INFLUENCE OF HABITAT CHARACTERISTICS ON AMPHIBIAN STRESS AND REPRODUCTIVE SUCCESS IN NORTH DAKOTA

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

As amphibians continue to decline, conservation efforts are a necessity in management plans. It is essential to determine the correlation between water characteristics, stress, and habitat alteration with anuran losses. Large portions of diverse wetlands across the state of North Dakota are being lost to agriculture at unprecedented rates and as a result, habitat for anurans is declining. Larval and visual encounter surveys were conducted to distinguish the essential habitat characteristics that are crucial during each stage of amphibian reproduction. In addition to collection of amphibian data, macro-and micro-habitat data were recorded at each site. Captured individuals had their blood drawn and water-borne corticosterone samples collected to assess this environmental stress. This study found that surrounding developed area impacts larval suitability of a habitat and stress levels. It provides an updated suitability model and baseline levels of corticosterone and white cell profiles for a native anuran species.

ACKNOWLEDGMENTS

I would like to thank my supportive advisor Dr. Matthew Smith for all of his invaluable feedback and guidance. Thank you to my committee members Dr. Tim Greives, Dr. Britt Heidinger, and Dr. Shawn DeKeyser for dealing with an answering my many questions. This project would not have been possible without the help of my fantastic field technicians and undergraduate volunteers. A special thanks to my pets, friends, and family that helped motivate me throughout this process. Finally, I would like to thank all of the frogs that let me capture them in order to do my research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC .....Area Under Curve.

BCE.....Bioclimatic Envelope Model.

BRT.....Boosted Regression Tree.

CORT.....Corticosterone.

CV .....Coefficient of Variation.

ENM.....Environmental Niche Model.

GAM .....Generalized Additive Model.

GIS .....Geographic Information Systems.

GLM.....Generalized Linear Model.

HSM.....Habitat Suitability Model.

M/T .....Metamorph/Tadpole (reproductive success).

MARS .....Multivariate Adaptive Regression Splines.

MaxEnt.....Maximum Entropy.

MODIS.....Moderate Resolution Imaging Spectroradiometer.

N/L .....Neutrophil to Lymphocyte Ratio.

NDSU.....North Dakota State University.

NDVI.....Normalized Difference Vegetation Index.

NLF .....Northern Leopard Frog *(Lithobates pipiens)*.

PCA.....Principal Components Analysis.

RF.....Random Forest.

ROC .....Receiver Operating Characteristic Curve.

SDM.....Species Distribution Model.

SVM.....Support Vector Machine.

TSS.....Total Sum of Squares.

USDA.....United State Department of Agriculture.

WMA .....Wildlife Management Area.

YOY .....Young of the Year.

LIST OF APPENDIX FIGURES

CHAPTER 1. INFLUENCE OF HABITAT CHARACTERISTICS ON AMPHIBIAN PRESENCE IN NORTH DAKOTA

1.1. Abstract

Assessments of habitat suitability provide a necessary framework to guide conservation decisions. This is particularly true for amphibians, as they are experiencing unprecedented population declines and extinction events. Northern Leopard frogs *(Lithobates pipiens)* are no exception, as they have been observed declining in the central and eastern regions of North America. In North Dakota, with a large agricultural presence, it is vital to establish the macro- and microenvironmental variables required for Northern Leopard frogs to be present and successfully reproduce. By creating an ensemble habitat suitability model for the state of North Dakota, the areas where agencies can focus amphibian management efforts are shown. Predicted suitability percentages across the state based on macroenvironmental characteristics range from 30-100%, providing an optimistic picture for conservation efforts. When comparing two life stages, adult and larval, the microenvironmental variables which influenced presence varied. Future research addressing possible connections between larval presence with phosphate and surrounding habitat are required to find adequate trends in predicted presence. These results reiterate that suitability models should include multiple life stages to get a more complete understanding of reproductively relevant habitat.

1.2. Introduction

The global decline of amphibians was first recognized in 1989 with the First World Conference of Herpetology (Stuart et al. 2004). A high percentage of anurans (frogs and toads) and caudata (salamanders) are extinct or threatened with extinction, 32% and 47%, respectively (Stuart 2008). These current extinction rates are immense compared to historical rates, anywhere

from 211 to 45,474 times the previously recorded rates (McCallum 2007). Throughout the literature, there is not one driving factor that can be pointed to which explains the large decline in these populations (Blaustein and Wake 1990; Blaustein et al. 1994). Drivers of this amphibian extinction and decline include disease, toxicants, climate change, and habitat loss/destruction (Alford and Richards 1999; Beebee and Griffiths 2005). This combination of drivers needs to be understood to conserve these species.

Diseases affecting amphibians have been found to destroy populations in certain regions. Therefore, it is important to understand the type of impact various diseases have on amphibians. The most lethal of diseases being *Batrachochytrium dendrobatidis*, commonly known as chytrid fungus. One study found that 42% of amphibian species observed had chytrid fungus and the disease to be associated with rapid declines (Olson et al. 2013). Other common diseases affecting amphibians that are not as clearly associated with declines include: ranavirus, red leg syndrome, Lucke herpesvirus, mycobacteriosis, chlamydiosis, and zygomycoses (Daszak et al. 1999; Beebee and Griffiths 2005; Densmore and Green 2007). While disease is a concern for decline in all taxa, toxicants in particular have large impacts on amphibians.

Since amphibians have semi-permeable skin, many heavy metals, chemicals, and pesticides have severe impacts on populations (Carey and Bryant 1995). One of the most well-known of these toxicants is atrazine, an herbicide which was widely applied across the globe (Solomon et al. 1996). In adult amphibians, exposure to atrazine has been correlated to demasculinized and then feminized male African clawed frogs (*Xenopus laevis*) (Hayes et al. 2010). While this example does not directly correlate to declines, these manipulations of the development can have confounding effects. Examples of these are increased susceptibility of disease, inhibition of development, and increased predation (Carey and Bryant 1995).

Currently, climate change presents multiple concerns for amphibians. Similar to the toxicants, there are direct and indirect effects of climate change that lead to declines. The increasing warm and dry conditions resulting from climate change, where waterbodies are disappearing before larvae can complete metamorphosis, directly leading to tadpole mortality (Pounds et al. 1999; Daszak et al. 2005). There are also some direct effects that do not result in immediate mortality but affect overall fitness, such as earlier breeding, changes to geographic locations, and smaller body size (Li et al. 2013). Indirect, additive effects on mortality include increases in predation, pathogens, and UV-B radiation (Beebee and Griffiths 2005; Corn 2005; Li et al. 2013). Climate change, in addition to anthropomorphic changes to the environment, result in amphibian habitat loss and destruction.

Of all the drivers of extinction and declines previously discussed, habitat loss/destruction is the one associated with the greatest losses. A majority of studies related to habitat loss found that fragmentation of wetland systems resulted in the greatest decrease in amphibian biodiversity (Lehtinen et al. 1999; Cushman 2005; Becker et al. 2007). Considering that amphibians need at least three types of habitat; breeding, active, and overwintering, it is clear they need connectivity between wetlands. Areas which are impacted by fragmentation of landscapes are limiting the dispersal of amphibians, impacting both population size and species diversity. As areas are increasingly fragmented and lost, understanding which habitat is suitable for a species is vital.

Throughout the literature, there are many ways to assess habitat suitability, with various methods currently disputed for vocabulary choice, accuracy of predictions, and type of interpretations made (Elith and Graham 2009; Peterson and Soberón 2012). In order to understand the debate surrounding habitat suitability models, it is important to start by defining relevant terms. Habitat suitability models (HSMs) use species occurrence data in combination

with relevant environmental variables to predict potential or actual distributions and forecast probable suitable habitat (Franklin 1995). HSMs, as a term, are used interchangeably with species distribution models (SDMs), ecological niche models (ENMs), and bioclimatic envelope models (BEMs), to list a few (Bradley et al. 2012). Following the recommendation from Bradley et al. (2012) and to reduce confusion, HSMs will be used to describe these predictive models henceforth. The next divide across literature is deciding the best statistical method the HSMs use.

