660	Non-normal	10,724	4,770 - 22,688	259	157,995
Monocytes (μL)	660	Non-normal	384	0 - 1,201	0	4,259
Eosinophils (μL)	660	Non-normal	2,378	912 - 5,145	0	17,600
Basophils (μL)	660	Non-normal	1,436	385 - 3,787	0	16,520
Heterophil / Lymphocyte	660	Non-normal	0.255	0.089 - 0.715	0.011	12
Glutamate Dehydrogenase (U/L)	255	Non-normal	11.3	4 - 43	1.5	690
Bile Acids (μmol/L)	571	Non-normal	6.3	2.8 - 15	1	71.3
Uric Acid (mg/dL)	563	Non-normal	1.4	0.9 - 2	0.8	5
Calcium (mg/dL)	579	Non-normal	11.1	8.5 - 18.1	2.5	37.3
Phosphorous (mg/dL)	579	Non-normal	3.7	2.4 - 5.3	1.4	10.5
Calcium / Phosphorous	579	Non-normal	3.33	2.32 - 4.43	1.2	6.44
Aspartate Aminotransferase (U/L)	579	Non-normal	69	39 - 138	16	1230
Creatine Kinase (U/L)	576	Non-normal	313	114 - 807	7	30,600
Total Protein (g/dL)	307	Non-normal	4.4	2 - 7.2	0.2	10.1
Prealbumin (g/dL)	307	Non-normal	0.07	0 - 0.23	0	0.43
Albumin (g/dL)	307	Non-normal	0.99	0.42 - 1.74	0.04	2.61
Alpha 1 Globulins (g/dL)	307	Non-normal	0.34	0.17 - 0.6	0.02	0.83
Alpha 2 Globulins (g/dL)	307	Non-normal	1.09	0.48 - 1.97	0.06	3.27
Beta Globulins (g/dL)	307	Non-normal	1.54	0.75 - 2.61	0.07	4.78
Gamma Globulins (g/dL)	307	Non-normal	0.4	0.17 - 0.66	0.01	1
Albumin / Globulin	307	Normal	0.328	0.085	0.13	0.56

Clinical pathology parameters differed by year, season, study site, sex, age class, and the presence of physical exam abnormalities. All parameters except PCV, total leukocyte count (WBC), lymphocyte count, the calcium to phosphorous ratio (Ca:P), creatine kinase (CK), and relative albumin significantly varied by year ($p < 0.05$). Seasonal differences are summarized in Figure 5. Generally, heterophils, monocytes, H:L, and Ca:P were lowest in summer, PCV was highest in summer, eosinophils, bile acids, and calcium rose throughout the active season, and relative albumin and A:G were higher in summer than spring. Turtles from Forbes had the lowest heterophil counts, heterophil to lymphocyte ratios (H:L), and relative beta globulins and the highest relative albumin and albumin to globulin ratio (A:G) compared to all other study sites ($p < 0.0001$, Figure 6). Turtles from Kickapoo had the highest TS ($p < 0.0001$, Figure 6). Male turtles had higher PCV, relative albumin, relative alpha 2 globulins, relative gamma globulins, and A:G, while females had higher calcium, phosphorous, and beta globulins (Figure 7). Adult turtles had higher calcium, Ca:P, and beta globulins while juveniles had higher lymphocyte counts, relative albumin, relative alpha 2 globulins, and A:G (Figure 8). The H:L was higher in turtles with abnormal physical exam findings (effect size = 0.04, 95% CI = 0.005 – 0.07, $p = 0.02$) and in turtles with aural abscesses compared to turtles with normal examinations (effect size = 0.12, 95% CI = 0.02 – 0.2, $p = 0.03$). Relative albumin concentrations were higher in turtles with normal examinations vs. those with asymmetrical nares (effect size = 1.4%, 95% CI = 0.05 – 2.7%, $p = 0.04$) and soft tissue injuries (effect size = 5.3%, 95% CI = 2.2 – 8.4%, $p = 0.001$). Relative beta globulins were lower in turtles with abnormal physical exam findings (effect size = 1.8%, 95% CI = 0.39 – 3.2%, $p = 0.01$), asymmetrical nares (effect size = 2.1%, 95% CI = 0.04 – 4.1%, $p = 0.04$), and soft tissue injuries (effect size = 6.4%, 95% CI = 1.7 – 11.1%, $p = 0.008$) compared to animals with no physical exam abnormalities.

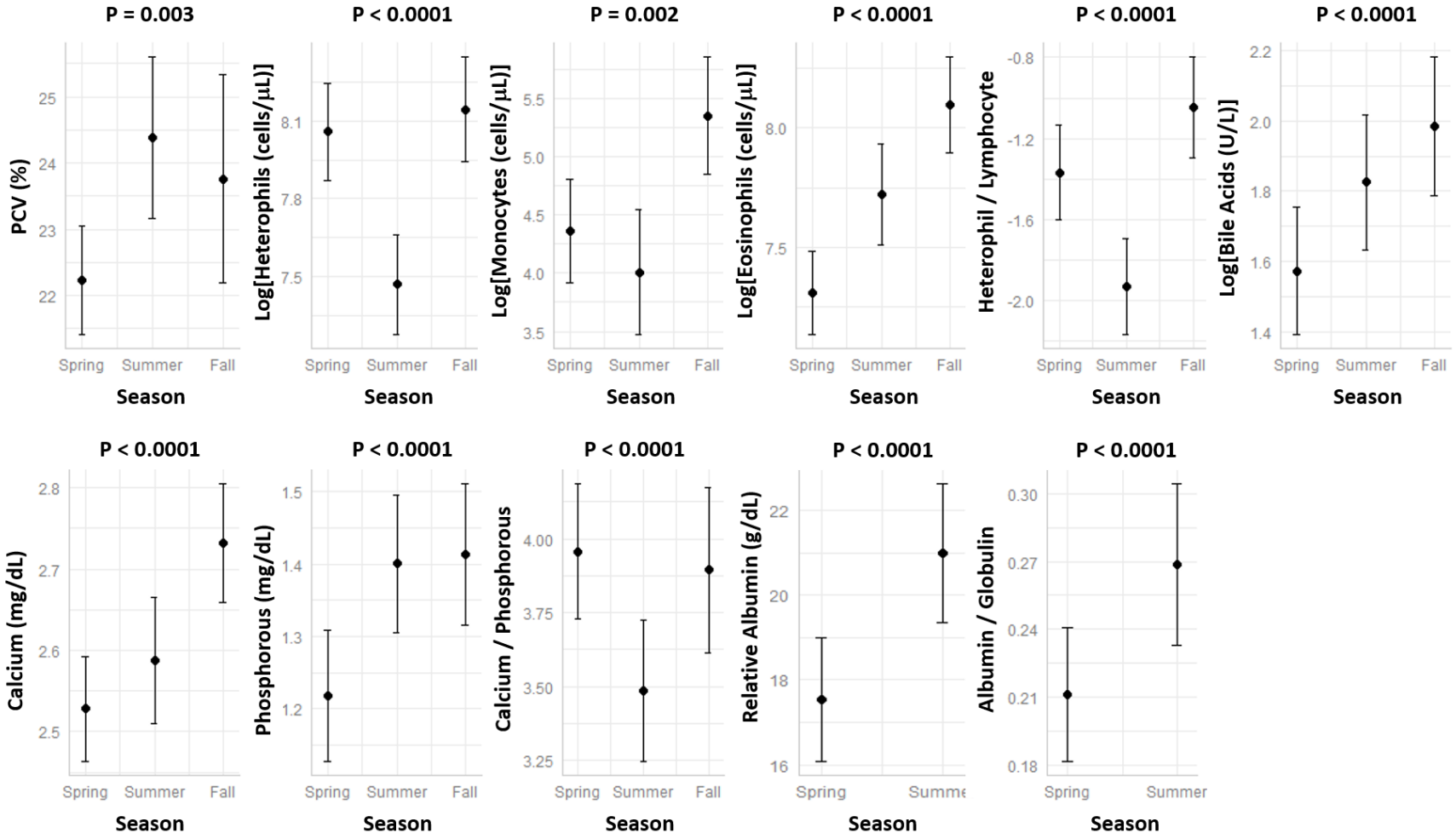

Figure 5. Statistically significant differences in clinical pathology parameters by season for eastern box turtles 2016 – 2019. Model estimates and 95% confidence intervals are presented.

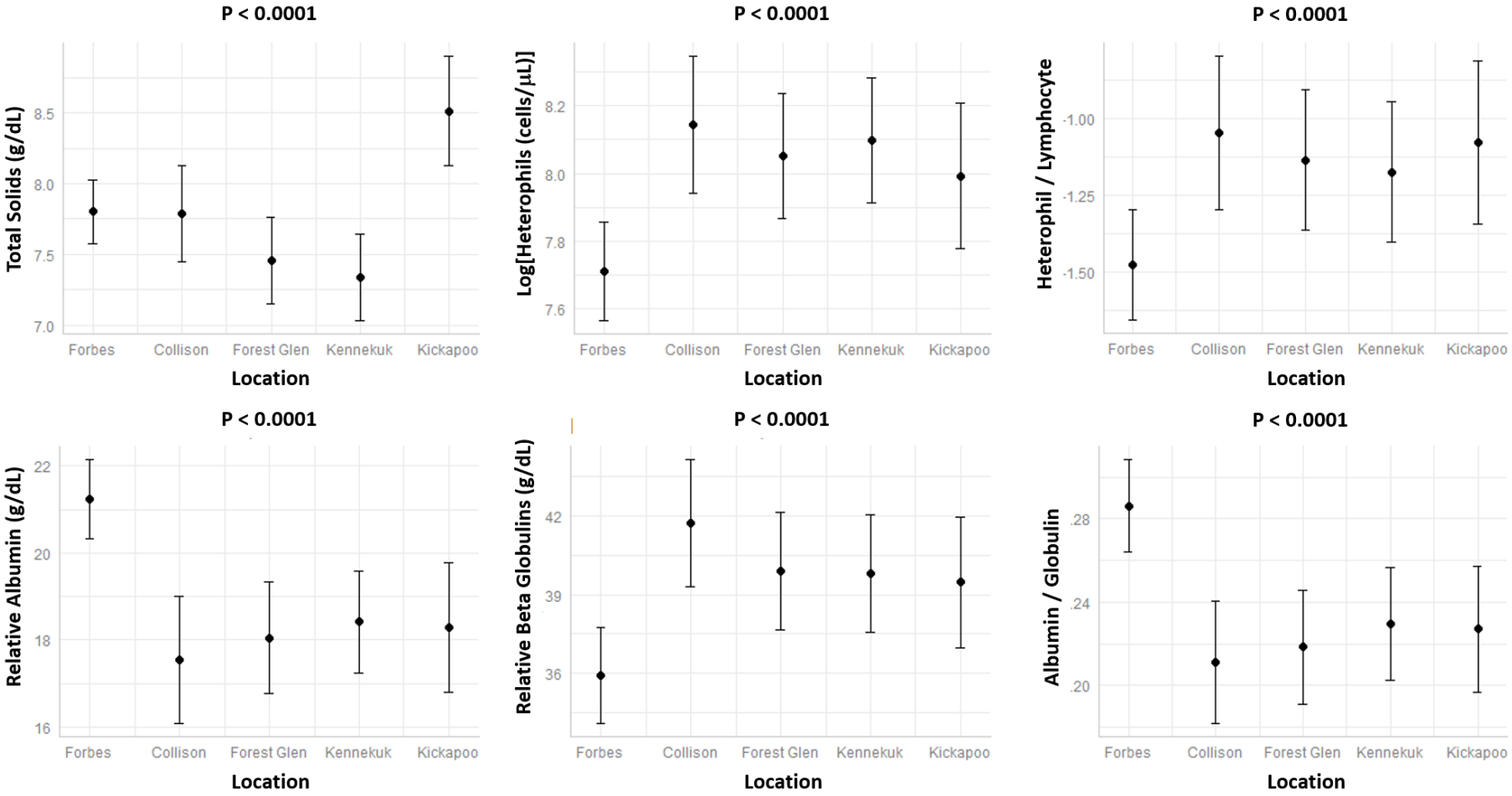

Figure 6. Statistically significant differences in clinical pathology parameters by study site for eastern box turtles 2016 – 2019. Model estimates and 95% confidence intervals are presented.

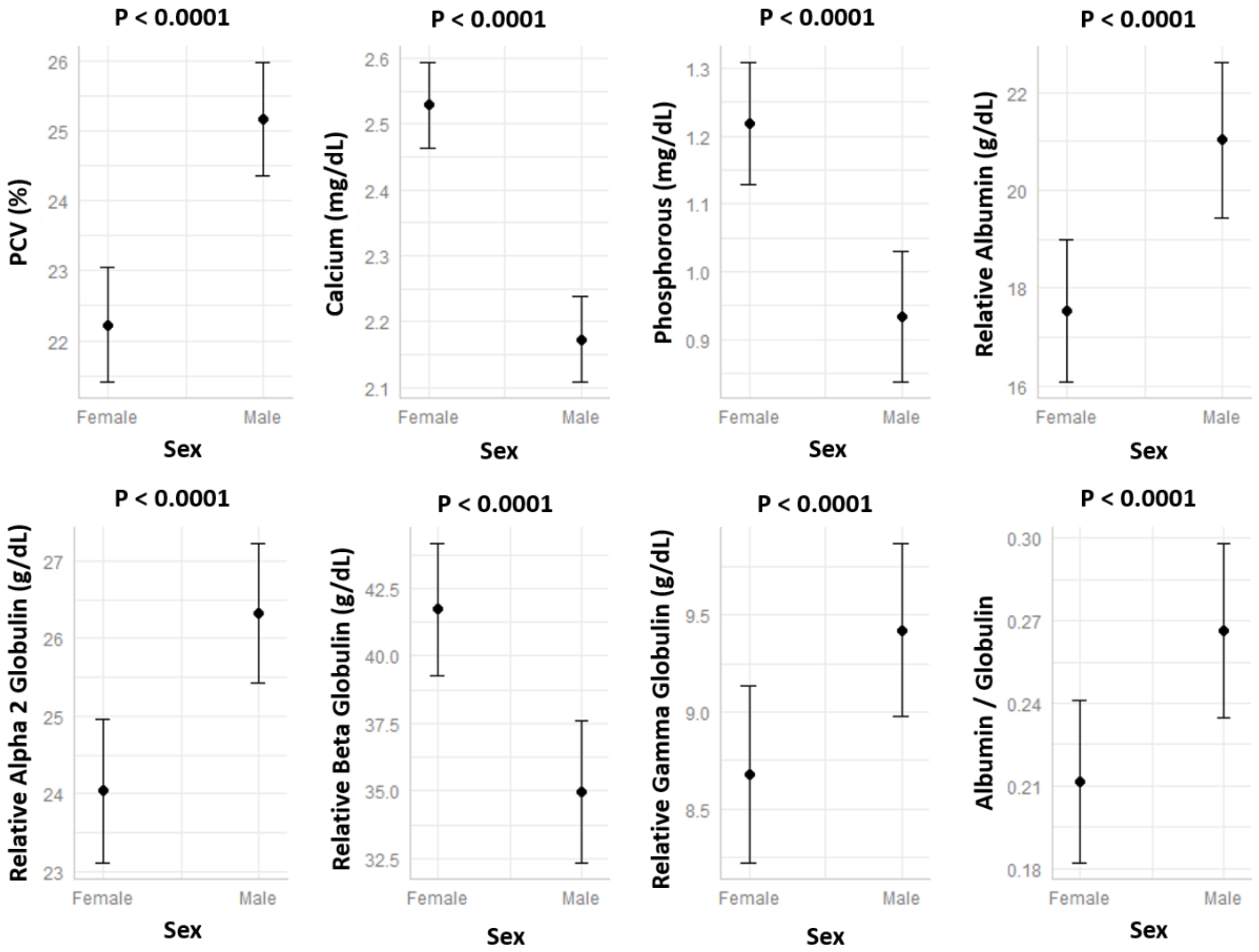

Figure 7. Statistically significant differences in clinical pathology parameters by sex for eastern box turtles 2016 – 2019. Model estimates and 95% confidence intervals are presented.