Statistical methods can be split into two groups: presence-only or presence-absence techniques (Elith et al. 2006). Presence-only methods include BEMs, ENMs, and Principal Component Analysis (PCAs). The more frequently used being presence-absence, also known as pseudo-absence or background. This includes methods used in HSMs, such as, maximum entropy (MaxEnt), generalized linear models (GLMs), generalized additive models (GAMs), boosted regression trees (BRT), and random forest (RF). MaxEnt uses artificial intelligence to create potential distributions based on the maximum entropy of the occurrence data (Phillips et al. 2006). GLMs link, using a transformation function selected based on the data, the mean value of the distribution with the weighted sum of the features (Nelder and Wedderburn 1972). GAMs are related to GLMs but use smoothers to fit the data to a regression function (Hastie and Tibshirani 1987). BRT fits multiple linear regression trees and is boosted to account for the error of the trees' prediction (Elith et al. 2008). RF also uses tree predictors but includes many of them for a more accurate prediction (Breiman 2001).

Throughout literature, there tends to be three different processes when it comes to selecting the statistical method for HSMs. MaxEnt has been shown to perform better than other models; while there are caveats, many people opt to use it on its own (Elith et al. 2011; Hallgren et al. 2019). Other researchers decide to use all methods and select the one with the greatest

accuracy using area under a receiver operating characteristic (ROC) curve (AUC) (Allouche et al. 2006, Shabani et al. 2018). A relatively new ensemble method is starting to gain traction in the literature. Ensemble models calculate the weighted averages of all the predicted suitabilities to create one model (Kindt 2018). While all these options can be appropriate, the settings, amount and location of pseudo-absence/background points, and assessment technique need to be selected on a case-by-case basis for each dataset (Barbet-Massin et al. 2012; Merow et al. 2013). Prediction area and number of presence points are important to consider when creating pseudo-absence points to correct for sampling bias, particularly when there is limited land for conservation plans. Management plans need to focus on specific regions to create plans best suited to benefit amphibians in wetlands that have not been destroyed or altered.

In North Dakota, more than 93% of land is privately owned, and as a result, there is limited area to implement management plans (North Dakota Game and Fish Department 2020). Fortunately, the state has over 200 different Wildlife Management Areas (WMAs) that contain around 230,000 acres of land. Within these WMAs, it is crucial to preserve the habitat that incorporates the essential environmental characteristics to maintain amphibian populations and provide suitable breeding habitat. There is little information on amphibian habitat requirements in the upper Great Plains. However, a recent study in central North Dakota used Ecological Niche Theory to address habitat preferences by several amphibian species (Mushet et al. 2012). Using call survey data from 196 km^2 buffers, they found that distance from trees was significant for most amphibians studied, including the Northern Leopard Frog *(Lithobates pipiens)*, the Boreal Chorus Frog *(Pseudacris maculate)*, and the Woodhouse's Toad *(Anaxyrus woodhousii)* (Mushet et al. 2012). They also found that distance to overwintering wetlands was significant for the Northern Leopard Frog as well as every other species except the Boreal Chorus Frog. These

findings present a strong correlation, displaying that anurans prefer wetlands with longer hydroperiods. Also, there was grassland preference in the Boreal Chorus Frog and Wood Frog (Mushet et al. 2012). With the preference for grasslands, this shows the importance in conserving specific areas of the state.

The previous habitat suitability models focused on core habitat requirements for calling adults (Mushet et al. 2012); however, amphibians require both aquatic and terrestrial habitats to complete their life cycle. Amphibians need at least three distinct habitats: breeding, active, and overwintering including the aquatic and terrestrial habitats. Thus, it is also important to evaluate characteristics of aquatic habitats since these areas are critical for breeding and early life stages. In fact, Dodd and Buchholz (2018) showed that amphibians may call and lay eggs in areas that are unsuitable for offspring, thus calling-based survey methods may not reflect habitat requirements for early life stages. It is vital to increase knowledge on habitat suitability throughout all life stages. It is also important to understand which biotic and abiotic characteristics influence early survival in amphibians; surveys should include assessments of tadpole and metamorph populations throughout the breeding season.

Another aspect of the habitat that is important for amphibians is the hydroperiod, which is the amount days that land has enough water for amphibian larvae to metamorphose. Euliss and Mushet compared the effects of excavated and natural wetlands on amphibian populations in western North Dakota (Euliss and Mushet 2004). Excavated wetlands are deeper and have longer hydroperiods, which were found to influence the species of amphibians at these sites. The Plains Spadefoot was one species that was only found in natural wetlands, while most were found in both types (Euliss and Mushet 2004). Tiger salamanders, on the other hand, were found in mostly excavated sites, and their presence influenced whether Chorus Frogs and Northern

Leopard Frogs were present. This shows how the water depth/hydroperiod can influence amphibians' presence, but also how the presence of Tiger Salamanders can influence species presence within North Dakota.

One study, based in Australia, started to look into how habitat characteristics can be used to predict presence. They were able to predict the occupancy of *Litoria raniformis* based on a combination of habitat variables with up to 75% accuracy. Results from this study show that seven of the eleven habitat variables were significant and were used for a univariate logistic regression. Of these variables, the number of layers of aquatic vegetation was the best at predicting occupancy of the species. However, mean height of vegetation, number of layers of fringing vegetation, turbidity, water temperature, and the interaction between hydrology and layers of aquatic vegetation were also factors that were able to predict occupancy (Wassens et al. 2009). Overall, this research began looking into how habitat variables can predict the presence of amphibians, but more work needs to be done in order to understand other species and habitat variables to protect indispensable wetlands.

Currently, most states use a 15-30 m buffer around aquatic habitats to protect wetland species. Buffers are important to maintain terrestrial habitat, but the current regulations do not match the range required for most amphibian species. In a paper by Semlitsch and Bodie (2003), they assessed the criteria of such buffer zones and found they need to be expanded. The core habitat, or range used by a population to travel between wetlands and for foraging, is actually around 159 to 290 m (Semlitsch and Bodie 2003). Similar to the Wassens et al. (2009), terrestrial aspects of habitat are vital for amphibians and need to be included in future assessments to deem a habitat suitable. With published literature focused on Northern Leopard frog habitat being

scarce, research conducted on related species in the Ranidae family can provide a foundation to assess habitat requirements of this species.

One such study was conducted on the Caucasian frog *(Rana macrocnemis)* to determine which environmental characteristics impact distribution in western Asia. Employing two different modeling methods, they found that the Caucasian frog preferred grassland and deciduous forests in higher elevations with lower temperatures (Najibzadeh et al. 2017). Another paper assessed the common frog *(Rana temporaria)* and found that the use of a water body was influenced by the habitat type, water body areas, altitude, invertebrate presence, and grazing. Breeding probability, as assessed by their models, was high, but the common frog did not breed in a large amount of the water bodies that it was present in (Băncilă et al. 2017). However, this is not uncommon; other amphibian species have been known to call and lay eggs in areas unsuitable for offspring (Dodd and Buchholz 2018). Băncilă et al. (2017) infer the models factoring in other life stages will differ when compared to the presence-only data. It indicates a gap in research when it comes to modelling habitat suitability at different reproductive stages instead of creating models solely based on adult presence.

This study will determine potential distribution and suitable habitat for the Northern Leopard frog, *L. pipiens*, across North Dakota. Occurrence data includes detection data from eastern North Dakota and citizen science data across the entire state. Macroenvironmental data will be pulled from online databases in addition to data collected in the field. HSMs will be created using an ensemble technique of the statistical methods with the highest predictive accuracy in addition to GAMs comparing the microenvironmental characteristics influencing presence in two different life stages, adult and larval.