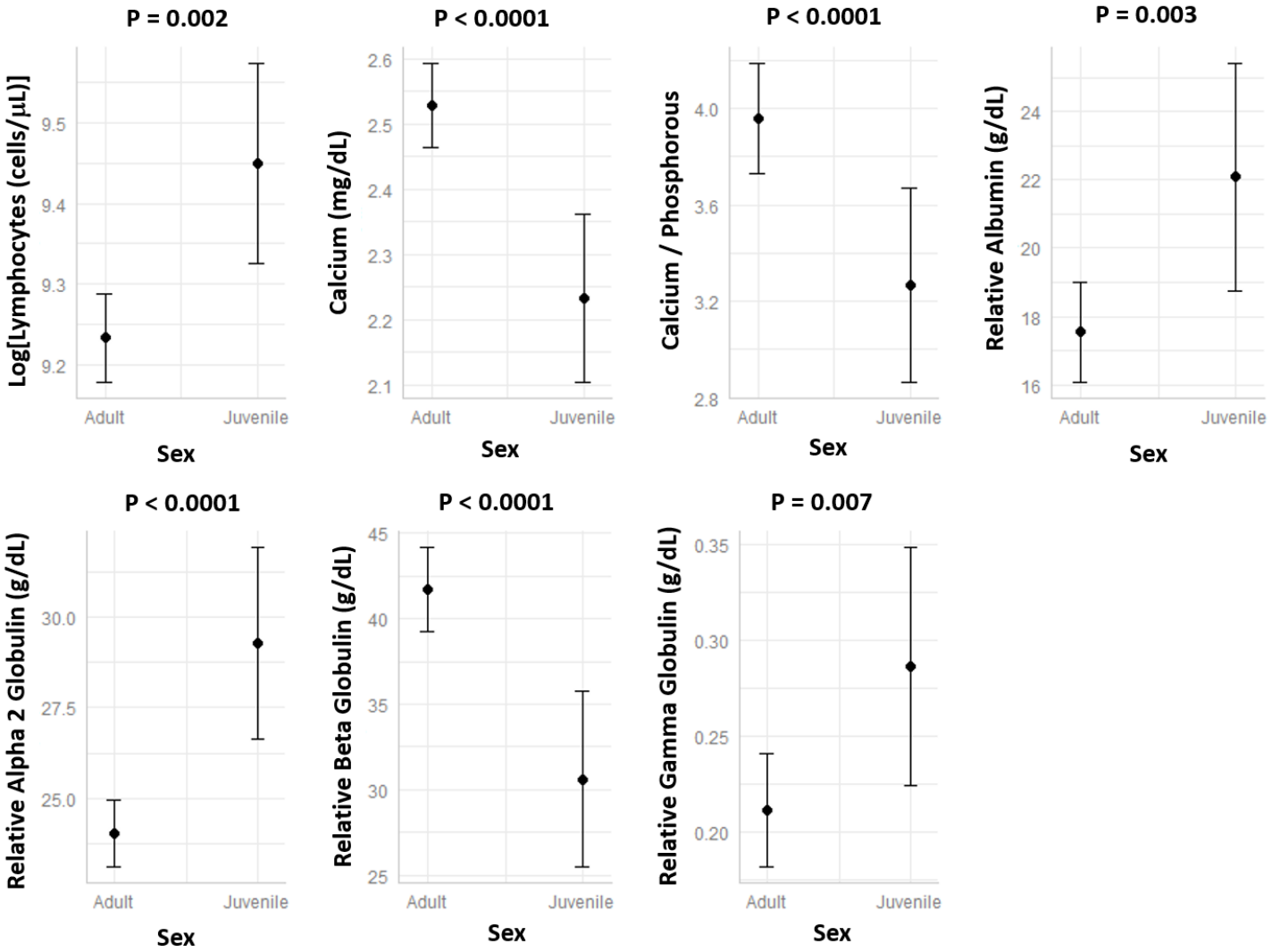

Figure 8. Statistically significant differences in clinical pathology parameters by age class for eastern box turtles 2016 – 2019. Model estimates and 95% confidence intervals are presented.

Individual Health Model

Prior to generating the health model, clinical pathology parameters for each turtle were converted to the absolute value of the distance from year, age class, and sex-specific median values. This was done for two reasons: 1) to capture the relationship between poor health and extreme clinical pathology values at both the low and high end of the reference interval, without introducing non-linear terms, and 2) to control for the effects of potential confounders and reduce the total number of variables to include in the final health model. Resulting median-based values were highly right-skewed, and were box-cox transformed to better support modeling assumptions.

The following predictors were associated with health status at a liberal alpha value of 0.15: WBC, relative heterophil count, absolute heterophil count, relative lymphocyte count, absolute lymphocyte count, absolute basophil count, H:L, relative alpha 2 globulins, absolute beta globulins, shell classification (Shell; AL, IL, WNL), tympanum classification (WNL vs. Aural Abscess), nares classification (WNL vs. Asymmetrical), presence of upper respiratory disease (URD) signs (Yes vs. No), and classification of appendages (WNL vs. Missing Digits vs. Missing Feet/Limbs). H:L was used to represent information for all heterophil and lymphocyte measures in the final health model.

Relative alpha 2 globulins and beta globulins were available for significantly fewer turtles (N = 75) than the rest of the predictor variables (N = 339). Case-wise deletion was performed to evaluate the contribution of these variables and a set of candidate models was ranked using AIC. The top-performing model in the reduced dataset did not contain relative alpha 2 globulins or beta globulins (data not shown), so further model ranking was performed using the full dataset without these variables.

The most parsimonious health model contained the additive effects of *WBC, H:L, Shell, and URD*, though there was a high degree of model selection uncertainty associated with omission of several other physical examination variables (Table 5). Internal and external validation were performed for the top three models separately and averaged (data not shown), and predictive capability of the model omitting Tympanum, Nares, and Appendages was superior, therefore this model was selected for full description. The top model including each of its parameter estimates and p-values is reported in Table 6, model predictions are displayed in Figure 9, and performance metrics produced by internal and external validation are reported in Table 7. External validation revealed a tendency to over-identify “unhealthy” animals, represented by a slight decrease in specificity, however, overall model performance remained acceptable with an accuracy of 90% and an AUC of 0.87.

Table 5. Model selection parameters for generalized linear models predicting health status in free-living eastern box turtles. H:L = heterophil : lymphocyte, WBC = total leukocyte count, URD = presence of upper respiratory disease clinical signs.

Model	N	K	AIC_c	ΔAIC_c	w_i
WBC + H:L + Shell + URD	339	6	165.9	0	37
WBC + H:L + Shell + URD + Tympanum + Nares + Appendages	339	10	166.4	0.43	29.8
WBC + H:L + Shell + URD + Nares	339	7	167	1.11	21.3
WBC + H:L + Shell + URD + Tympanum + Nares	339	8	168.3	2.42	11.1
WBC + H:L + Shell + URD + Tympanum + Nares + Appendages + Location	339	14	174.1	8.2	0.6
H:L + Shell + URD	339	5	175.8	9.87	0.3
Shell + URD + Tympanum + Nares + Appendages + Location	339	8	186.7	20.76	0
WBC + H:L + Shell	339	5	232.8	66.86	0
WBC + H:L + URD	339	4	251.4	85.47	0
WBC + H:L	339	3	312.2	146.31	0
Null	339	1	321.6	155.63	0

Table 6. Parameter estimates for the most parsimonious model predicting health status in free-living eastern box turtles (*Terrapene carolina carolina*). WNL = within normal limits, IL = inactive lesion, H:L = heterophil : lymphocyte, WBC = total leukocyte count, URD = presence of upper respiratory disease clinical signs.

Healthy = Shell + URD + WBC + H:L				
	β	**SE**	**Z value**	**p-value**
Intercept	3.96	1.93	2.05	0.04
Shell: AL	7.63	1.74	4.38	< 0.0001
Shell: IL	0.69	1.74	1.63	0.104
URD: Yes	6.6	1.65	4.01	< 0.0001
WBC	0.105	0.032	3.26	0.001
H:L	0.914	0.258	3.54	0.0004

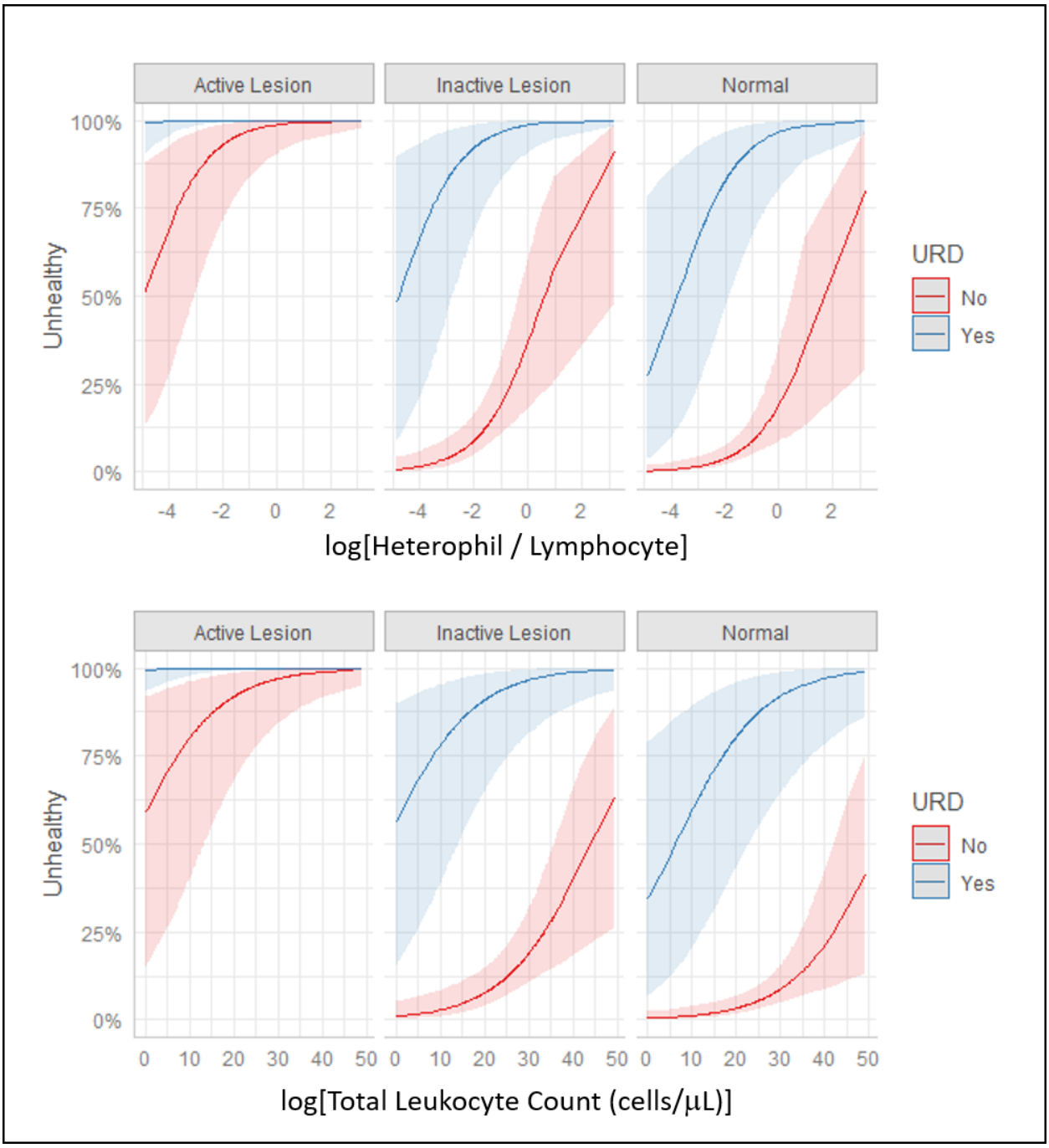

Figure 9. Predictions with 95% confidence intervals for individual health status in eastern box turtles.

Table 7. Model fit metrics from internal and external validation of the most parsimonious model predicting health status in free-living eastern box turtles.

Metric	Internal	External	Scale	Ideal
Brier Score	0.062[a]	0.082	0 - 1	Close to 0
AUC	0.889	0.869	0.5 - 1	Close to 1
Accuracy (%)	93.2	90.3	0 - 1	Close to 1
Sensitivity	1	1	0 - 1	Close to 1
Specificity	0.62	0.57	0 - 1	Close to 1
Somer's Delta	0.754[a]	0.738	-1 - 1	Close to -1 or 1

[a] Optimism-corrected values based on internal validation via bootstrapping

Spatial Epidemiology

Significant spatial clusters were not identified for any parameter tested, indicating that pathogens and poor health indices were distributed homogeneously within the population. Turtles did not cluster by age class or sex, at least at the spatiotemporal scale evaluated in this study.

Population Health

The Schumacher-Eschmeyer population size estimates from smallest to largest were Kickapoo: 161 (120 – 246), Forest Glen: 257 (220 – 308), Collison: 353 (242 – 647), Kennekuk: 521 (347 – 926), and Forbes: 1037 (758 – 1555). Using vital parameter estimates from the literature all sampled populations tended to be stable over the simulated 100-year period. Baseline extinction probability was zero for all sites except Kickapoo, where the extinction probability was 0.2%. Sensitivity testing revealed that annual removal of adult females impacted every population, but the effect was dependent upon population starting size (Figures 10 & 11). Kickapoo was the most affected, with removal of one adult female per year leading to a high probability of population extinction within the next century. Forest Glen and Collison could tolerate loss of two females per year and Kennekuk could lose three before the probability of extinction rose above 90%. Forbes could lose up to 5 females per year with an 83% probability of extinction, revealing an overall superior resiliency to disturbance in this population compared to the Vermilion County sites.

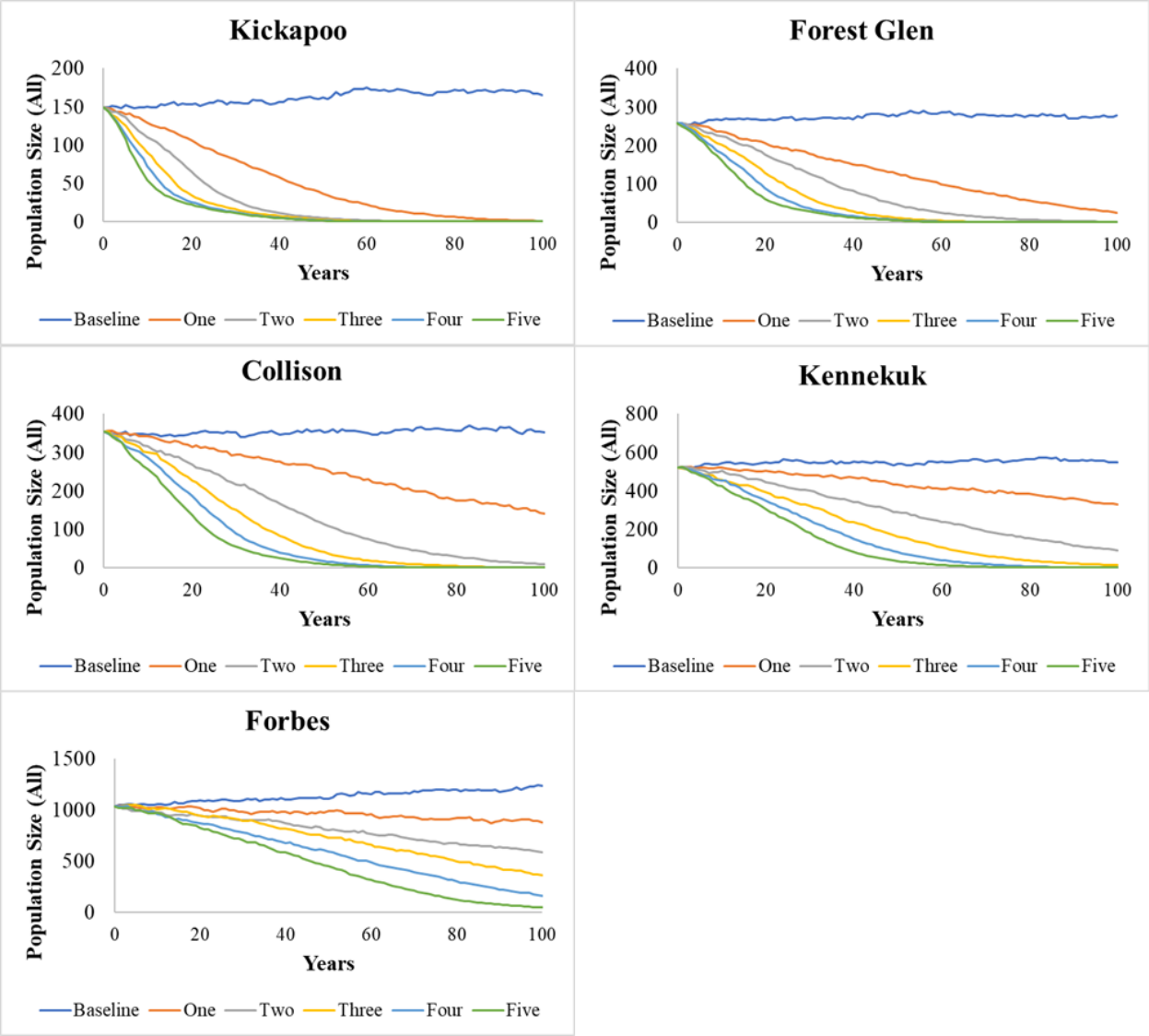

Figure 10. Simulated mean population size over time in populations of eastern box turtles experiencing different levels of adult female removal each year (0 – 5 turtles/year).

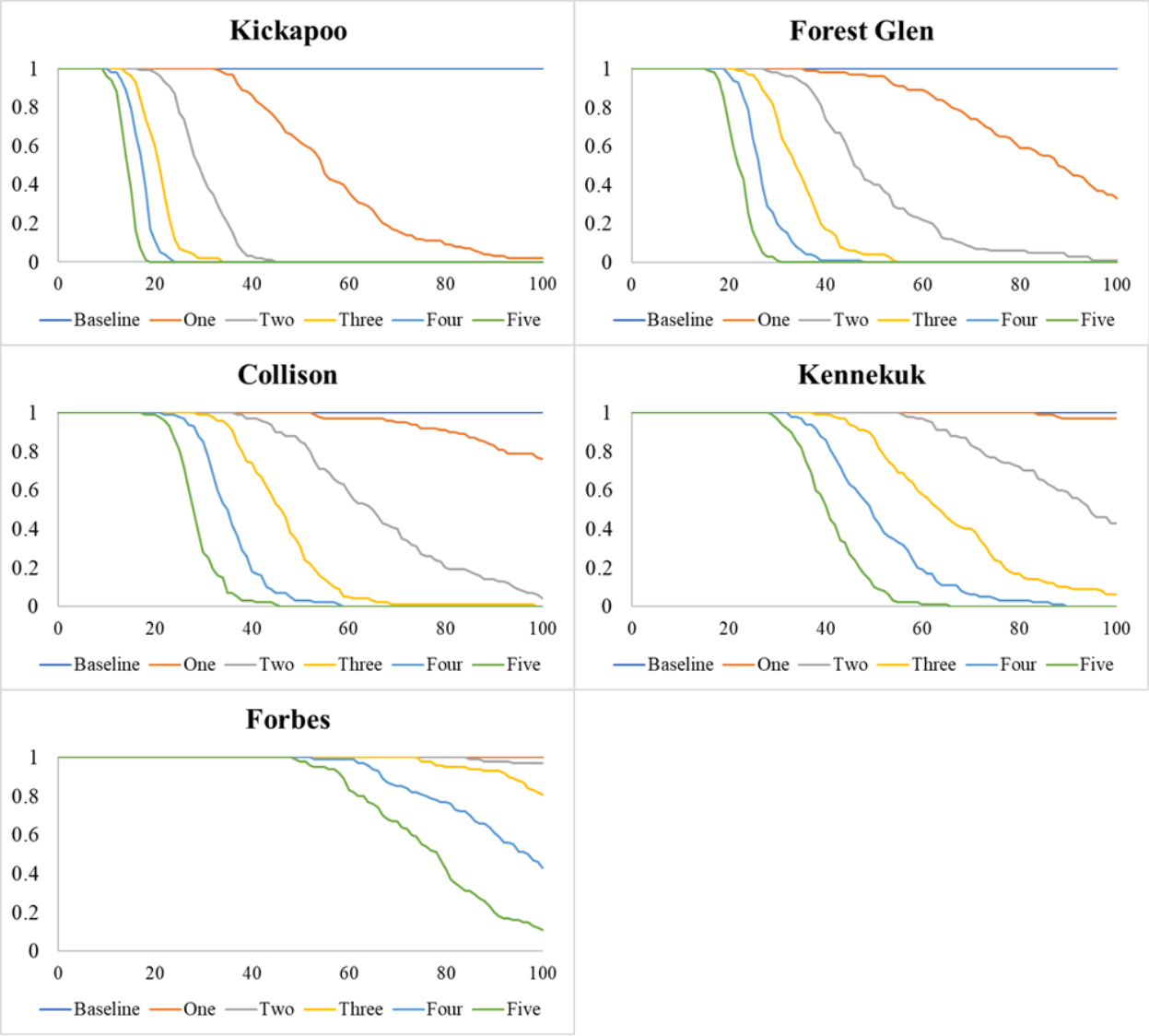

Figure 11. Simulated survival probability over time in populations of eastern box turtles experiencing different levels of adult female removal each year (0 – 5 turtles/year).

Ornate Box Turtles

Two hundred ninety-two ornate box turtle health assessments were performed at the Nachusa Grasslands between 2016 and 2019 (Figure 12). Demographic data, physical examination abnormalities, and pathogen presence is summarized in Table 8 and Figure 13. A male bias was observed at the Orland Track in 2016 and 2017 (32% female in 2016, $p = 0.001$; 33% female in 2017, $p = 0.003$) and juvenile turtles were found each year, reflecting active recruitment. Eight deceased turtles were collected over the last four years, all tested negative for FV3. Clinical pathology data are summarized in Table 9.

Microhabitat use varied by age class and sex; specifically, juvenile turtles were found in soil more frequently compared to adults ($p = 0.007$) and adult males were found in brambles more often than females ($p = 0.002$). The prevalences of TerHV1 ($p < 0.0001$) and adenovirus ($p < 0.0001$) were significantly higher in 2016 than in any other year. Juvenile turtles were significantly more likely to test positive for TerHV1 than adults (OR = 7.1, 95% CI = 2.1 - 24, $p = 0.002$) and males were more likely to test positive for adenovirus than females (OR = 3.5, 95% CI = 1.03 – 11.6, $p = 0.04$), however, pathogens were not associated with clinical signs of illness.

Physical examination findings were generally similar between years and sites. Shell lesions were the most frequent and striking physical exam abnormality with a prevalence from 50 – 60% each year. The prevalence of these lesions was significantly higher in adult turtles than juveniles (OR = 9.1, 95% CI = 3.4 – 24.4, $p < 0.0001$). Turtles with active shell lesions had higher eosinophil counts, H:L, and creatine kinase and lower relative albumin and A:G than turtles with normal shells (Figure 14). Turtles with inactive shell lesions had lower relative albumin and A:G than turtles with normal shells (Figure 14). Male turtles had higher PCV, glutamate dehydrogenase (GLDH), uric acid (UA), AST, relative albumin, relative alpha 1 globulins, and A:G, while female turtles had higher calcium, phosphorous, Ca:P, relative alpha 2 globulins, and beta globulins (Figure 15). Seasonal differences were noted in heterophils, lymphocytes, eosinophils, H:L, prealbumin, alpha 1 globulins, gamma globulins, and A:G. The nature and degree of these changes is reviewed in the annual report for T-104-R2. All clinical pathology parameters differed by year except eosinophils, basophils, GLDH, bile acids, and UA ($p < 0.05$).

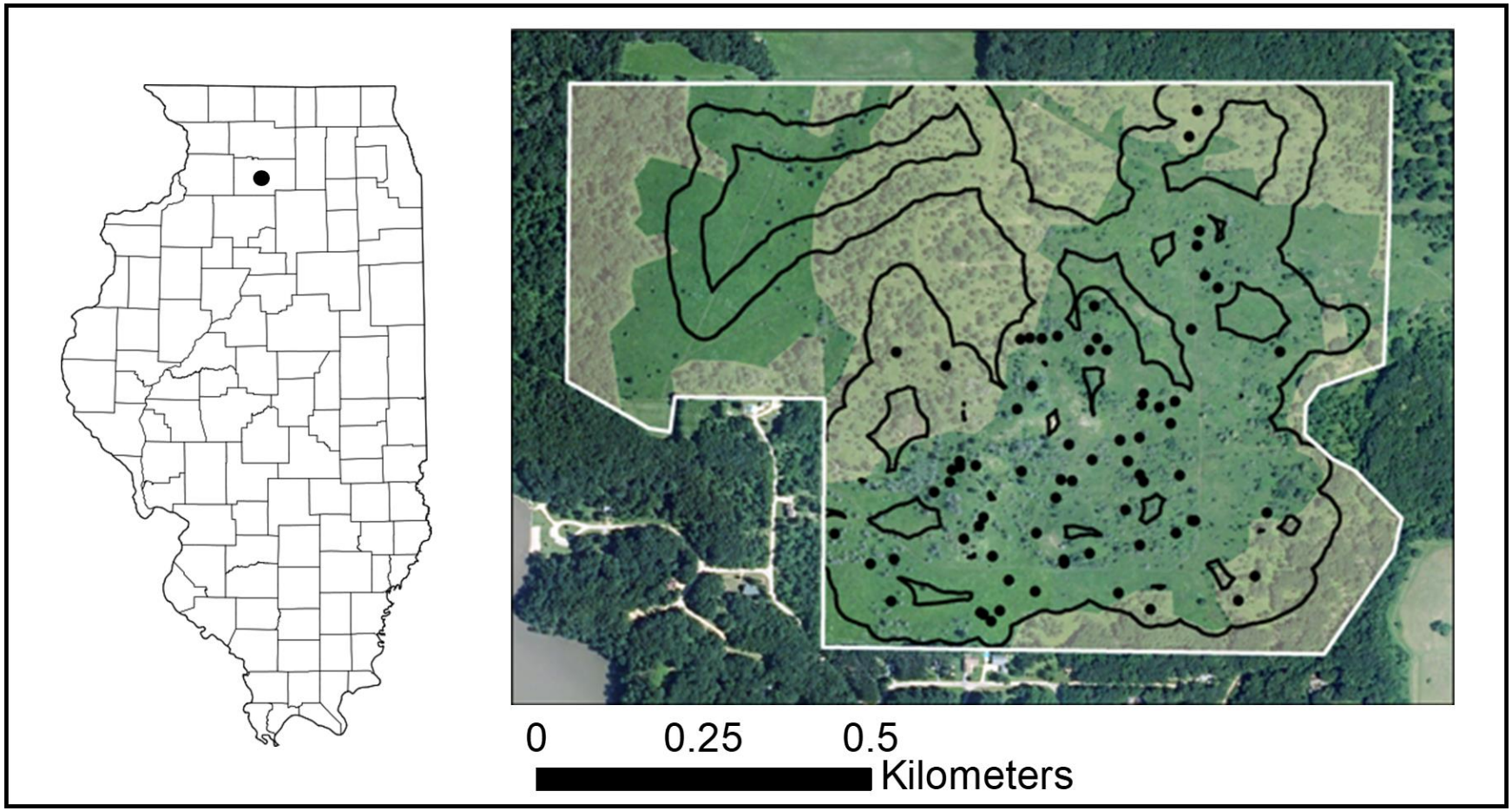

Figure 12. Nachusa Grasslands Orland Track (outlined in white) – main study site for ornate box turtle health assessments. A GPS track of one box turtle search (black lines) and several box turtle capture sites (black dots) are also displayed.

Table 8. Demographics, physical exam abnormalities, and pathogen presence in ornate box turtles.

		2016	2017	2018	2019
Site	**Orland Track**	72	74	56	66
	South Bison Unit	0	14	9	0
Season	**Spring**	72	88	65	51
	Summer	0	0	0	15
Sex	**Male**	44	42	37	32
	Female	19	29	27	26
	Unknown	9	17	1	8
Age Class	**Adult**	65	68	63	59
	Juvenile	7	18	2	7
Clinical Signs	**Blepharoedema**	0	1 (1%)	0	0
	Nasal Discharge	0	0	1 (1.5%)	0
	Asymmetrical Nares	2 (3%)	1 (1%)	1 (1.5%)	4 (6%)
	Burn Injury	2 (3%)	0	2 (3%)	1 (1.5%)
	Developmental Abnormality	1 (1.5%)	2 (2%)	4 (6%)	0
	Head, Limb, or Tail Trauma	8 (11%)	5 (6%)	4 (6%)	4 (6%)
	Inactive Shell Lesion	34 (47%)	29 (33%)	20 (31%)	18 (27%)
	Active Shell Lesion	8 (11%)	19 (22%)	19 (29%)	15 (23%)
Pathogens	**# Turtles Tested**	72	44	49	44
	Adenovirus	16 (22%)	1 (2%)	1 (2%)	1 (2%)
	TerHV1	21 (30%)	0	0	0
	TerHV1 + Adenovirus	11 (15%)	0	0	0

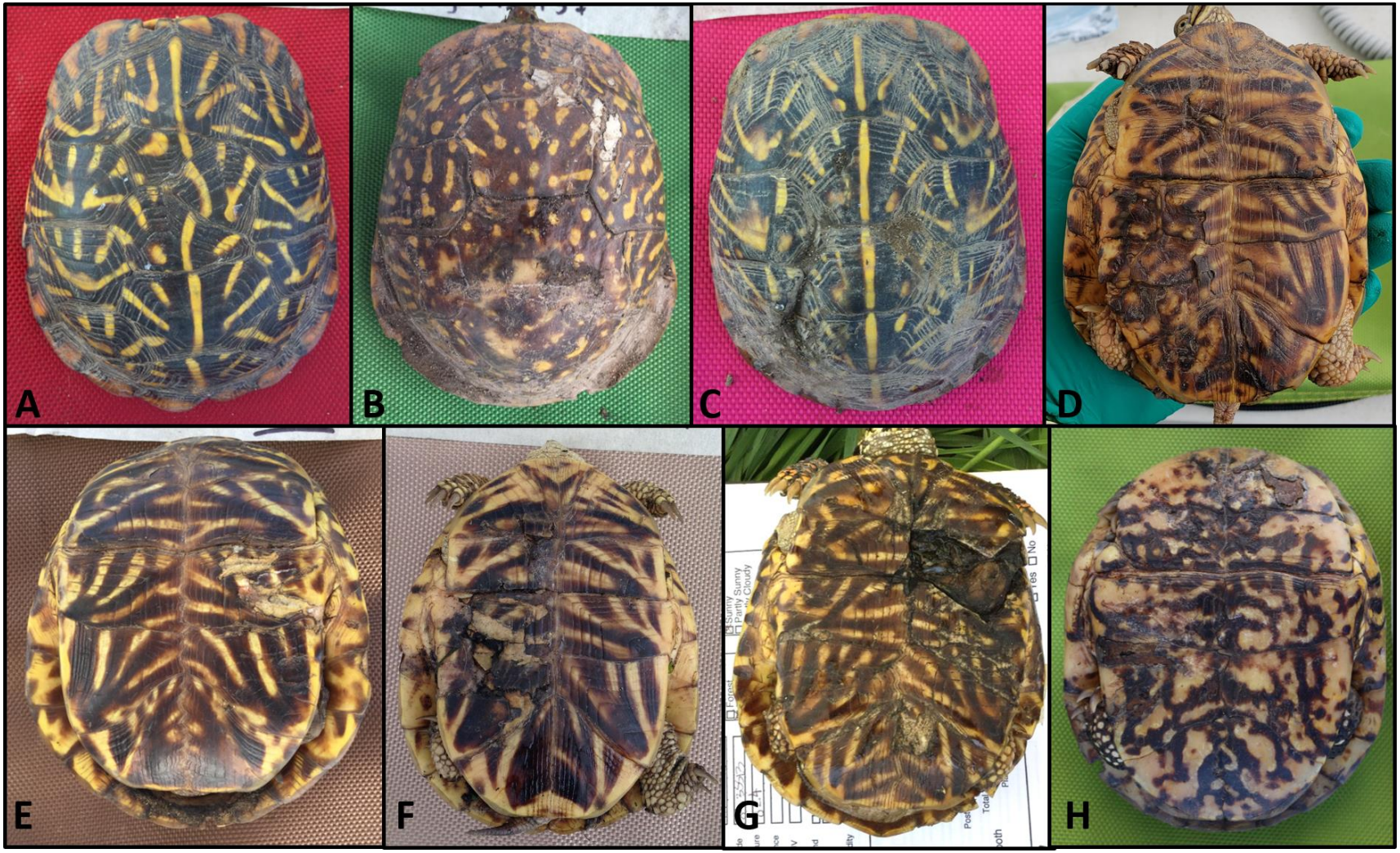

Figure 13. Common ornate box turtle shell abnormalities. A) Developmental abnormality – supranumerary vertebral scutes. B) Healed burn injury. C & D) Inactive shell lesions including a healed fracture (C) and healed shell trauma (D). E – H) Active shell lesions characterized by moist, soft foci and/or bleeding upon manipulation.