1.3. Methods

The study was carried out in 24 WMAs across the eastern portion of North Dakota, for a total of 38 sites (Figure 1). Sites were selected based on ability to access the land, the aquatic habitat available, and distance to other WMAs. Many of the sites were smaller bodies of water, as they are easier to survey and more likely to support amphibian breeding. All sites were sampled at least once every summer month (April-August) for one year, with sites proximate to NDSU being sampled over all three years (2018, 2019, 2020). During the summer of 2018, the sites near NDSU, in the center of the state, were sampled. Sites in the south were sampled during 2019, and the north was sampled in 2020. While some sites were sampled multiple years, duplicate presence data was removed in order to remove possible bias.

All procedures in these methods followed protocol #A18024 and was approved by the North Dakota State University Animal Care and Use Committee.

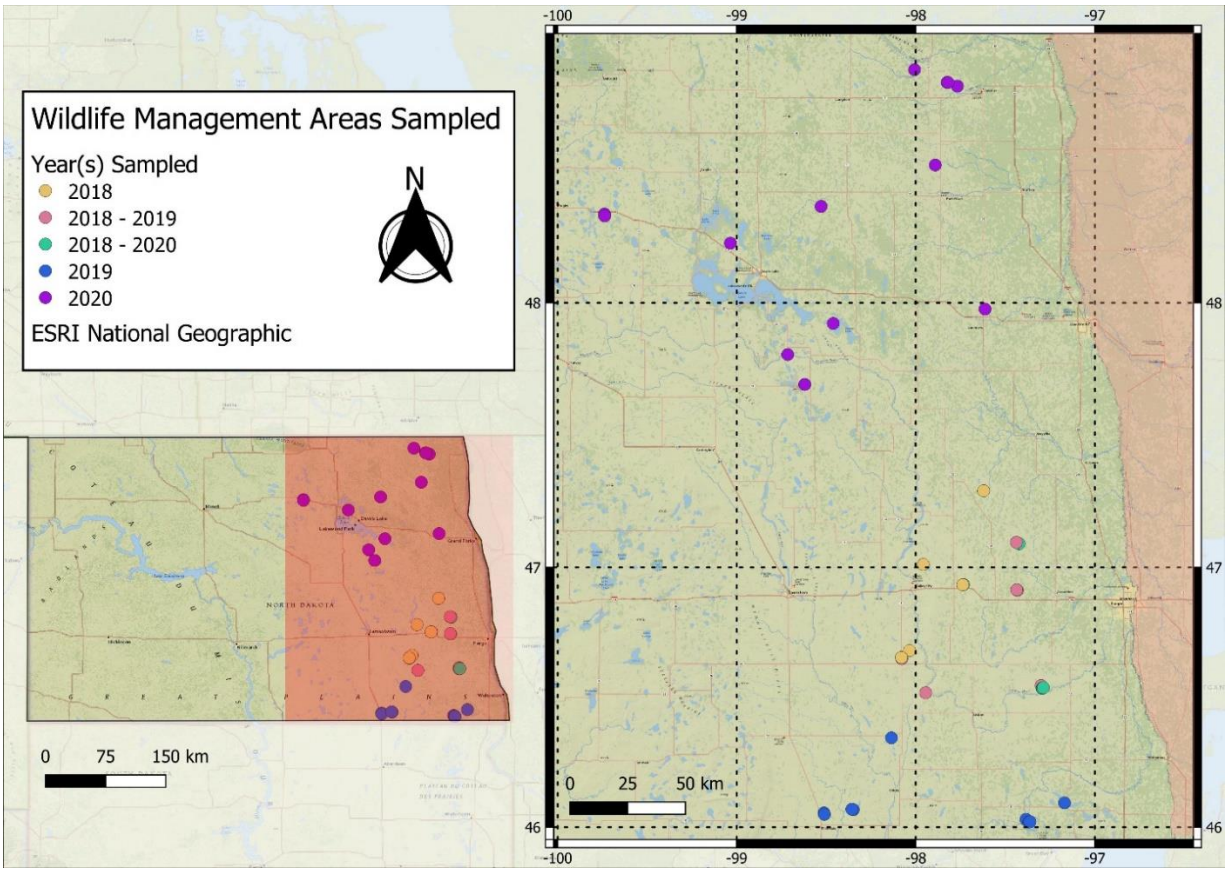

Figure 1: Map of Wildlife Management Areas part of the survey in Eastern North Dakota grouped by year.

At each sample site, larval sampling and visual encounter surveys were used to assess anuran populations. Larval sampling consisted of trapping using multiple activity, minnow, and hoop net traps at each site. At each site, at least 6 minnow traps, 2 activity traps, and one hoop net were used. After 24 to 48 hours, traps were checked, and amphibians were removed, counted, and staged for developmental state. Aquatic invertebrate predators captured were recorded and included dragonfly nymphs (*Sympetrum spp.*), water beetles (*Dytiscidae spp.*), and water scorpions (*Ranatra spp.*), to name a few. All fish that were captured had species determined and the total numbers recorded before being released. The presence of any invertebrate predators and/or fish at a site were considered to have predators present. Larval amphibians were also recorded by the number of each species and estimated developmental stage. Approximate Gosner

stage was recorded for tadpoles, and metamorphs (end of larval stage, with tail resorption) were recorded as young-of-year (YOY) (Gosner 1960).

Each site was assessed using a visual encounter survey. This consisted of the field crew walking around the site/wetland with nets, capturing and recording individuals' approximate age and species. If an individual was not caught but observed, it was recorded as present but not captured. In addition to the occurrence data collected in the field, North Dakota records from the citizen science program, HerpMapper (2021), were used to increase the distribution of presence data in the western portion of the state. Sampling bias was considered when creating pseudo-absence points by random selection within a 30km radius of the presence data (Barbet-Massin 2012).

Physio-chemical water variables were collected at least once a month at each site including pH, conductivity, and dissolved oxygen along three random locations at a water depth of 1 meter. These variables were collected using Oakton Instruments© DO 450 meter and Hanna© Instruments HI 9811-5. The three water samples were separated and placed into 12 vials to test phosphate, nitrate, nitrite, and ammonia levels, using API© aquarium water test kits. Before these tests were run, these water samples were used to collect information on lead, copper, and iron with test strips.

Aerial photos from the USDA and State Water Commission at 0.3-0.6 meter pixel resolution were imported into ArcMap 10.7 to create polygons of habitat type (North Dakota GIS Hub 2019; North Dakota GIS Hub 2020; North Dakota State Water Commission 2018). In ArcMap, buffers of 2km in diameter from the WMA site were added. Habitat within those buffers were categorized as either: wetland, open water, agriculture, prairie/grassland, developed, or wooded/forest. Wetlands were standing water, not defined as open water, or an indication of

standing water, and the shallow vegetative area surrounding the water. Lakes, large ponds, rivers, creeks, and streams that were not stagnant and/or lacked vegetation were categorized as open water. The category of agriculture included crop and hay fields in addition to active pastures, based on field experience and aerial evidence of grazing herds. Prairie/grassland included all grassland, prairie, un-used pastures, and fallow fields, which could include small tree areas (< 3 meters). Developed areas were all man-made features including farmsteads, parking lots, paved roads, quarries, and any area with heavy human traffic/use. Wooded/forest included areas with trees covering more than three meters and including tree lines/shelterbelts.

Two examples of digitized buffer areas can be found in Figure 2. The total number of polygons used to digitize the buffer around each site was added as a measure of heterogeneity. Water characteristics (Table 1), in addition to land cover areas (in square meters) calculated from aerial photographs are considered microenvironmental variables (Table 2).