Table 9. Descriptive statistics for ornate box turtle hematology, plasma biochemistries, and protein electrophoresis. Central tendency = mean or median, Dispersion = standard deviation or 10th – 90th percentiles depending upon data distribution.

Analyte	N	Distribution	Central Tendency	Dispersion	Min	Max
Packed Cell Volume (%)	286	Non-Normal	25.4	19 - 29	5.5	37.5
Total Solids (g/dL)	286	Non-Normal	6.4	5.34 - 7.7	2.4	9.6
Total Leukocyte Count (/μL)	286	Non-Normal	18,560	10,704 - 27,245	7,323	50,789
Heterophils (μL)	286	Non-Normal	4,858	2,678 - 8,018	620	17,424
Lymphocytes (μL)	286	Non-Normal	8,931	4,640 - 17,209	1,837	40,123
Monocytes (μL)	286	Non-Normal	678	204 - 1,585	0	2,889
Eosinophils (μL)	286	Non-Normal	1,911	534 - 4,471	0	8,718
Basophils (μL)	286	Non-Normal	483	77 - 1,346	0	5,697
Heterophil / Lymphocyte	286	Non-Normal	0.54	0.214 - 1.241	0.053	2.9
Glutamate Dehydrogenase (U/L)	90	Non-Normal	11.5	4.5 - 55	1.3	136.8
Bile Acids (μmol/L)	202	Non-Normal	4.2	2.6 - 8.5	1.1	21.7
Uric Acid (mg/dL)	200	Non-Normal	1.4	0.9 - 1.8	0.8	5.6
Calcium (mg/dL)	202	Non-Normal	9.3	7.7 - 16.3	6	28.4
Phosphorous (mg/dL)	202	Non-Normal	2.8	1.9 - 4	1.4	7.6
Calcium / Phosphorous	202	Non-Normal	3.8	2.78 - 4.84	2.1	8.64
Aspartate Aminotransferase (U/L)	202	Non-Normal	94	47 - 179	20	420
Creatine Kinase (U/L)	201	Non-Normal	269	70 - 985	10	12,930
Total Protein (g/dL)	216	Non-Normal	5	3 - 7.5	1	9.8
Prealbumin (g/dL)	216	Non-Normal	0.07	0.03 - 0.17	0	0.46
Albumin (g/dL)	216	Non-Normal	1.28	0.71 - 2.11	0.32	2.92
Alpha 1 Globulins (g/dL)	216	Non-Normal	0.38	0.23 - 0.67	0.07	0.98
Alpha 2 Globulins (g/dL)	216	Non-Normal	1.3	0.71 - 2.07	0.26	3.05
Beta Globulins (g/dL)	216	Non-Normal	1.44	0.82 - 2.27	0.21	3.95
Gamma Globulins (g/dL)	216	Non-Normal	0.39	0.21 - 0.66	0.08	0.93
Albumin / Globulin	216	Normal	0.4	0.07	0.22	0.6

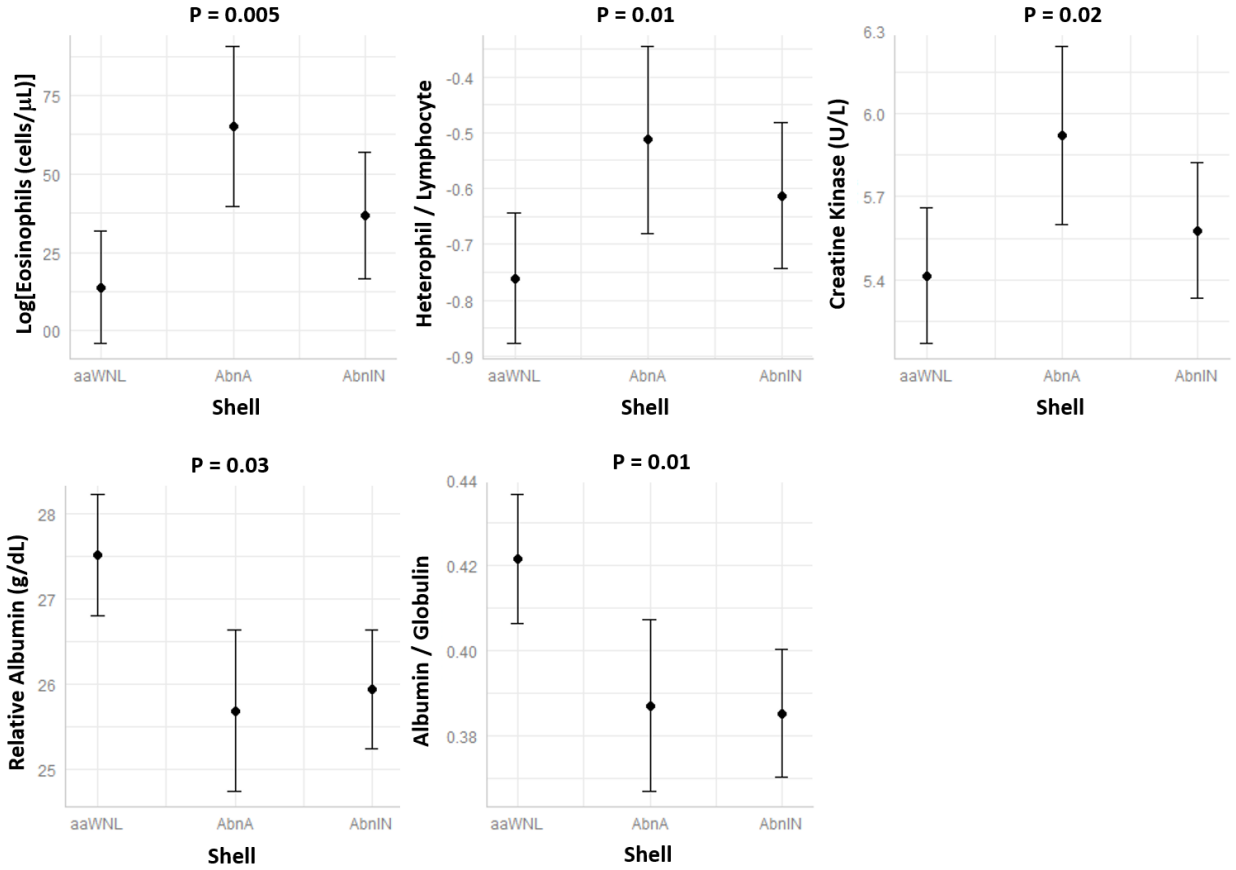

Figure 14. Statistically significant differences in clinical pathology parameters by shell condition in ornate box turtles 2016 – 2019. Model estimates and 95% confidence intervals are presented. aaWNL = normal shell, AbnA = active shell lesion, AbnIN = inactive shell lesion.

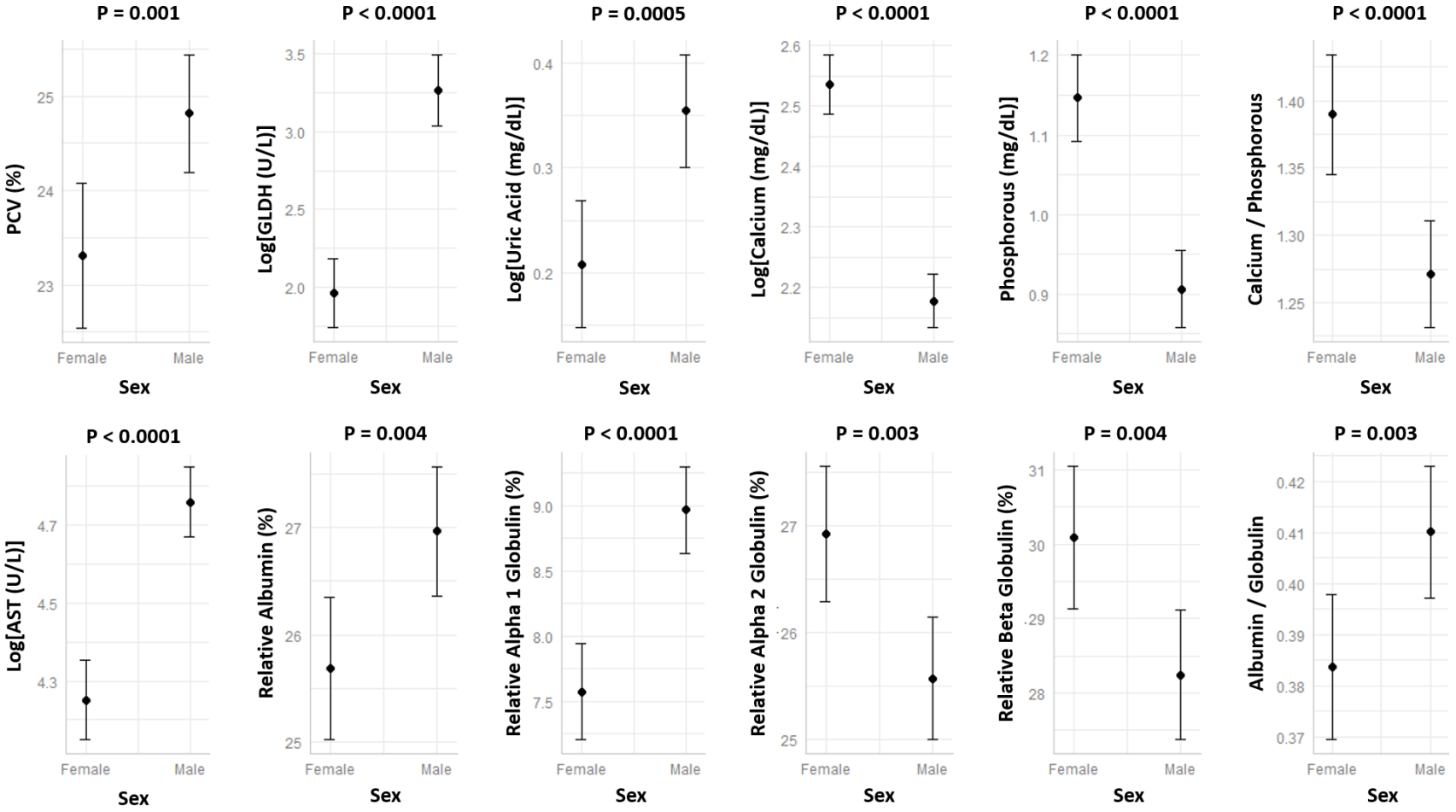

Figure 15. Statistically significant differences in clinical pathology parameters by sex in ornate box turtles 2016 – 2019. Model estimates and 95% confidence intervals are presented.

Individual Health Model

Prior to health modeling, clinical pathology parameters were normalized in a similar manner to the eastern box turtle data and box-cox transformed. The following predictors were associated with health status at a liberal alpha value of 0.15: WBC, absolute heterophil count, relative heterophil count, absolute lymphocyte count, relative lymphocyte count, H:L, absolute basophil count (Baso), absolute eosinophil count (Eo), and shell classification (Shell; AL, IL, WNL). The H:L is calculated from both heterophil and lymphocyte counts, so H:L was used to represent information for all heterophil and lymphocyte measures in the final health model.

The most parsimonious model for predicting health contained the additive effects of *Shell, WBC, Eo, Baso, and H:L*, though inclusion of the Eo variable resulted in a high degree of model selection uncertainty (Table 10). Internal and external validation were performed for the top two models separately and averaged (data not shown), and predictive capability of the model including Eo was superior, therefore this model was selected for full description. The top model is reported in Table 11, model predictions are displayed in Figure 16, and performance metrics produced by internal and external validation are reported in Table 12. External validation revealed a tendency to under-identify "unhealthy" animals, represented by a slight decrease in sensitivity, however, overall model performance remained acceptable with an accuracy of 68% and an AUC of 0.87.

Table 10. Model selection parameters for generalized linear models predicting health status in free-living ornate box turtles. H:L = heterophil : lymphocyte, WBC = total leukocyte count, Baso = absolute basophil count, Eo = absolute eosinophil count.

Model	N	K	AIC_c	ΔAIC_c	w_i
Shell + H:L + WBC + Baso + Eo	155	7	151.46	0	0.49
Shell + H:L + WBC + Baso	155	6	151.62	0.16	0.45
Shell + H:L + WBC	155	5	155.91	4.45	0.06
Shell + H:L	155	4	161.62	10.16	0
Shell * H:L * WBC * Baso * Eo	155	18	173.61	22.15	0
Null	155	1	216.84	65.39	0

Table 11. Parameter estimates for the most parsimonious model predicting health status in free-living ornate box turtles. AL = active lesion, IL = inactive lesion, H:L = heterophil : lymphocyte, WBC = total leukocyte count, Baso = absolute basophil count, Eo = absolute eosinophil count. Model: Healthy = Shell + WBC + H:L + Baso + Eo.

	β	SE	Z value	p-value
Intercept	-3.263	1.3	-2.514	0.01
Shell: AL	3.664	0.77	4.762	0.000002
Shell: IL	1.602	0.464	3.452	0.0006
WBC	0.024	0.009	2.681	0.007
H:L	1.603	0.364	4.391	0.00001
Baso	0.106	0.041	1.49	0.009
Eo	0.084	0.056	2.592	0.136

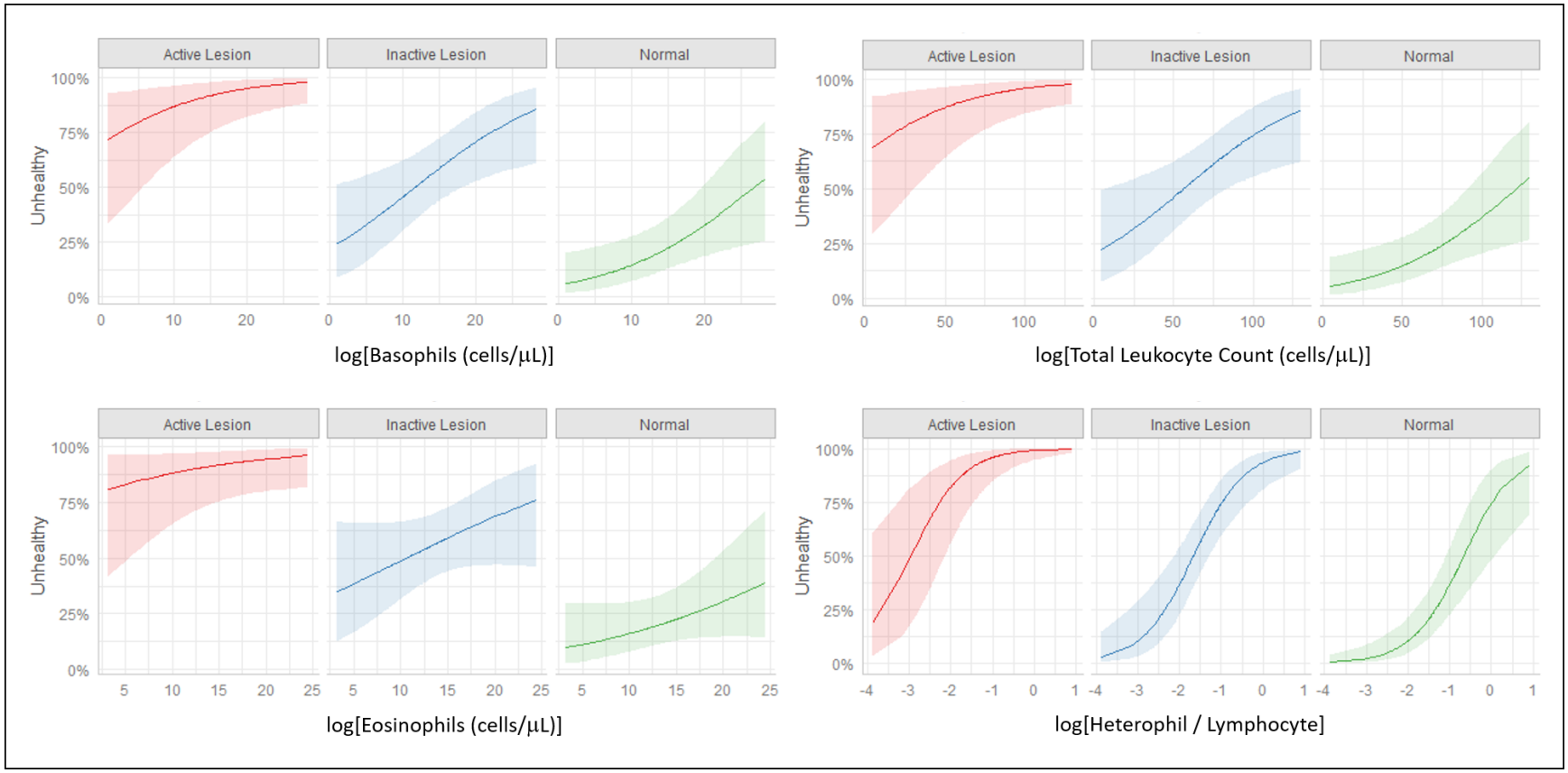

Figure 16. Predictions with 95% confidence intervals for individual health status in ornate box turtles.

Table 12. Model fit metrics from internal and external validation of the most parsimonious model predicting health status in free-living ornate box turtles.

Metric	Internal	External	Scale	Ideal
Brier Score	0.156[a]	0.23	0 - 1	Close to 0
AUC	0.865[a]	0.872	0.5 - 1	Close to 1
Accuracy (%)	78	68	0 - 1	Close to 1
Sensitivity	0.81	0.57	0 - 1	Close to 1
Specificity	0.75	0.91	0 - 1	Close to 1
Somer's Delta	0.716[a]	0.745	-1 - 1	Close to -1 or 1

[a] Optimism-corrected values based on internal validation via bootstrapping

Spatial Epidemiology

Significant spatial clusters were not identified for any parameter tested, indicating that pathogens and poor health indices were distributed homogeneously within the population. Turtles did not cluster by age class or sex, at least at the spatiotemporal scale evaluated in this study.

Population Health

The Schumacher-Eschmeyer population size estimate was 268 turtles, with a 95% confidence interval from 184 – 493 individuals. Starting population sizes for the three baseline scenarios were therefore 268 (estimated), 493 (optimistic), and 184 (pessimistic). Populations tended to decline slightly over the simulated 100-year period as evidenced by negative growth rates, but extinction probability was zero for all three baseline scenarios (Figure 17). Sensitivity testing revealed that annual removal of 2 adult females beyond background mortality rates elevated the probability of population extinction to over 50% in every scenario (Figure 18). In the pessimistic scenario, removal of 1 adult female per year was enough to increase the probability of population extinction to over 80%. Removal of up to five adult males per year was also impactful, but much less so than adult female removal (Figure 18). In contrast, removal of hatchling females was much less destabilizing compared to adult turtle removal (Figure 18). Population health of Nachusa ornate box turtles appears extremely sensitive to the loss of relatively small numbers of adult females. Strategies to protect this demographic may improve population stability over time.

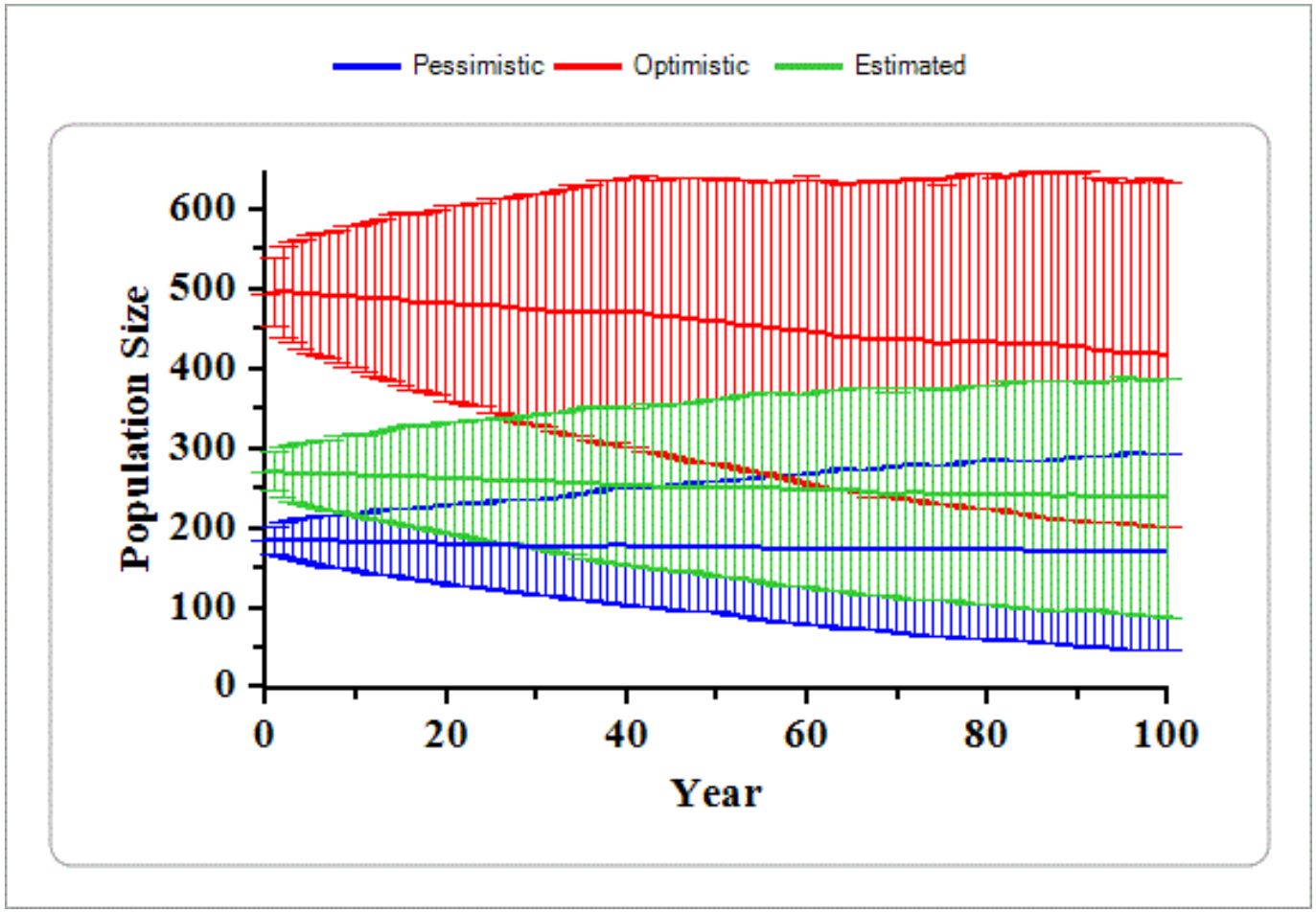

Figure 17. Population size estimates +/- standard deviation simulated over 100 years for free-living ornate box turtles at three different starting population sizes: Estimated = 268 turtles, Pessimistic = 184, Optimistic = 493 turtles.

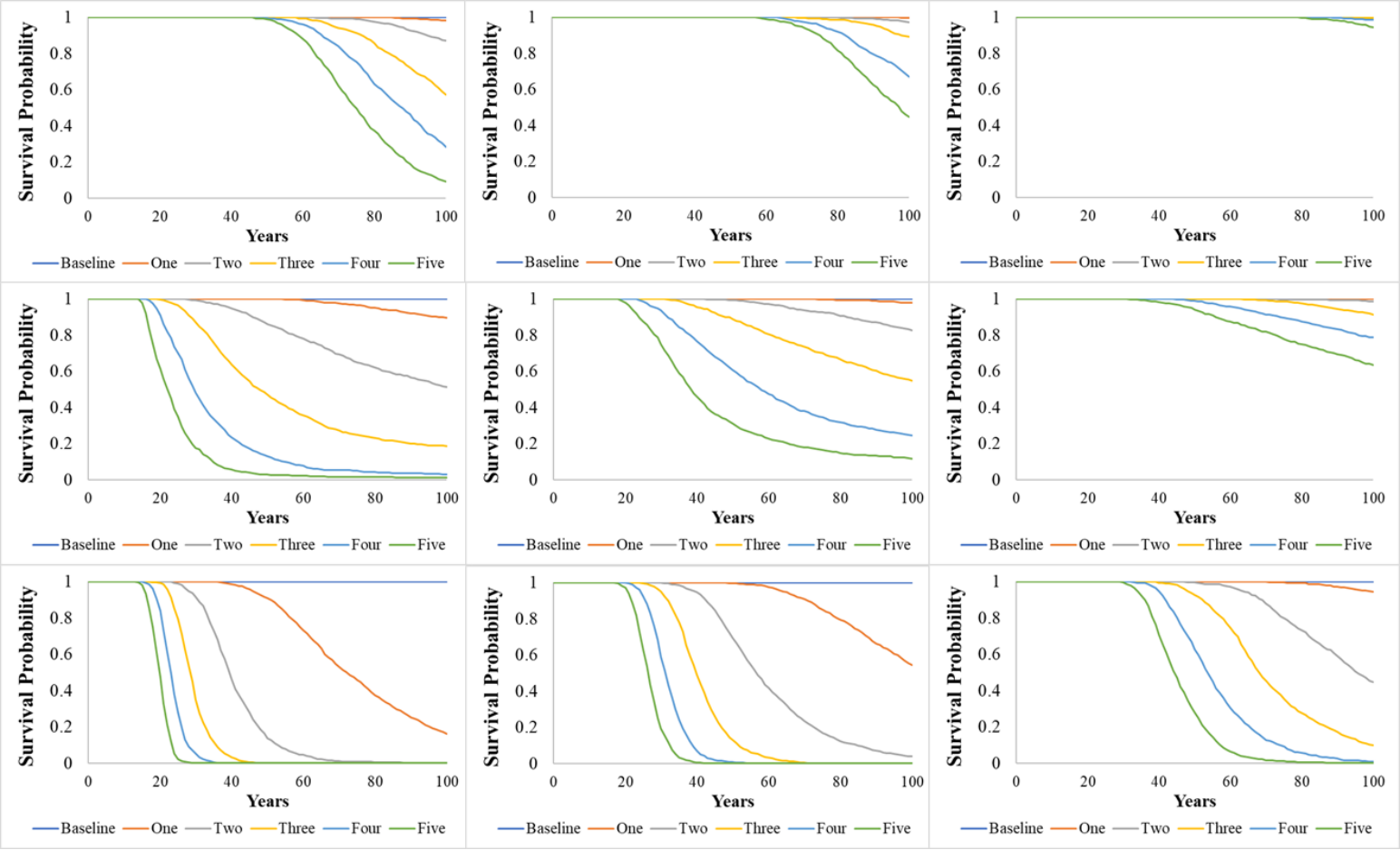

Figure 18. Simulated mean survival probability over time in a population of ornate box turtles (*Terrapene ornata ornata*) experiencing annual loss of 0 – 5 female hatchlings (top row), adult males (middle row), or adult females (bottom row). Starting population sizes: left column = 184 turtles, middle column = 268 turtles, right column = 493 turtles.

Silvery Salamanders

Nine hundred and seven silvery salamander health assessments were performed between 2016 and 2019. Demographic data, physical examination abnormalities, and pathogen presence during routine health surveillance is summarized in Tables 13 & 14 and Figure 19.

Throughout the sampling period, the most common physical examination abnormalities included developmental abnormalities of the jaw and/or limbs, healed and active injuries, hemorrhages, yellow skin nodules consistent with *Clinostomum* sp. (a.k.a. "yellow grub") infection, and white skin nodules consistent with *Dermotheca* sp. infection, which were initially identified in silvery salamanders in 2017 (Figure 20). In 2018 and 2019 white skin nodules consistent with *Dermotheca* sp. were also identified in two small-mouth salamanders (*Ambystoma texanum*) from pond 283. Necropsy with histopathology confirmed that both small-mouthed salamanders were infected with a mesomycetozoean parasite, and that sporangia were confined to the skin. Sequencing of the 18S rRNA gene revealed identical sequence from both silvery and small-mouthed salamanders, indicating that this parasite is capable of infecting multiple species. The small-mouthed salamander sequence was also identical to sequences recovered from silvery salamanders in 2017 and 2018. Phylogenetic reconstruction confirmed this parasite as a *Dermotheca* sp. that is closely related to *Dermotheca viridescens* – a parasite of red-spotted newts (*Notophthalmus viridescens*) from the eastern United States first described in 2008 (Raffel et al., 2008, Adamovicz et al. 2019).

Both body condition and weight varied by year ($p < 0.05$), but not consistently by study site (Kickapoo, Middle Fork, Collison) or pond. The odds of healed injuries were 2 times higher at Collison than Kickapoo (95% CI = 1.2 – 3.3, $p = 0.02$). Hemorrhages were significantly more common in larvae than adults (OR = 12.5, 95% CI = 4.2 – 36.9, $p < 0.0001$). Salamanders with visible *Dermotheca* sp. nodules were lighter than those with no evidence of infection (effect size = 1g, 95% CI = 0.02 – 2g, $p = 0.04$).

In 2016 FV3 was detected during routine sampling in seven adult silvery salamanders from ponds 75 and 284 and five larvae from ponds 67, 69, and 73. In 2017, FV3 was detected in a single larva from pond 285, and FV3 was not detected in any salamander during 2018. FV3 was identified in association with larval mortality events in 2016 and 2017, and 2019 (detailed below), but no mortality event was observed in 2018, potentially due to warmer temperatures inhibiting viral replication. FV3 prevalence varied by year ($p < 0.0001$) and was significantly more common in larvae and metamorphs than adults (OR = 8, 95% CI = 1.9 – 34.5, $p = 0.005$). FV3 detection was also more likely in salamanders with hemorrhages (OR = 10, 95% CI = 1.8 – 55.6, $p = 0.008$). Bd, Bsal, and the Chlamydiaceae family were not detected during the study period.