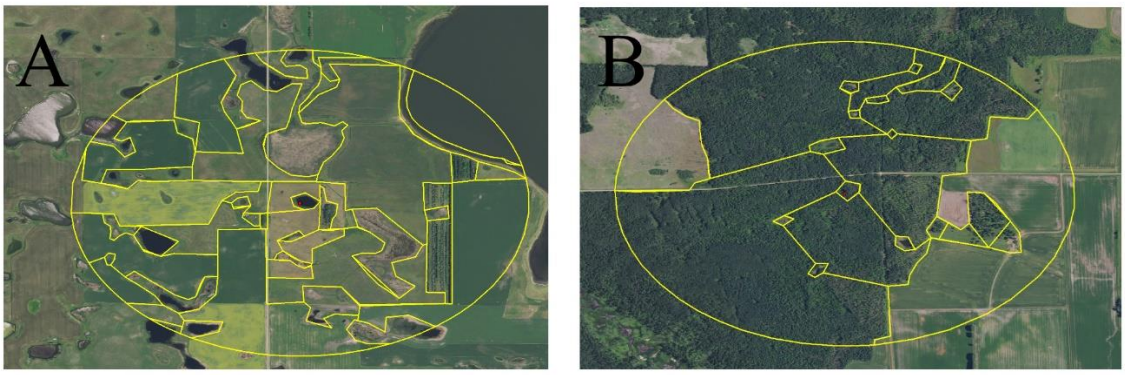

Figure 2: Buffer area with habitat polygons for sites Knox Slough B (A) and Jay V. Wessels B (B).

Table 1: Microenvironmental water variables for each site (mean ± standard deviation).

WMA	Year(s)	NLF	M/T	PO_4^{3-} mg/L	NO_3- mg/L	NO_2^- mg/L	Cu mg/L	NH_3 mg/L	Fe mg/L	Pb mg/L	DO_2 ppm	pH	Cond mS/cm
Black Swan	2020	Yes	Yes	4.44±1.26	0±0	0±0	0±0	0.33±0.14	0±0	6.67±11.55	0.91±0.07	7.8±0.2	853±125
Bluestem Prairie A	2019	Yes	Yes	0.61±0.34	0±0	0±0	0±0	0.06±0.1	0±0	0±0	0.93±0.09	7.1±0.1	320±131
Bluestem Prairie B	2019	Yes	Yes	0.25±0.22	0±0	0±0	0.22±0.38	0.03±0.05	0±0	0±0	1.23±0.5	7.2±0.2	193±106
Brewer A	2018 - 2020	Yes	Yes	1±1.12	0±0	0±0	0.4±0.3	0.07±0.11	0.33±0.75	0±0	0.96±0.14	7.8±1	193±156
Brewer B	2018 - 2019	Yes	Yes	0.47±0.5	0±0	0±0	0.39±0.35	0.14±0.24	0±0	6.67±11.55	0.81±0.32	7.1±0.1	299±104
C. C. Cook	2020	No	No	0.25±0.25	0±0	0±0	0.17±0.29	0.25±0	0±0	0±0	0.93±0.05	7.8±0.1	1567±387
Camp Grafton	2020	Yes	Yes	0.67±0.17	0±0	0±0	0.17±0.29	0.25±0	0±0	0±0	0.92±0.04	7.2±0.1	238±68
Eldon Hillman	2020	Yes	Yes	0.25±0.25	0±0	0±0	0.17±0.29	0.17±0.14	0±0	0±0	0.88±0.03	7.3±0.1	404±71
Grand Forks	2020	Yes	Yes	1.25±1.23	0±0	0±0	0.22±0.25	0.31±0.1	0±0	0±0	0.91±0.12	7.5±0.3	952±175
Jay V. Wessels A	2020	Yes	Yes	0.44±0.51	0±0	0±0	0.17±0.29	0.56±0.29	0±0	0±0	0.78±0.17	7.4±0.2	379±44
Jay V. Wessels B	2020	Yes	Yes	0.47±0.13	0±0	0±0	0.06±0.1	0.11±0.19	0±0	0±0	0.74±0.23	7.4±0.1	415±47
Kenner Marsh	2020	No	No	0.42±0.14	0±0	0±0	0.33±0.29	0.25±0	0±0	0±0	0.9±0.14	7.8±0.3	1646±150
Knox Slough A	2020	Yes	Yes	0.14±0.13	0±0	0±0	0.39±0.35	0.17±0.14	0±0	0±0	0.96±0.05	8±0.2	1349±120
Knox Slough B	2020	Yes	Yes	0.14±0.24	0±0	0±0	0.17±0.29	0.25±0	0±0	0±0	0.93±0.07	8.3±-.1	1787±127
Koldok	2018 - 2020	No	No	0.55±0.18	1.33±1.39	0±0	0.3±0.22	0.17±0.12	0±0	1.33±2.98	1.09±0.63	7.3±0.3	956±526
Magnolia A	2018 - 2019	Yes	Yes	0.56±0.25	2.78±4.81	0.08±0.14	0.44±0.1	0.17±0.29	0.56±0.96	4.44±7.7	1.55±0.87	7.1±0.1	849±371
Magnolia B	2018 - 2019	No	No	0.47±0.05	2.22±3.85	0.08±0.14	0.39±0.54	0.33±0.3	0±0	6.67±11.55	0.83±0.16	7.1±0.1	1014±535
Maple River A	2019	Yes	Yes	1.58±1.31	0±0	0±0	1.5±2.18	0.08±0.14	0±0	0±0	0.8±0.1	7.1±0.1	291±143
Maple River B	2019	Yes	Yes	0.83±0.24	0±0	0±0	0.5±0.71	0.08±0.12	0±0	0±0	1.09±0.3	7.1±0	164±9
Mirror Pool A	2018 - 2019	Yes	Yes	0.39±0.35	0±0	0±0	0±0	1.42±2.24	0±0	4.44±7.7	1.14±0.29	7.2±0.1	172±73
Mirror Pool B	2019 - 2020	Yes	Yes	0.32±0.29	0±0	0±0	0.2±0.22	0.08±0.12	0±0	1.33±2.98	1.04±0.22	7.8±0.9	269±93
Mirror Pool C	2018 - 2020	Yes	Yes	0.32±0.12	0±0	0±0	0.57±0.43	0.13±0.15	0±0	0±0	0.81±0.2	7.3±0.3	231±103
Mirror Pool D	2018 - 2020	Yes	No	0.37±0.25	0±0	0±0	0.1±0.09	0.05±0.11	0±0	0±0	1.17±0.21	7.6±0.6	233±119
Olson	2018 - 2019	Yes	No	0.83±0.29	0±0	0±0	0.33±0.44	0.17±0.14	0±0	6.67±11.55	1.44±1.14	7±0.1	1258±224
Pembina Hills	2020	Yes	No	0.14±0.1	0±0	0±0	0.22±0.25	0.17±0.14	0±0	0±0	0.99±0.01	8.2±0.2	615±40
Ransom	2018 - 2019	Yes	Yes	0.72±0.25	0±0	0±0	0.33±0.29	0.17±0.14	0.56±0.96	6.67±11.55	1±0.12	7.1±0.1	578±65
Seth Gordon	2019	Yes	Yes	0.81±0.27	0.44±0.77	0±0	0.28±0.25	0.33±0.38	0±0	6.67±11.55	0.51±0.4	7.1±0	655±54
Stacks	2019	Yes	Yes	0.78±0.19	0±0	0±0	0.17±0.17	0.08±0.14	0±0	0±0	0.39±0.17	7.1±0	682±44
Tewaukon A	2019	Yes	Yes	2.5±2.29	0±0	0±0	0.06±0.1	0.25±0.17	0±0	2.22±3.85	0.85±0.29	7.1±0	791±446
Tewaukon B	2019	Yes	Yes	1.44±1.36	0±0	0±0	0.11±0.19	0.11±0.13	0±0	0±0	0.87±0.18	7.2±0	350±98
Tewaukon C	2019	Yes	Yes	0.69±0.39	0±0	0±0	0±0	0.08±0.08	0±0	0±0	1.07±0.13	7.1±0.1	1042±329
Warwick Springs	2020	Yes	No	0.75±0.43	0±0	0±0	0±0	0.44±0.57	0±0	0±0	1±0.1	7.8±0.3	431±84
Wild Prairie	2020	No	No	0.86±0.55	0±0	0±0	0.33±0.29	0.17±0.14	0±0	0±0	0.78±0.2	7.6±0.3	806±287

NLF is whether any life stage of Northern Leopard frogs (Lithobates pipiens) was present at a site. M/T stands for metamorphs or tadpole's presence at a site (Yes/No).

Table 2: Microenvironmental habitat variables in square meters for each habitat type.

WMA	Year(s)	NLF	M/T	Prairie/Grassland	Wood/Forest	Developed	Agriculture	Wetland	Open Water	Hetero
Black Swan	2020	Yes	Yes	896256	182567	5847	1214331	519005	312292	35
Bluestem Prairie A	2019	Yes	Yes	1238005	0	10276	518410	1368954	0	93
Bluestem Prairie B	2019	Yes	Yes	1558941	0	0	214072	1368504	0	96
Brewer A	2018 - 2020	Yes	Yes	571648	273215	29418	1906212	110145	230293	36
Brewer B	2018 - 2019	Yes	Yes	1260344	507711	0	862801	480772	26410	37
C. C. Cook	2020	No	No	1115380	62370	67871	862340	1027180	0	34
Camp Grafton	2020	Yes	Yes	2354714	43456	216242	481864	28659	0	26
Eldon Hillman	2020	Yes	Yes	1431561	925219	62006	560755	153528	0	50
Grand Forks	2020	Yes	Yes	174752	809040	73350	1718389	152309	206008	57
Jay V. Wessels A	2020	Yes	Yes	17255	2491368	0	559025	63225	0	25
Jay V. Wessels B	2020	Yes	Yes	17861	2217754	22986	797172	74033	0	24
Kenner Marsh	2020	No	No	1139834	78311	19233	62988	1835857	0	51
Knox Slough A	2020	Yes	Yes	1048193	120644	17052	1055812	576015	308103	49
Knox Slough B	2020	Yes	Yes	977592	141478	4849	1284380	590312	0	47
Koldok	2018 - 2020	No	No	528668	98999	71916	1922800	508841	0	38
Magnolia A	2018 - 2019	Yes	Yes	405906	316602	0	2128144	254338	0	45
Magnolia B	2018 - 2019	No	No	433891	404546	0	1958142	338537	0	39
Maple River A	2019	Yes	Yes	1489197	24412	17172	402520	1204471	0	70
Maple River B	2019	Yes	Yes	1702224	53304	29612	198759	1153282	0	96
Mirror Pool A	2018 - 2019	Yes	Yes	2417033	598716	0	0	118900	0	26
Mirror Pool B	2019 - 2020	Yes	Yes	651923	1645314	2862	378230	202010	247500	61
Mirror Pool C	2018 - 2020	Yes	Yes	657830	1624989	2764	419363	224252	209971	73
Mirror Pool D	2018 - 2020	Yes	No	619625	1448667	2945	541272	340278	186080	57
Olson	2018 - 2019	Yes	No	1307634	0	50262	1383608	392306	0	30
Pembina Hills	2020	Yes	No	107496	1261832	64742	1510112	33123	156709	29
Ransom	2018 - 2019	Yes	Yes	1367975	599716	159477	918433	89918	3389	60
Seth Gordon	2019	Yes	Yes	244958	49675	40685	695135	716737	1388856	48
Stacks	2019	Yes	Yes	457335	166767	59545	914242	1100846	442963	31
Tewaukon A	2019	Yes	Yes	409996	51081	3660	1558962	593123	518225	39
Tewaukon B	2019	Yes	Yes	1288925	158664	0	354913	944844	387334	78
Tewaukon C	2019	Yes	Yes	1449074	142110	0	617033	519243	411408	104
Warwick Springs	2020	Yes	No	2159384	408609	77460	0	319142	169871	37
Wild Prairie	2020	No	No	1444179	35476	22140	503861	1122682	0	27

And the last column denotes heterogeneity, or number of polygons within in the 1 km buffer. NLF is whether any life stage, M/T is reproductive stages of Northern Leopard frogs *(Lithobates pipiens)* was present at a site (Yes/No).

Principal component analysis (PCA) is a method used to analyze a large dataset by creating new variables called principal components from the original data (Wold et al. 1987; Abdi and Williams 2010). The microenvironmental data is exhaustive with 17 variables, and PCA scores are used to represent the data in fewer variables to be used in the GAMs. When considering the first four PCA scores, 55% of the proportion of variance in the 17 original variables are explained. Binomial GAMs using the four PCA scores were ran for two life stages: denoted by NLF and M/T in Tables 3 and 4. NLF refers to sites where only adults were present, but there was no reproduction detected, meaning that they either didn't use the wetland to reproduce or that breeding was missed. M/T denotes that metamorphs and/or tadpoles were present at a site, inferring that there was successful reproduction. All four PCAs were run with smoothers first, and those with empirical distribution functions (EDF) of 1 were changed to be fitted as linear.

Environmental datasets used as macro- variables in model building include the online WorldClim data, North Dakota GIS hub data for hydrologic features and landcover and moderate resolution imaging spectroradiometer (MODIS) normalized difference vegetation index (NDVI) (Hijmans et al. 2005; North Dakota State Water Commission 2020; North Dakota Game and Fish Department 2019; Didan 2015). These macroenvironmental variables were selected based on perceived importance and data availability (Table 3). From this list, variables were removed that were highly correlated using variance inflation factors. As a result, X2, X5, X6, X7, X9, X10, X11, bio12, X16, X17, and X19 were excluded from the remaining analyses for multicollinearity issues.

Table 3: List of macroenvironmental variables used to assess habitat suitability in *L. pipiens* (Hijmans et al. 2005; North Dakota State Water Commission 2020; North Dakota Game and Fish Department 2019; Didan 2015).

Code	Variable Description	Unit
X1	Annual Mean Temperature	Degree Celsius (°C)
X2	Mean Diurnal Range	Degree Celsius (°C)
X3	Isothermality (X2/X7)	Percentange (%)
X4	Temperature Seasonality	Percentage (%)
X5	Maximum Temperature of Warmest Month	Degree Celsius (°C)
X6	Minimum Temperature of Coldest Month	Degree Celsius (°C)
X7	Temperature Annual Range (X5-X6)	Degree Celsius (°C)
X8	Mean Temperature of Wettest Quarter	Degree Celsius (°C)
X9	Mean Temperature of Driest Quarter	Degree Celsius (°C)
X10	Mean Temperature of Warmest Quarter	Degree Celsius (°C)
X11	Mean Temperature of Coldest Quarter	Degree Celsius (°C)
bio 12	Annual Precipitation	Millimeter (mm)
bio 13	Precipitation of Wettest Month	Millimeter (mm)
bio 14	Precipitation of Driest Month	Millimeter (mm)
X15	Precipitation Seasonality	Percentage (%)
X16	Precipitation of Wettest Quarter	Millimeter (mm)
X17	Precipitation of Driest Quarter	Millimeter (mm)
X18	Precipitation of Warmest Quarter	Millimeter (mm)
X19	Precipitation of Coldest Quarter	Millimeter (mm)
NDh2o1	Hydrologic Features	Hydrologic Unit Code (HUC)
LCD	Landcover	Minimum Mapping Unit (MMU)
NDVI	Vegetation Index	NDVI

Habitat suitability of Northern Leopard frogs was predicted using the presence, pseudo-absence and macroenvironmental variables in the sdm package via R 4.0.4 (Naimi and Araújo 2016; R Core Team 2021). Statistical modeling methods included BRT, FDA, GAM, GLM, multivariate adaptive regression spline (MARS), MaxEnt, RF, and support vector machine (SVM) were assessed using five-fold cross-validation with 30% of the presence data used for training. Based on the AUC (>0.75), TSS (>0.5), and Kappa (>0.1) measures of accuracy the top

models were selected, and an ensemble of suitability predictions was created (Figure 3). All R-Script for the HSM and GAMs can be found in Appendix A.