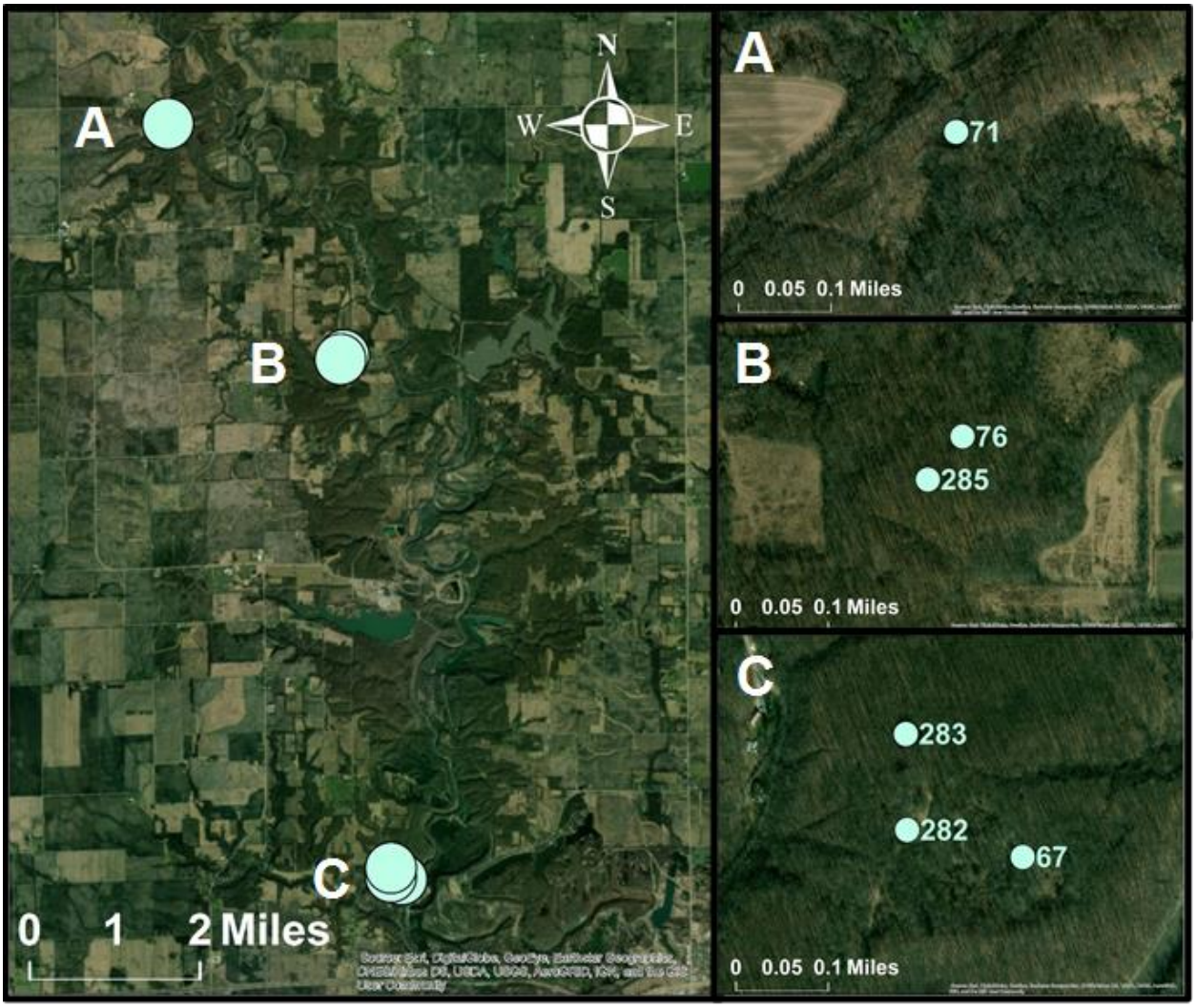

Figure 19. Locations of the six most commonly-sampled ephemeral ponds containing silvery salamanders in the Vermilion County COA during the 2016-2019 sampling seasons. A) Collison, B) Middle Fork, C) Kickapoo State Park.

Table 13. Demographics, physical exam abnormalities, and pathogen presence in adult silvery salamanders. Medians are presented for weight and BCS. KSP = Kickapoo State Park, MF = Middle Fork Fish and Wildlife Area, COL = Collison, BCS = body condition score, FV3 = frog virus 3.

Site	Pond	Year	N	Weight (g) (Range)	BCS (Range)	White Nodules	Yellow Nodules	Develop Abnormality	Fresh Injury	Healed Injury	Hemorrhage	FV3
KSP	66	2016	23	14 (9-21)	3.25 (3-4)	0	0	0	0	0	0	0
KSP	67	2016	1	14	3	0	0	0	0	0	0	0
		2017	53	12.1 (9.3-18.6)	3 (1.5-5)	4 (7.5%)	0	3 (5.7%)	2 (3.8%)	6 (11%)	0	0
		2018	15	13 (9-15)	3.5 (3-4)	0	0	2 (13%)	0	3 (20%)	0	0
		2019	42	14.5 (8-19)	3.5 (1.5-5)	2 (7%)	1 (3%)	5 (18%)	3 (10%)	8 (29%)	2 (7%)	0
KSP	282	2016	1	13	3	0	0	0	0	0	0	0
		2017	30	12.4 (8.3-19.1)	3.25 (2-5)	1 (3.3%)	0	2 (6.7%)	2 (6.7%)	2 (6.7%)	0	0
		2018	19	13.5 (10-17)	3.8 (3-5)	0	1 (5%)	2 (10%)	0	1 (5%)	0	0
		2019	21	14.8 (9.5-23)	3.7 (2.5-5)	0	0	2 (7%)	3 (10%)	4 (14%)	0	0
KSP	283	2016	21	14.2 (9-21)	3.3 (3-5)	0	0	0	0	0	0	0
		2017	108	13.1 (8.2-22.2)	3 (2-5)	12 (11%)	3 (2.8%)	3 (2.8%)	4 (3.7%)	12 (11%)	0	0
		2018	70	14 (10-19)	3.8 (2-5)	5 (7%)	0	8 (11.4%)	0	3 (4.3%)	0	0
		2019	54	14.2 (7-20)	3.5 (2-5)	4 (7%)	0	8 (13%)	3 (5%)	6 (10%)	0	0
COL	71	2016	0	-	-	-	-	-	-	-	-	-
		2017	99	13 (8.9-18.4)	3 (2-5)	0	4 (4%)	8 (8%)	3 (3%)	23 (23%)	2 (2%)	0
		2018	57	13 (7-18)	3.5 (2-5)	0	3 (5%)	11 (19%)	2 (3.5%)	6 (10.5%)	1 (1.8%)	0
MF	69	2016	2	13 (11-15)	3 (3-3)	0	0	0	0	0	0	0
MF	75	2016	17	13 (8-18)	3 (2-4)	0	0	1 (6%)	0	2 (12%)	0	3 (18%)
MF	284	2016	4	14.8 (11-19)	3.25 (3-4)	0	0	0	0	0	0	4 (100%)
MF	290	2016	1	10	3	0	0	0	0	0	0	0
MF	76	2016	12	12 (7-17)	2.83 (2-4)	0	0	0	0	0	0	0
		2017	81	13.9 (7.5-20.7)	3 (2-5)	1 (1.3%)	1 (1.3%)	13 (16%)	1 (1.3%)	12 (15%)	2 (2.5%)	0
		2018	39	12 (6-19)	3.3 (3-4)	0	0	1 (2.6%)	3 (7.7%)	1 (2.6%)	0	0
MF	285	2016	2	12.5 (12-13)	3 (3-3)	0	0	0	0	0	0	0
		2017	19	14.7 (9.1-19.5)	3.5 (3-5)	0	0	1 (5.3%)	1 (5.3%)	5 (25%)	0	0
		2018	20	11.5 (9-16)	3.2 (2-4)	1 (5%)	0	1 (5%)	0	2 (10%)	0	0

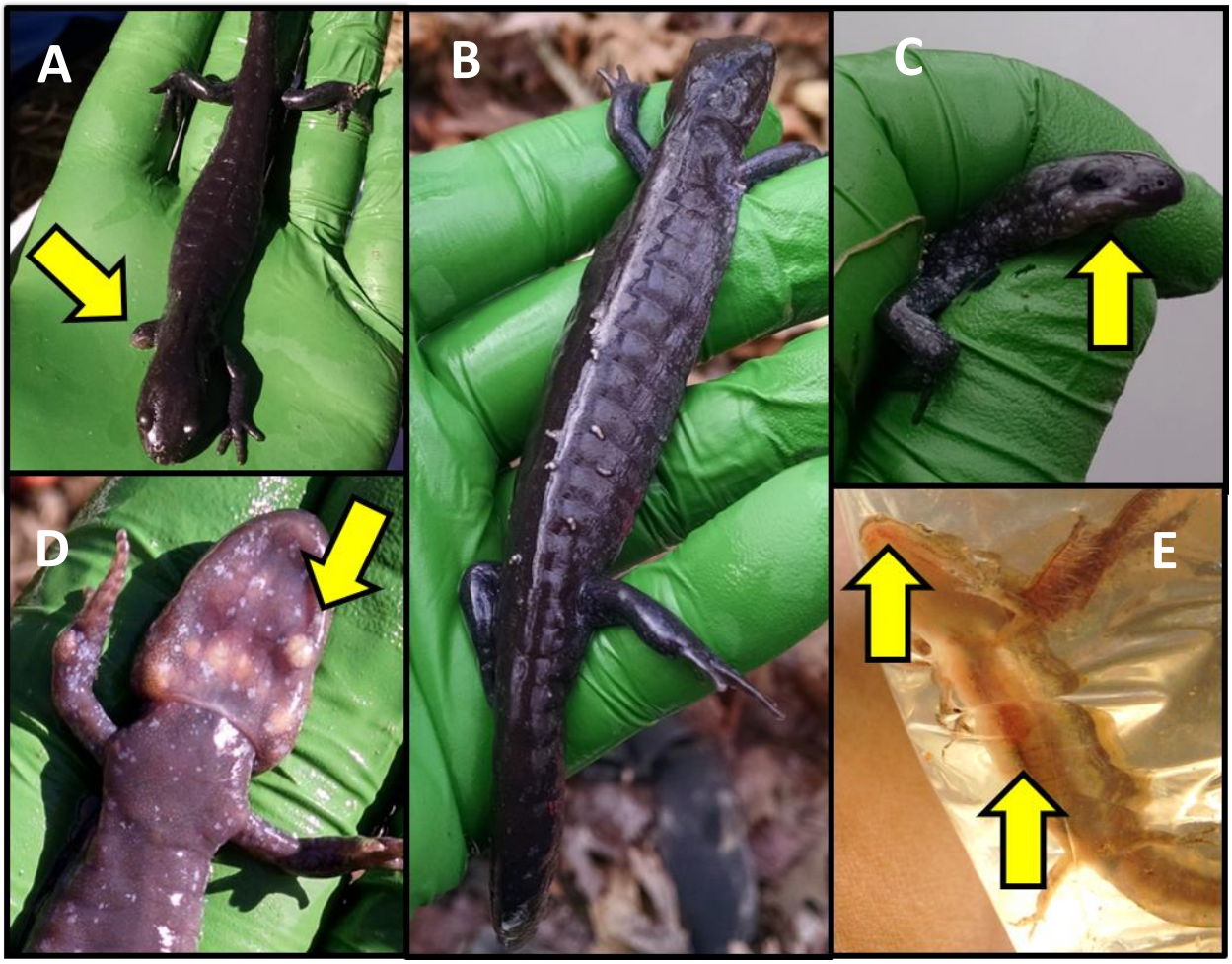

Figure 20. Common physical examination abnormalities in silvery salamanders from Vermilion County, IL during 2016-2019. A) Trauma, B) White cutaneous nodules consistent with *Dermotheca* sp. infection, C) Developmental abnormality of the mandible, D) Yellow subcutaneous nodules consistent with *Clinostomum* sp. infection, E) Hemorrhages.

Table 14. Demographics, physical exam abnormalities, and pathogen presence in larval silvery salamanders. Medians are presented for weight and BCS. KSP = Kickapoo State Park, MF = Middle Fork Fish and Wildlife Area, COL = Collison, BCS = body condition score, FV3 = frog virus 3.

Site	Pond	Year	N	Weight (g) (Range)	BCS (Range)	Develop Abnormality	Fresh Injury	Healed Injury	Hemorrhage	FV3
KSP	66	2016	0	-	-	-	-	-	-	-
KSP	67	2016	4	2.5 (2-3)	3.13 (2-4)	0	1 (25%)	0	0	3 (75%)
		2017	7	2 (1-2)	3 (2.5-3.5)	0	0	2 (29%)	1 (14%)	0
		2018	0	-	-	-	-	-	-	-
		2019	6	1 (1-1)	2 (1-2.5)	0	0	0	0	0
KSP	282	2016	0	-	-	-	-	-	-	-
		2017	1	4	3	0	0	0	0	
		2018	5	2.8 (2-4)	3.1 (2-4)	0	0	0	0	0
		2019	8	1.8 (1-3)	3 (2.5-4)	0	0	0	0	0
KSP	283	2016	0	-	-	-	-	-	-	-
		2017	8	2 (1-4)	3 (1.5-4)	0	1 (13%)	2 (25%)	0	0
		2018	7	1.7 (1-3)	1.5 (1-2)	0	0	0	2 (29%)	0
		2019	6	3 (2-4)	3.5 (2.5-5)	0	0	0	0	0
COL	71	2016	5	3.8 (2-5)	2.8 (2-3)	0	1 (20%)	0	2 (40%)	1 (20%)
		2017	14	1.36 (1-2)	2.5 (2-3)	1 (7%)	0	2 (14%)	1 (7%)	0
		2018	9	1.9 (1-3)	2.7 (1.5-3.5)	0	1 (11%)	0	0	0
MF	69	2016	1	3	3	0	1 (100%)	0	0	1 (100%)
MF	75	2016	2	3 (3-3)	3 (3-3)	0	1 (50%)	0	0	0
MF	284	2016	0	-	-	-	-	-	-	-
MF	290	2016	0	-	-	-	-	-	-	-
MF	76	2016	0	-	-	-	-	-	-	-
		2017	0	-	-	-	-	-	-	-
		2018	10	2.4 (1-4)	2.4 (2-3)	0	1 (10%)	0	0	0
MF	285	2016	0	-	-	-	-	-	-	-
		2017	1	4	3	0	0	0	1 (100%)	1 (100%)
		2018	0	-	-	-	-	-	-	-
MF	289	2016	1	3	3.5	0	0	0	0	0
MF	70	2016	1	3	4	0	0	0	0	0

Silvery Salamander Mortality Events

Mortality events resulting in the deaths of over 300 silvery salamander larvae were identified in four ponds in 2016. Approximately 80% of the estimated silvery salamander larval population was killed during these outbreaks (Low, 2019). Two general epidemic patterns were present: a point source pattern starting at the beginning of May in ponds 66 and 283 and rapidly resolving within 10 days, and a more spread out distribution lasting several weeks beginning in May in pond 282 and June in pond 73. This pattern may be consistent with either a common-source, multiple event epidemic pattern or a propagating pattern slowed by a decreased transmission rate between hosts – perhaps secondary to decreased larval density, impaired viral replication due to alterations in temperature or pond conditions, or changes in host immunity due to developmental stage. The largest outbreak occurred in pond 66 and resulted in 237 documented deaths within eight days.

Deceased larvae at each pond frequently displayed hemorrhaging and edema, and moribund larvae were often uncoordinated with inconsistent righting reflexes. Liver and spleen samples from fifty larvae were tested for FV3 during each outbreak using qPCR, and all were positive. Virus isolation was attempted from multiple pools of organs at each pond, but cytopathic effects were not observed after up to three blind passages. Ranavirus conventional PCR was pursued on DNA extracted from liver and spleen samples collected from Kickapoo to enable comparison between the virus affecting silvery salamanders (SS-RV) and a historic ranavirus isolate (EBT-RV) associated with mortality in eastern box turtles at Kickapoo in 2014 (Adamovicz 2018).

Mortality of small numbers of silvery salamanders was observed in 2017 at ponds 76 and 285. Twelve deceased silvery salamanders, eleven of which displayed hemorrhages and edema, were recovered from pond 76 from April 26th – May 11th. Pooled tissues tested qPCR positive for FV3. A single clinical silvery salamander was observed in pond 285 on June 2nd following a ranavirus mortality event in wood frog (*Rana sylvaticus*) larvae. This individual was FV3 positive via qPCR. Larval mortality was not observed in 2018 for any amphibian species, including silvery salamanders.

In 2019, approximately 50 silvery salamander, wood frog (*Rana sylvaticus*), and small-mouthed salamander (*Ambystoma texanum*) metamorphs were recovered deceased with clinical signs of FV3 infection (hemorrhage, edema) from pond 283. Ten dead wood frog and two silvery salamander metamorphs were also collected from pond 67. The timing of this mortality event ranged from May 30 – July 18th in pond 283 and May 30th – June 12th in pond 67. Ranavirus testing of pooled organ samples was strongly positive, however, virus isolation was again unsuccessful.

Three deceased adult salamanders were collected and necropsied in 2019, the details of which are summarized in the TR-104-R2 annual report. Deaths were attributable to parasitism with *Clinostomum* sp., poor condition, and unknown causes.

Ranavirus Genetics

PCR of the MCP produced a 1509bp fragment for EBT-RV and a 1485bp fragment for SS-RV, DNApol PCR produced a 519bp fragment for EBT-RV and a 504bp fragment for SS-RV, and vIF-2α PCR produced a 211bp fragment for EBT-RV and a 928bp fragment for SS-RV. Aligned portions of the EBT-RV and SS-RV MCP were identical except for two base pairs. The EBT-RV sequence was 100% identical to multiple FV3 sequences in Genbank, while the SS-RV isolate was 99% identical to the same sequences (accession numbers MG953520.1, MG953519.1, MG953518.1, KJ175144.1). Aligned portions of the EBT-RV and SS-RV DNApol sequences were identical except for three base pairs, and both the EBT-RV and SS-RV sequences were 99% homologous to multiple FV3 sequences (accession numbers MG953520.1, MG953519.1, MG953518.1, KJ175144.1). There was a 23 base pair difference between

the aligned portions of EBT-RV and SS-RV vIF-2 α, making this the most divergent gene sequenced (Figure 7.4). The EBT-RV vIF-2 α was 100% identical to multiple FV3 sequences, while the SS-RV sequence was 99% similar to multiple amphibian ranaviruses (e.g. KM516750.1, KF512820.1, KF033124.1, KX397571.1), indicating that the ranavirus affecting silvery salamanders may be distinct from that historically affecting sympatric box turtles at KSP.

Individual Health Model

Twenty-two salamanders fit the criteria to be classified as "unhealthy" during the four-year study period, including 13 adults and nine larvae. Adults were classified as unhealthy due to poor BCS (N = 3) active injuries (N = 8), heavy parasitism (N = 2), and hemorrhages (N = 1) while larvae were classified as unhealthy due to poor BCS (N = 4), extensive hemorrhaging (N = 4), and active injuries (N = 1). The following variables were significantly associated with health at a liberal alpha value of 0.15: BCS, presence of active traumatic injuries ("Trauma"), and hemorrhages. Inclusion of the FV3 variable necessitated case-wise deletion of all salamanders which were not tested for pathogens via qPCR, resulting in a final sample size of 351 (including only 15 unhealthy salamanders) for model ranking.

The most parsimonious model predicting silvery salamander health included the additive effects of *BCS, Trauma, and Hemorrhage* (Tables 15 & 16). Performance metrics produced by internal validation via bootstrapping with 500 replicates are reported in Table 17. External validation was not pursued due to the low number of unhealthy animals identified for model building.

Table 15. Model selection parameters for generalized linear models predicting health status in free-living silvery salamanders. BCS = body condition score, Trauma = presence of fresh traumatic injuries.

Model	N	K	AIC_c	ΔAIC_c	w_i
BCS + Trauma + Hemorrhage	351	4	73.4	0	0.952
BCS + Trauma	351	3	79.4	6.02	0.047
Trauma + Hemorrhage	351	3	86.5	13.12	0.001
BCS + Hemorrhage	351	3	99.1	25.67	0
Trauma	351	2	103.6	30.14	0
BCS	351	2	104.3	30.86	0
Hemorrhage	351	2	111.5	38.05	0
Null	351	1	125.9	52.53	0

Table 16. Parameter estimates for the most parsimonious model predicting health status in free-living silvery salamanders. KSP = Kickapoo State Park, MF = Middle Fork Fish and Wildlife Area, Trauma = presence of fresh traumatic injuries, BCS = body condition score.

Healthy = Year + Location + BCS + FV3 + Trauma				
	β	**SE**	**Z value**	**p-value**
Intercept	-1.17	1.54	-0.76	0.44
Hemorrhage: Yes	-2.68	0.91	-2.94	0.003
Trauma: Yes	-4.1	0.81	-5.06	0.0008
BCS	1.95	0.58	3.35	< 0.0001

Table 17. Model fit metrics from internal validation of the most parsimonious model predicting health status in free-living silvery salamanders.

Metric	Internal	Scale	Ideal
Brier Score	0.028[a]	0 - 1	Close to 0
AUC	0.93	0.5 - 1	Close to 1
Accuracy (%)	0.97	0 - 1	Close to 1
Sensitivity	0.99	0 - 1	Close to 1
Specificity	0.4	0 - 1	Close to 1
Somer's Delta	0.86[a]	-1 - 1	Close to -1 or 1

[a] Optimism-corrected values based on bootstrapping

Mortality Events & Novel Disease Processes

We investigated a variety of mortality events in free-living herptiles and captive species of conservation concern from 2016 – 2019. In October 2016, we were presented with 14 live and one recently-deceased alligator snapping turtle (AST; *Macrochelys temminckii*) from a head-starting program in Chicago Public Schools operating through Operation Endangered Species. Initial clinical signs included paronychia (nail bed inflammation) and nail loss (N = 9), cutaneous ulceration (N = 7), plastron ulceration (N = 6), and rhinitis (N = 5) (Figure 21). All turtles tested negative for ranavirus, herpesvirus, and *Mycoplasma* sp. *Emydomyces testavorans*, an emerging fungal pathogen causing shell disease in multiple species of aquatic turtles, was diagnosed using polymerase chain reaction (PCR) and culture (Woodburn et al., 2019). Seven turtles died or were euthanized due to progressive clinical signs. Necropsy and histopathology revealed ulcerative dermatitis/rhinitis (N = 8) and rare osteomyelitis (N = 1), with no other significant findings. Lesions were PCR positive for *E. testavorans* and were colonized with morphologically consistent fungi. Most clinical signs in survivors resolved within several months. However, over the next three years turtles developed progressive hyperkeratosis with excessive skin and scute shedding, and experienced intermittent cutaneous ulceration of the extremities. All turtles remain *E. testavorans* PCR positive, though a treatment trial with nebulized antifungal medication (terbinafine) is ongoing. This case series illustrates that *E. testavorans* can cause significant cutaneous lesions in ASTs and demonstrates that spontaneous clearance is unlikely. *E. testavorans* should be considered a pathogen of concern for AST head-starting and reintroduction programs.

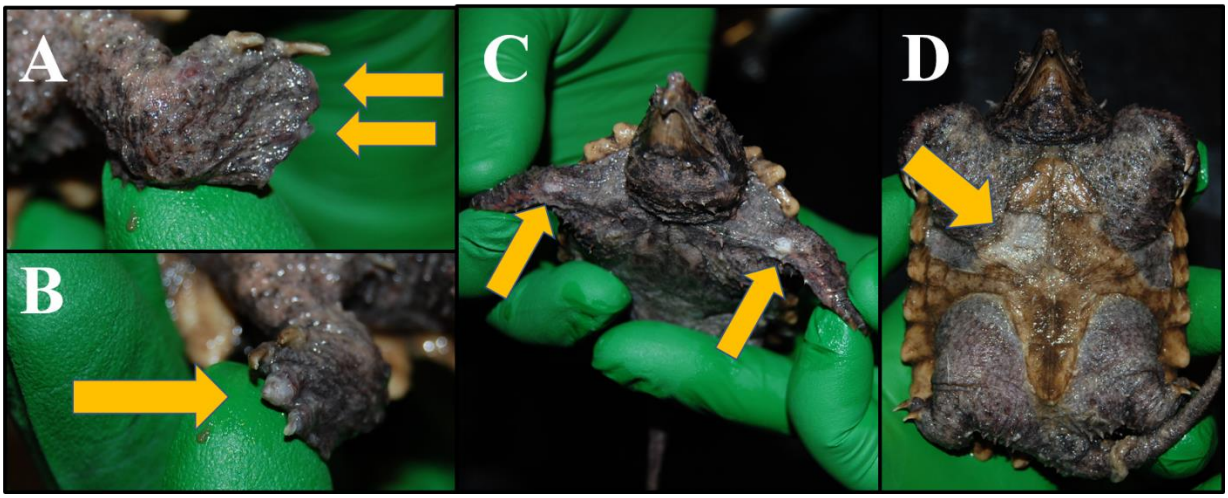

Figure 21. Clinical appearance of juvenile alligator snapping turtles infected with *Emydomyces testavorans*. A) Nail loss. B) Paronychia. C) Cutaneous ulceration. D) Plastron ulceration.

Multiple necropsies were conducted on a radiotelemetered box turtle population at Kickapoo State Park from 2016 – 2019 (Rayl et al. 2019). Six animals presented dead in late February 2017. Necropsy findings revealed emaciation and muscle atrophy consistent with death during overwintering. This was most likely related to unusual weather patterns during the winter of 2016. Six more turtles died from April – November, 2017. Necropsy revealed severe ulcerative gastritis with associated hepatitis and serositis (N=1), marked necrotizing meningoencephalitis (N=1), egg binding (N=1), and sepsis (N=1). Necropsy findings were open in two other cases. While half of box turtle deaths appeared attributable to unsuccessful brumation, the other half died due to a variety of causes. These findings support our antemortem assessments which indicate poorer overall health status at Kickapoo State Park. Our PVA analysis indicates that this population is fairly unstable, and continued losses at this rate may compromise the long-term persistence of box turtles at this site.

A smaller number of necropsies were conducted for a radiotelemetered Blanding's turtle (*Emydoidea blandingii*) population in Lake County, Illinois from 2016 – 2019. Two turtles were found dead unexpectedly, while the other rapidly declined during hospitalization for pneumonia and was euthanized. Necrohemorrhagic enterocolitis was a major finding at each necropsy, and while autolysis prevented thorough lesion characterization in two cases, fungal sepsis was identified as the cause in one animal. This case series will help improve antemortem health assessment in this species by directing the inclusion of tests assessing enteric health.

Investigation of novel or unusual disease presentations was also pursued. Mycobacteriosis was diagnosed using PCR and histopathology in a 16lb AST presenting with pharyngeal and cutaneous mass lesions (Figure 22). A necrotizing bacterial infection was diagnosed via culture in an adult male eastern box turtle from Forest Glen. Interestingly, we had previously characterized an outbreak of necrotizing bacterial infections in box turtles at this site six years prior (Adamovicz 2018). Infection with *Amphibiothecum penneri* was diagnosed in an American toad from Kickapoo via histopathology and PCR, and a similar diagnostic approach was used to characterize *Dermotheca* sp. infection in silvery salamanders and small-mouthed salamanders (Adamovicz et al., 2019). Our diagnosis of *Dermotheca* sp. represents the first detection of a Mesomycetozoean parasite in midwestern salamanders. Continued diagnostic assessment of unusual morbidity and mortality events in Illinois herptiles will provide useful information about the prevalence, etiology, and potential management implications of different disease processes and is an essential component of holistic health assessments.

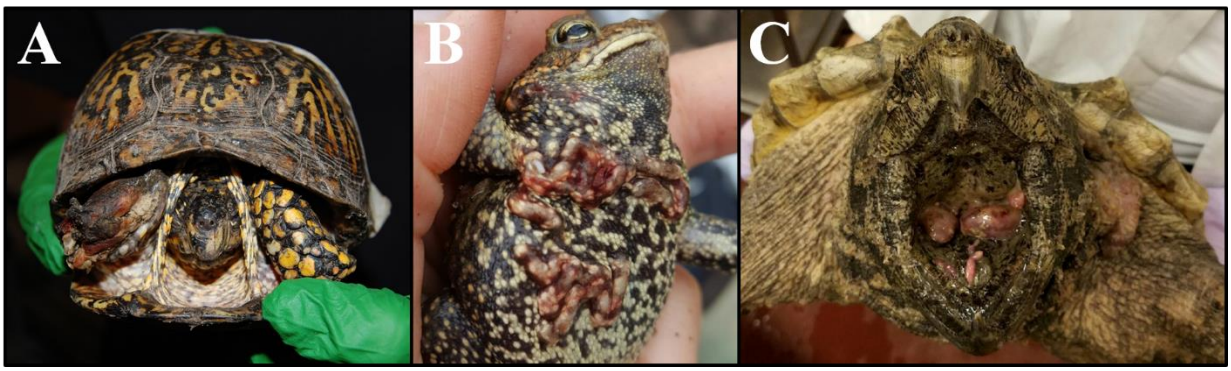

Figure 22. Unusual disease presentations in Illinois herptiles. A) Necrotizing bacterial infection in an eastern box turtle. B) *Amphibiothecum penneri* infection in an American toad. C) Pharyngeal and cutaneous Mycobacteriosis in an alligator snapping turtle

DISCUSSION

This project set out to characterize the health of 3 herptile SGNC in 3 separate IWAP campaigns by developing general health profiles, identifying infectious and non-infectious causes of morbidity and mortality, and modeling the wellness of individuals and populations. We have generated a tremendous amount of data about the role of diagnostic testing and pathogen surveillance in herptile health assessments, and have established valuable baseline health parameters for future monitoring. Our findings provide a strong foundation for understanding wild herptile health, and can be used to inform management strategies supporting positive health and conservation outcomes.

Box Turtle Clinical Pathology, Pathogen Surveillance, and PVA

Several previous studies have examined clinical pathology parameters in free-living and managed-care box turtles (Adamovicz et al., 2015, Flower et al., 2014, Harden et al., 2018, Kimble and Williams 2012, Lloyd et al., 2016, Rose and Allender 2011), however, our study is the largest to incorporate seasonal, annual, and population-level associations and therefore provides the most complete understanding of normal variability. Our findings demonstrate differences in most clinical pathology values based on year, season, age, and sex; consistent with previous studies in multiple chelonian species (Anderson et al., 1997, Andreani et al., 2014, Bielli et al.,2015, Brenner et al., 2002, Chaffin et al., 2008, Christopher et al., 1999, Chung et al., 2009, Dickinson et al., 2002, Eshar et al., 2014, Eshar et al., 2016, Grioni et al., 2014, Harden et al., 2018, Innis et al., 2007 Kimble and Williams 2012, Lewbart et al., 2018, Lloyd et al., 2016, López et al., 2017, Raphael et al., 1994, Rose et al., 2011, Rosenberg et al., 2018, Scope et al., 2013, Yang et al., 2014). Historically, this significant physiologic variation has hindered interpretation of reptile bloodwork and prevented confident differentiation of healthy and unhealthy animals. However, our work demonstrates that physiologic drivers of clinical pathology analytes can be controlled using modeling, and that relevant differences in health status are detectable using routine bloodwork. Specifically, total leukocyte counts (WBC) and H:L values are useful indicators of stress and illness in both eastern and ornate box turtles. Hematology is therefore an important component of box turtle health assessment protocols, and monitoring leukocyte counts may facilitate the identification of populations in need of intervention.