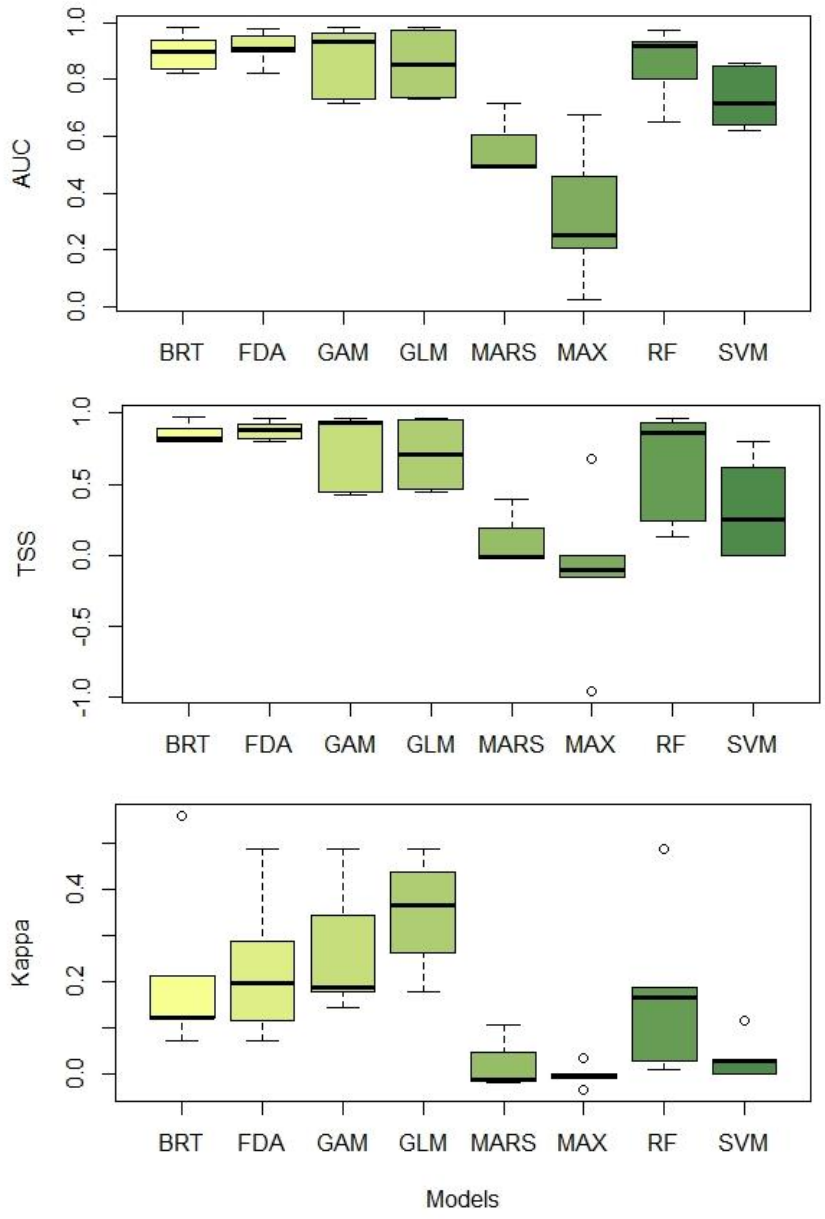

Figure 3: Model performance (AUC, TSS, and Kappa) for each model type based on 5 iterations.

1.4. Results

The HSM for the Northern Leopard frog provides predictions of suitability on a scale from 0-1, with 0 being unsuitable and 1 being suitable (Figure 3). Forecasted suitability across the state of North Dakota ranged from 0.3 to 1. This model is an ensemble of the highest performing statistical methods, GLM, GAM, BRT, RF, FDA (Table 4). The model with the greatest predictive accuracy was BRT, with an AUC of 0.87. However, when looking at TSS, the RF model performed better, reiterating the need for ensemble methods. Average receiver operating characteristic (ROC) curves for each of the statistical methods, which convey diagnostic ability, can be found in Appendix B, Figure B1. All of the ROC curves show fair to excellent accuracy of the training and test data. Selecting the BRT model, the relative importance of the macroenvironmental show variables X8 (mean temperature of wettest quarter) and bio13 (precipitation of wettest month) as influential to the areas with greater forecasted suitability (Figure 5). An ecological niche of the two most influential variables plotted in two-dimensional space can be found in Appendix B, Figure B2. Plots of each variable show environmental niche areas ranging from 0.4 to 1 and do not add any additional information not provided by the suitability map.

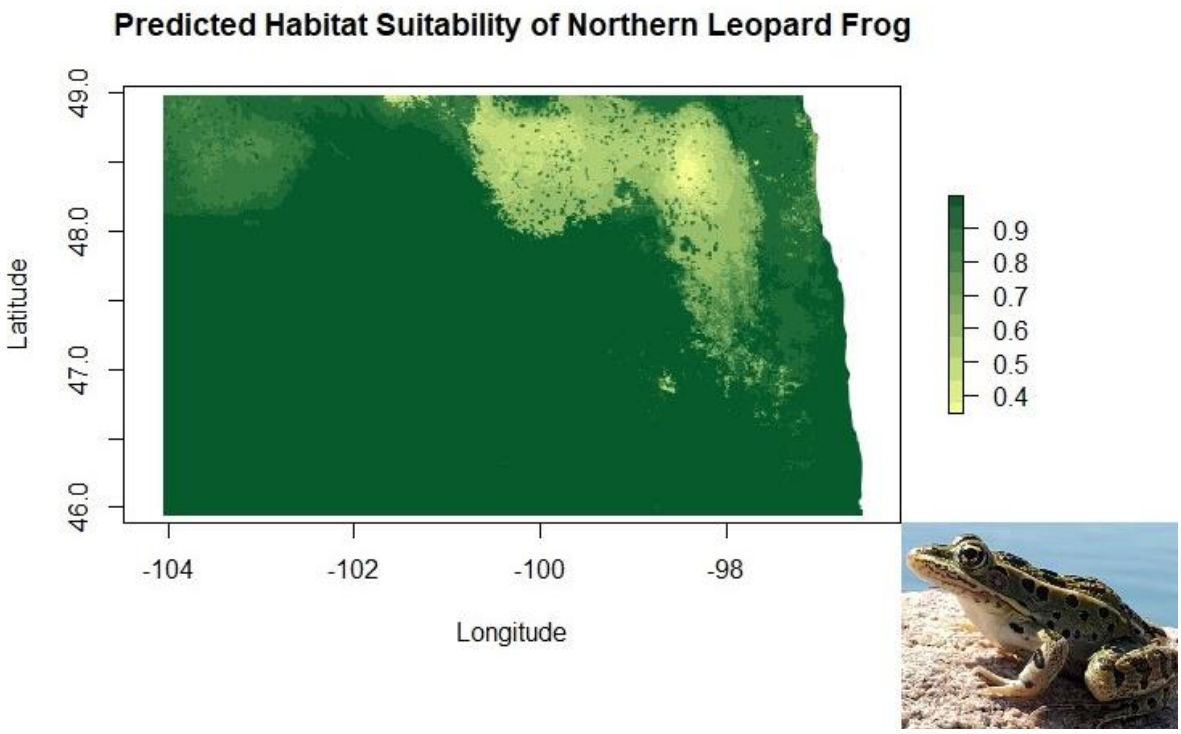

Figure 4: The predicted suitable habitat of the Northern Leopard frog across North Dakota.

Table 4: The performance of the models used in the ensemble to create the predicted habitat suitability model.

Method	AUC	COR	TSS	Deviance
GLM	0.77	0.12	0.58	1.74
GAM	0.76	0.17	0.55	1.80
BRT	0.87	0.17	0.80	0.13
RF	0.86	0.14	0.82	0.14
FDA	0.86	0.17	0.84	0.15

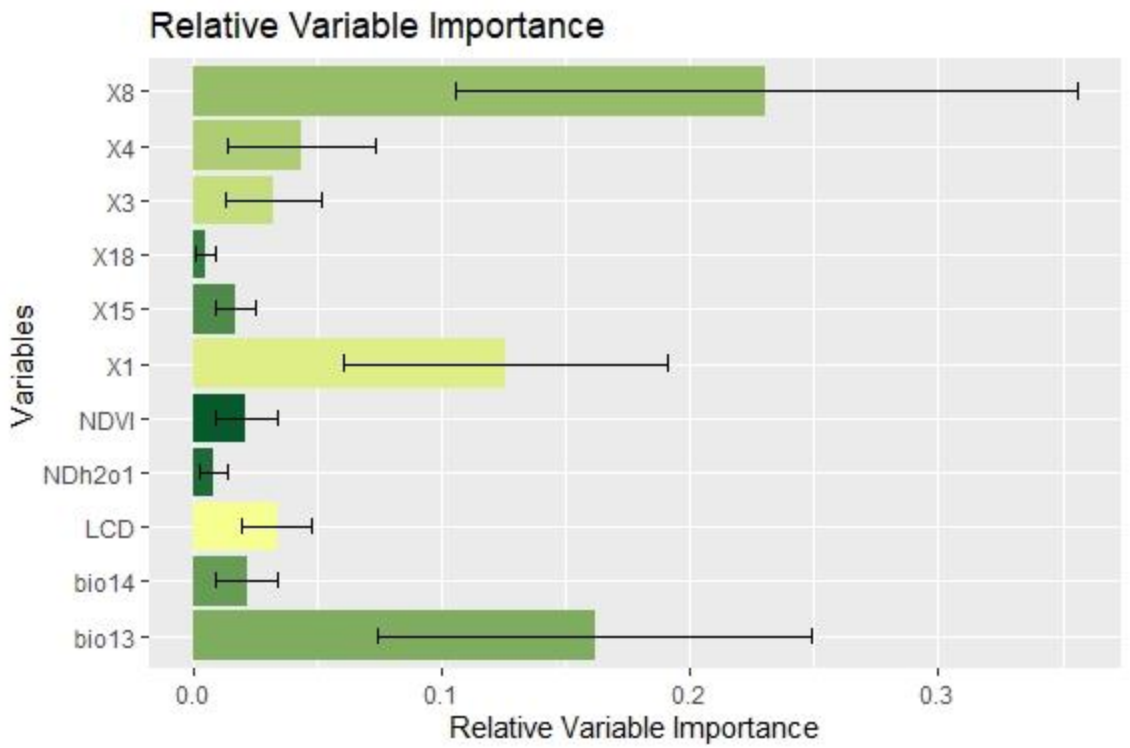

Figure 5: Relative variable importance of the highest performing model (BRT).

The first four principal components cumulatively explain around 55% of the variance in the microenvironmental variables (Table 5). Each of the principal components contain a percent of contribution from each of the micro-variables (Table 6). Nitrate and Nitrite, Wooded/Forest and Wetland, Prairie/Grassland, and Developed variables contribute the greatest to PC1, PC2, PC3, and PC4, respectively. Utilizing the four principal components as predictors with the response variables of NLF (adults, but no reproduction detected) and M/T (reproductive stages present), GAMs were created (Table 7). Plots of smooth functions can be found in Appendix B, Figure B3. These plots show that the effect of PC4 on the NLF model is greater at lower values. For the M/T plots, show the effect of PC3 is greater at both the lower and higher ends of the principal component. This conveys that the extreme values found in PC3 have a greater impact on the presence of reproductive stages.

Table 5: Importance of each principal component used in the GAMs.

Importance of Components	PC1	PC2	PC3	PC4
Standard Deviation	1.92	1.50	1.37	1.25
Proportion of Variance	0.22	0.13	0.11	0.09
Cumulative Proportion	0.22	0.35	0.46	0.55

Table 6: Loadings of each of the microenvironmental variables to each of the principal components.

Micro-Variable Contribution	PC1	PC2	PC3	PC4
Phosphate	0.13	0.64	12.61	15.48
Nitrate	20.15	1.51	0.01	8.59
Nitrite	19.04	1.54	0.00	9.69
Copper	0.67	7.14	1.90	0.14
Ammonia	0.31	0.21	15.49	0.88
Iron	7.15	0.00	2.22	0.53
Lead	6.36	0.78	3.89	2.91
DO2	6.59	2.27	2.42	0.00
pH	0.03	6.00	0.07	0.13
Conductivity	11.81	4.33	2.77	0.04
Prairie/Grassland	4.53	16.02	18.02	0.19
Wooded/Forest	0.45	25.91	0.02	15.76
Developed	0.01	0.04	14.25	20.27
Agriculture	16.76	1.72	2.12	7.21
Wetland	2.49	23.95	6.54	0.10
Open Water	0.12	1.34	10.29	8.77
Hetero	3.39	6.61	6.73	9.30

Table 7: Results from the GAMs, first equation is for the adults, second is for larval stages.

Family: Binomial, Link Function: Logit Formula: NLF ~ PC1 + PC2 + s(PC3) + s(PC4)				
Parametric Coefficients	Estimate	Standard Error	Z-Value	Pr>|Z|
(Intercept)	10.11	16.15	0.63	0.53
PC1	-0.42	0.38	-1.10	0.27
PC2	0.02	0.29	0.08	0.94
Smooth Terms	Edf	Ref.df	Chi.sq	P-Value
s(PC3)	3.37	3.82	0.10	0.90
s(PC4)	2.10	2.67	1.52	0.59
R-sq. (adj) = 0.21, Deviance explained = 36.5%, AIC = 48.05				

Formula = M/T ~ PC1 + s(PC2) + PC3 + s(PC4)				
Parametric Coefficients	Estimate	Standard Error	Z-Value	Pr>|Z|
(Intercept)	3.05	3.53	0.87	0.39
PC1	-0.72	0.74	-0.98	0.33
PC3	2.01	1.18	1.70	0.09
Smooth Terms	Edf	Ref.df	Chi.sq	P-Value
s(PC2)	4.97	5.99	5.67	0.46
s(PC4)	5.28	6.08	3.99	0.67
R-sq. (adj) = 0.52, Deviance explained = 62.9%, AIC=47.87				

1.5. Discussion

In this study, predictive suitability models were created for the Northern Leopard frog *(Lithobates pipiens)* using an ensemble of the most accurate statistical models. Results show that a majority of North Dakota provides potentially suitable habitat for this species, with all of the state showing at least 30% suitability. One concerning aspect of this prediction is that the areas with lower suitability across the northeastern part of the state overlaps with the Prairie Pothole Region. While sampling bias was considered in the analysis by specifying pseudo-absence points near the presence data, it is possible that more presence data in the southwest could increase

homogeneity of the model. A possible biological explanation for this prediction is that Northern Leopard frogs require permanent waterbodies for over-wintering. Larger rivers and lakes can be found in the western side of the state, considering they migrate around 1.6 km a year, it is expected that they stay near these over-wintering sites (Kendell 2002).

When assessing statistical methods for the HSM, there is not a consistent framework for performance cutoffs across the literature. AUCs, which are above 0.7, are generally considered reasonable, but there have been arguments for only looking at the TSS or Kappa measures (Swets 1988; Leroy et al. 2018). There are similar inconsistencies for a majority of aspects of these predictive models. The best approach currently is to use an ensemble of methods, but it is clear that there is still more work that needs to be done in this field to create the most effective models (Shabani et al. 2016). Additionally, the greater the presence and absence data, the more accurate the model, so effort should be made to increase sample size and dispersion.

Macroenvironmental variable selection is extremely important in HSMs. While the variables that were the most important to the BRT model, precipitation and temperature of the wettest quarter/month, are biologically relevant; it is likely that some crucial variables were missed in the analysis (Austin and Van Niel 2011; Mod et al. 2016). The wettest quarter/month of the year in North Dakota is in and around June, during Northern Leopard frog breeding season. As a result, the temperature and precipitation during this time is important for suitable reproductive habitat in addition to suitable overwintering habitat with increased hydroperiods. To combat this possibility that variables were missed, variance in microenvironmental variables were used to assess habitat preference between adults and larval individuals.