We detected four pathogens in eastern box turtles (TerHV1, *Terrapene* adenovirus, *Mycoplasma* sp., and *S. typhimurium*) and two pathogens in ornate box turtles (TerHV1, *Terrapene* adenovirus) over the course of four years. None of these pathogens significantly predicted wellness, however, our findings establish baseline prevalence data for future monitoring and help characterize aspects of disease epidemiology in box turtles. TerHV1 was originally detected in association with pneumonia in a hatchling eastern box turtle, but subsequent screening of wild turtles prior to and during our study has revealed high prevalence and no associated clinical signs of illness (Sim et al., 2015, Kane et al., 2017). We therefore propose that TerHV1 is a host-adapted pathogen in eastern box turtles, and that clinical signs are rare except in young and immunosuppressed animals (Kane et al., 2017). *Terrapene* adenovirus was first identified in a captive ornate box turtle that died during brumation and had inclusion body hepatitis at necropsy (Farkas and Gal, 2009). It has also been detected in captive and rehabilitating eastern box turtles, with apparently limited clinical effects (Doszpoly et al., 2013, Franzen-Klein, 2020). Our findings indicate that clinical illness in adenovirus-positive box turtles is uncommon, and that like TerHV1, this is likely a host-adapted pathogen.

The results of the present study support a limited role for TerHV1 and adenovirus in individual health outcomes. However, both herpesvirus and adenovirus infections are characterized by viral latency within neural tissues and recrudescence associated with periods of physiologic stress (Maclachlan et al., 2017a, Maclachlan et al., 2017b). Changes in TerHV1 and *Terrapene* adenovirus prevalence may therefore indicate shifts in health status, and long-term monitoring of these pathogens may prove useful for assessing trends in wild box turtle population wellness. Our study also identified significant associations between the detection of *Mycoplasma* sp. and clinical signs of URD, consistent with previous literature in box turtles and the described pathophysiology of *Mycoplasma agassizii* and *M. testudineum* in North American tortoises (Feldman et al., 2006, Jacobson et al., 2014, Ossiboff et al., 2015, Palmer et al., 2016). While *Mycoplasma* 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 may be useful for understanding shifts in individual and population 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 definitely be considered a severe threat to eastern box turtles (Adamovicz et al., 2018). Other pathogens, such as RNA viruses, have also recently emerged as significant threats to free-living chelonians (Waltzek et al., 2019, Zhang et al., 2018). Performing surveillance for a wider range of potential box turtle pathogens may improve our understanding of herptile health threats, and enable the development of targeted disease prevention and control strategies when needed.

Population health is defined in terms of resiliency in the face of stressors. Healthy populations are characterized by stability and persistence despite continual challenge (Deem et al., 2008; Hanisch et al, 2012; Stephen, 2014; Stephen, 2017). Population viability analysis is a useful tool to simulate and explore response to stressors, and was used as a proxy to evaluate population health in this study. PVA revealed that eastern and ornate box turtle populations are highly sensitive to the removal of adult females. This is consistent with other chelonian studies, and is likely due to a combination of several biological characteristics including delayed reproduction, low fecundity, low recruitment, and high adult survivorship – all of which make the survival of reproductively active adults (especially females) crucial for population stability (Brooks et al., 1991; Congdon et al., 1993; Congdon et al., 1994; Crouse et al., 1987; Dodd et al., 2016; Heppell, 1998). In eastern box turtles, the large population size of Forbes made it relatively resistant to extinction. However, the smaller populations in Vermilion County were much more likely to be rapidly driven extinct by the loss of adult females. This was especially obvious for Kickapoo, which was significantly affected by annual removal of a single adult female turtle. The Vermilion county populations are therefore more likely to be negatively impacted by stochastic events such as disease

introduction, and conservation activities may be more impactful in these sites. The ornate box turtle population at Nachusa is also relatively small and susceptible to stochastic events, making it an ideal target for the ongoing management programs which are already underway at that site.

Eastern Box Turtle Health & Management Recommendations

Health assessment of 700 turtles from five populations revealed that individual health was best predicted by a combination of physical exam findings and hematology values. Specifically, turtles with clinical signs of URD (nasal discharge, blepharoedema, and/or ocular discharge), active shell lesions, and deviations from population median values for total leukocyte count and H:L were more likely to be classified as "unhealthy". Prior to modeling, clinical pathology values were normalized based on age, sex, season, site, and year-specific median values. This ensured the model would be generalizable between populations and years, however, it did remove the possibility of evaluating site-specific impacts on individual health.

Several clinical pathology differences were noted between turtles from Forbes and the Vermilion county sites. Generally, these differences (lower heterophil counts, H:L, and relative beta globulins and higher albumin and A:G) indicated a better plane of health in turtles from Forbes. In contrast, turtles from Kickapoo had the highest total solids of all sites, which may be consistent with the production of more inflammatory proteins (Campbell, 2013). Population size was also significantly different between sites, with Forbes having the largest and Kickapoo the smallest box turtle populations. This is not necessarily surprising, as Forbes provides larger and more contiguous habitat options than the highly fragmented and degraded Vermilion county sites. Furthermore, the forest at Forbes is managed more intensively (summer and winter burns) to combat the ingress of invasive species and promote open woodland habitat, which is preferred by box turtles (Dodd, 2001). Management of the Vermilion county sites is less aggressive, and invasive plant species are present in varying degrees at each site. They are most problematic at Kickapoo, where they have significantly reduced the number, size, and quality of field habitats available for thermoregulation and nesting within the last 10 years. Fields at Kennekuk have also been focally heavily reduced by ingrowth of invasive autumn olive shrubs (*Elaeagnus umbellata*), however several large fields remain open and are heavily utilized by box turtles. We found that male turtles typically occupy forest while female and juvenile turtles frequent field habitats, suggesting that the availability of both habitat types is important to support the needs of different sexes and life stages. Our findings correlate positive individual health status and robust population size with improved habitat quality, indicating that habitat restoration may represent a practical approach to support the health of box turtles and other species within the same ecosystem.

In addition to small population size and poor habitat quality, we found that eastern box turtles at Kickapoo also experience a high mortality rate attributable to multiple causes. A radiotelemetry study at Kickapoo documented mortality in 22 turtles between 2016 and 2018 due to organ disease, bacterial infection, dystocia, and overwintering mortality secondary to an unusually warm winter (Rayl et al., 2019). In 2014 and 2015 we also documented the deaths of 10 adult turtles due to ranavirus infection, though many more may have died and not been recovered (Adamovicz et al., 2018). PVA indicates that the current rate of adult loss from Kickapoo is likely not sustainable. Because there is no singular cause of mortality, and many deaths were directly caused or precipitated by poor health, we suggest that management efforts should focus on promoting overall wellness through habitat restoration. Continued research on box turtle health and disease may facilitate the development of more targeted interventions against common causes of morbidity and mortality.

While box turtle health trended towards being better at Forbes, this was the only site where road mortality was documented for box turtles, potentially indicating a need for signage to alert motorists to turtle crossings. Similarly, Forbes, Forest Glen, and Kennekuk were the only sites where box turtles were

observed with burn injuries secondary to land management practices. A more judicious burning schedule which considers the activity patterns of box turtles may reduce the individual health impacts of this management strategy (Gibson, 2009; Howey and Roosenburg, 2013).

Ornate Box Turtle Health & Management Recommendations

Results from four years of ornate box turtle health assessments in 291 individuals at the Nachusa grasslands indicate that shell lesions associated with predator trauma are the most common threat to individual health. Active and inactive shell lesions were highly prevalent in this population (50-60%/year), and approximately half of these lesions were considered to negatively impact health status based on visual examination alone. Turtles with active shell lesions also had multiple clinical pathology abnormalities supporting negative health impacts, including elevations in eosinophil counts, H:L, and creatine kinase, and decreases in relative albumin and A:G. These changes are consistent with tissue damage and inflammation (Campbell, 2013). Interestingly, turtles with inactive shell lesions also had decreased relative albumin and A:G ratios compared to turtles with normal shells, reflecting prolonged physiologic changes that persist after clinical resolution of shell injury. This likely represents a diversion of resources away from growth and reproduction and towards wound healing, a shift that may temporarily or permanently affect fitness, fecundity, and survival.

Population viability analysis revealed that the ornate box turtle population at Nachusa is highly sensitive to the removal of adult females; similar to the eastern box turtle populations in Illinois and multiple published reports (Brooks et al., 1991; Congdon et al., 1993; Congdon et al., 1994; Crouse et al., 1987; Dodd et al., 2016; Heppell, 1998). A recent simulation study in Florida box turtles (*Terrapene carolina bauri*) showed that annual removal of 3.8% of the population would drive stable and declining populations extinct within 50 years (Dodd et al., 2016). One previous report from Nachusa documented seven predation-associated deaths out of 153 uniquely identified individuals (4.6% mortality) over the course of three years, which may be an unsustainable rate of loss for this population (Kim Schmidt, personal communication). Even worse, this is likely an underestimate of the true predation-specific mortality rate due to the poor detectability of ornate box turtles using visual encounter surveys (Refsnider et al., 2011). This is concerning for the wellness of the Nachusa ornate box turtle population, as it demonstrates that the loss of a small number of turtles each year due to depredation/disease/poor health/burn-related deaths is very possible. Furthermore, the overall estimated size of the Nachusa population is small, increasing the potential for stochastic events such as climatological phenomena, increasing predator abundance, or disease to negatively impact population stability.

Management recommendations for the ornate box turtles at the Nachusa Grasslands include strategies that support both individual and population health. Predator injuries are the most commonly identified threat to individual health, and mortality secondary to predator trauma may impact population stability. Therefore, identification and control of box turtle predators, specifically human-subsidized mesopredators present in unnaturally high abundances at the site, may rapidly improve individual health and population viability. Infectious diseases which negatively impact eastern box turtles, such as ranavirus, were fortunately not detected in the ornate box turtles at Nachusa. However, introduction of infectious diseases could negatively impact both individuals and the population. Therefore, if population supplementation methods such as head-starting or translocation are adopted, disease screening must be implemented to reduce the risk of novel pathogen entry.

Silvery Salamander Health & Management Recommendations

The results from the last four years of health assessment in 907 silvery salamanders indicate that parasitism and developmental abnormalities are common, however, the presence of fresh injuries,

hemorrhages, and poor body condition are the best predictors of individual health. The study of silvery salamander health is inherently complicated due to the cryptic nature of this species. Adult salamanders spend much of their time in burrows and only emerge in large numbers for breeding in February and March each year. Assessing health during this window is the best way to obtain large sample sizes, however, this population is likely biased towards animals which are healthy enough to attempt breeding. This may explain why so many of the adults in this study were categorized as "apparently healthy".

Assessing population health is also difficult, as basic biological knowledge such as lifespan, dispersal probability, dispersal distance from natal ponds, degree of philopatry, and survival probabilities of different age classes is uncharacterized or poorly documented in this species. This lack of background knowledge impedes assessment of population viability and prevents modeling the impacts of specific threats such as recurrent ranavirus outbreaks. Future studies on the biology and ecology of silvery salamanders are needed to contextualize health assessment findings and determine when management intervention is needed in response to different threats.

Despite these limitations, we did identify FV3 as a significant threat to silvery salamander larvae, with high morbidity and over 80% mortality (Low, 2019). Ranaviruses, including FV3, have been associated with mortality events in over 100 species of ectothermic vertebrates on every continent except Antarctica (Duffus et al., 2015). Recurrent outbreaks, such as those observed at Kickapoo State Park, can contribute to population declines and extirpations, which is especially concerning for state-endangered species with a limited geographical distribution like silvery salamanders (Teacher et al., 2010; Earl and Gray, 2013; Heard et al., 2013; Price et al., 2014). Within Vermilion County, green frog (*Rana clamitans*) tadpoles act as reservoir hosts by maintaining FV3 within wetlands over time, and wood frog (*Rana sylvatica*) tadpoles act as amplification hosts which precipitate multi-species mass mortality events if they are exposed to infected green frogs (Low, 2019). Potential control measures for ranavirus infection target factors that maintain this pathogen within the environment. Reducing green frog larval abundance can be accomplished through draining wetlands in the fall. This may eliminate a major mechanism of ranavirus persistence and decrease its threat to silvery salamanders. Increasing the distance between wetlands can also constrain the effects of ranavirus outbreaks, and may be an option to consider when planning artificial pond locations. In addition to threatening silvery salamanders, ranavirus infection is also important for box turtle conservation. FV3 has been previously identified in association with eastern box turtle mortality at Kickapoo, though comparison of three gene targets from the EBT-RV and SS-RV indicates that the viruses infecting box turtles and amphibians are genetically distinct. This likely means that multiple strains of FV3 are circulating at KSP, underscoring the need for additional research to understand ranavirus dynamics at this site. Continued health assessment in conjunction with ecological research will enhance our understanding of threats and enable development of effective conservation strategies for multiple herptile species at this and other locations.

Objectives for Future Research

1. Expand disease surveillance to include emerging pathogens which may threaten SGNC in Illinois.
    a. RNA viruses including picornaviruses, ferlaviruses, nidoviruses, arenaviruses, bunyaviruses, bornaviruses, reoviruses, coronaviruses, and parvoviruses.
    b. Parasitic infections including Perkinsus organisms and *Cryptosporidium* spp.
    c. Recently-described box turtle pathogens such as *Terrapene* herpesvirus 2 – a cancer-associated virus.
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. Incorporate these metrics into the health modeling framework to evaluate patterns of disease susceptibility and resistance.

    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
        ii. Eastern massasauga rattlesnakes (*Sistrurus catenatus*)
        iii. Blanding's turtles (*Emydoidea blandingii*)
        iv. Alligator snapping turtles (*Macrochelys temminckii*)
6. Apply health assessment protocols and the health modeling framework to herptile ecological assemblages which include SGNC to identify common causes of poor health and develop effective, community-wide interventions promoting wellness.
    a. Areas of interest include:
        i. The snake community at Carlyle Lake, home to the last viable population of eastern massasauga rattlesnakes in Illinois. Snake fungal disease, aka ophidiomycosis (*Ophidiomyces ophiodiicola*) has been detected in multiple snake species at this site for several years, and is responsible for documented morbidity and mortality in massasaugas. This community provides a unique opportunity to understand the dynamics of natural ophidiomycosis in multiple species, and studying this system may provide insights into effective disease prevention and control strategies which simultaneously benefit both rare and common species.
        ii. Aquatic chelonian and amphibian communities in Lake, Kane, and Dupage Counties. Many of these communities contain both rare and common herptile species which share diseases and serve as sentinels for ecosystem health. Understanding drivers of health in multiple species from the same study sites may elucidate pathogen epidemiology, clarify threats to species conservation, and improve the assessment of overall ecosystem wellness.
        iii. Chelonian and amphibian communities at Kickapoo State Park. This site contains poor quality habitat and at least two circulating strains of ranavirus, both of which degrade local herptile health and contribute to high rates of morbidity and mortality. Kickapoo is therefore an excellent study site for understanding how multiple threats work concurrently to impact herptile wellness. Learning from this system may help prevent similar situations from developing in other IDNR-controlled ecosystems.

Conclusions

This study demonstrates the feasibility of modeling health in wildlife and illustrates its use for identifying clinically useful diagnostic tests and informing practical conservation interventions. Comprehensive approaches to identifying and managing conservation threats are becoming more attractive as wildlife face an increasingly complex array of natural and anthropogenic stressors. The modeling framework developed in this project identifies ways to simultaneously support health and combat threats, and may represent one pathway for improving conservation outcomes in wild species of conservation concern. We have amassed a significant amount of data and new scientific knowledge within the four years covered by this grant. Our goals for Phase 2 are to continue our sampling strategy to further characterize the drivers of health in Illinois herptiles incorporate screening for RNA viruses, and to refine and improve our health models.

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