Unfortunately, neither of the two GAMs were significant, and they both had relatively large AIC values. The response variable selected (NLF or M/T) did change which terms required

smooths. PC3 was nearing significance for the larval stages at 0.09. The five micro-variables, which contributed the most to this principal component, were total areas of prairie/grassland, developed, and open water in the buffer and the variance of phosphate and ammonia concentrations in the waterbodies. It is not surprising that these habitat types could be indicative of presence. Northern Leopard frogs are commonly called grass frogs and are often found in grassland habitats. One exciting outcome is that there was a difference in the significance of the principal components between the two life stages, as one was nearing significance, showing that larval stage occurrence should be considered in future studies and suitability predictions.

It is not surprising to find phosphate as a possible predictor of metamorph or tadpole presence. Previous research on salamanders, show a preference for wetlands with lower phosphate (Ficetola et al. 2011). It is possible that Northern Leopard frogs in this region are acclimating to environments with changing phosphate and ammonia levels, due to agriculture, so a larger sample size may indicate a stronger relationship (Mann et al. 2009). Conductivity did not contribute greatly to PC3, where previous research has found high variance of conductivity levels in wetlands deterred presence (Sanzo and Hecnar 2006). One study found that high conductivity increased the baseline corticosterone in a related species, *Rana sylvatica*, impacting physiology (Chambers 2011).

This study can be utilized by local agencies to focus future survey and conservation work in areas of high suitability. With limited land available, the WMAs with over 90% suitability, according to our HSM, should be where conservation efforts are concentrated. Levels of phosphate and ammonia need to be assessed in the wetlands of the selected WMAs to determine if reproduction will occur. Additionally, the habitat/land types around the wetlands should be evaluated by areas in a buffer of prairie, developed, and open water. These can fit into the three

habitats required by amphibians: breeding, active, and over-wintering. The amount of open water can be correlated with overwintering habitat. Developed areas, with human impacts, can alter a site making it unsuitable for breeding. Finally, prairie/grassland habitat is useful for the active season.

Considering the complexity of the effects between presence and various environmental data, research with greater presence/absence data in the southwestern portion of the state is recommended. Additionally, agencies should consider the inconsistencies of the literature surrounding HSMs. When creating future models, it is important to be consistent but also revise methodology based on new recommendations. While increased wetland and amphibian surveys can add to the results of this study, this was all that was able to be accomplished within our time and budget constraints. This study provides a framework for future amphibian conservation goals and directions for possible trends in the state of North Dakota.

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CHAPTER 2. INFLUENCE OF HABITAT CHARACTERISTICS ON NORTHERN LEOPARD FROG *(LITHOBATES PIPIENS)* STRESS IN NORTH DAKOTA

2.1. Abstract

As amphibian decline continues, it is vital to assess the required habitat characteristics for persistence. While there are multiple methods for evaluation of suitable habitat, many do not consider the impacts of environmental variables on immune function/stress responses. Previous research has addressed the impacts of predation, pollutants, and competition on amphibian stress in laboratory settings, while few consider correlations with habitat variables in the field. To fill this gap in the research, this study determines if there are correlations between microenvironmental variables and two measures of stress: corticosterone levels and white blood cell profiles in larval Northern Leopard frogs *(Lithobates pipiens)*. In this study, water-borne corticosterone methods were used in conjunction with blood smears to minimize impact to populations in wildlife management areas across eastern North Dakota. Average white blood cell profiles show greater numbers of all leukocytes; except lymphocytes and the neutrophil to lymphocyte ratio is positively correlated with the area of surrounding developed land. While no significant correlations were found with the corticosterone levels, this study provides a baseline for larval Northern Leopard frog stress in North Dakota.

2.2. Introduction

Amphibian global decline has been associated with various stressors including habitat alteration and destruction (Delis et al. 1996; Pounds et al. 2006). One method to evaluate if habitat is suitable is to examine stress levels of amphibian populations in various habitats. Any change to the habitat, including habitat loss and increased pollutants, could negatively impact amphibian stress. One effect is on glucocorticoid levels, which is a steroid hormone that

regulates growth, reproduction, behavior, survival, cognition, cell proliferation, and immune function, through the hypothalamic-pituitary-interrenal axis (Chambers et al. 2011; Oakley and Cidlowski 2011). For amphibians in particular, the glucocorticoid that is involved in stress is corticosterone (CORT), which increases in response to a stressful stimulus. Examples of such stimuli include predators, disease, and environmental factors (Burraco et al. 2015).

Any type of altered environment, whether natural or human, has been found to increase interrenal and CORT dysfunction (Falso et al. 2015), but there are still gaps in our knowledge of acute and chronic stress in amphibians. CORT is measuring acute, or short-term stress, but white blood cell profiles measure chronic, or long-term stressors (Falso et al. 2015). By recording both CORT and leukocyte profiles, it provides a more complete picture of the individual's stress, instead of relying on one method. While there is some research on stress levels in the herpetological field, many are controlled laboratory experiments and lack the use of free-living amphibians responding to naturally occurring, chronic stressors (Moore et al. 1991; Falso et al. 2015).

Understanding stress response is necessary to provide insight into how amphibians are adapting to long-term stressors (Moore and Jessop 2003). Moore and Jessop (2003) point out that there is large variation in stress response due to varying physiological conditions such as sex, age, reproductive status, disease, and body condition, also known as adrenocortical modulation. Also, many changes during amphibian development are mediated by CORT including cell differentiation, hepatic enzyme activity, and tail fin absorption (Chambers et al. 2011). For this reason, it may be difficult to determine a single cause for abnormal CORT levels.

Recently there has been research addressing whether urbanization of wetlands can be correlated with elevated CORT levels. A study that assessed free-living salamanders (*Eurycea*

tonkawae) and their baseline CORT levels shows that the baseline levels of CORT were higher in the urban sites during 2012 and 2013 but were higher in the rural sites in 2014 (Gabor et al. 2018). This research used a general comparison between rural and urban sites but does not go into the specific habitat characteristics found within these sites that could be correlated with the elevated baseline CORT.

A second method that has been used to assess immune function and stress levels is through blood profiles. Of the circulating white blood cells or leukocytes, two are altered by stress with an increase in neutrophil and a decrease lymphocyte levels (Davis and Maerz 2008). One study suggests the release of CORT may cause a shift in the distribution of leukocytes and may affect the response of the immune system to stress (Dhabhar et al. 1996). While there is still a large gap in our knowledge of how these leukocyte levels are altered, it does occur predictably, and many researchers assess stress based on the neutrophil and lymphocyte ratios. One example by Davis and Maerz (2008) used blood profiles to study paedomorphic mole salamanders *(Ambystoma talpoideum)* that were exposed to an altered environment that resulted in a ratio of neutrophil to lymphocytes to be, on average, twice as high as the wild-caught salamanders.

However, there has been some concern as to whether glucocorticoid levels and leukocyte profiles are appropriate measures of physiological state, which is addressed in two reviews. These papers found that leukocyte profiles and glucocorticoid assessments both have their advantages; it is important to understand the parameters of these tests when drawing conclusions (Davis et al. 2008). Another problem that they address in these reviews is that stress induced by disease can be quantified by assessing the eosinophil levels, part of the leukocyte profiles (Davis et al. 2008). Eosinophils are known to be reduced in response to disease where monocytes will increase in cases of infection, providing a way to assess if other factors could be contributing to

the changes in leukocyte profiles. The second review concluded that the Cort-Adaptation Hypothesis, which includes reproduction, is more accurate than the Cort-Fitness Hypothesis (Bonier et al. 2009). Cort-Adaptation hypothesis makes the prediction that an increase in glucocorticoids will increase reproduction. Whereas the Cort-Fitness hypothesis predicts that with increased glucocorticoids, all measures of fitness will decline. This provides information on how to accurately assess the physiological state of amphibians substantiating the need for additional research to see if the Cort-Adaptation hypothesis can be substantiated in other species.

One recently published study looked at the effect of pesticides on both white blood cell profiles and CORT concentrations in the Northern Leopard frog *(Lithobates pipiens)* (Gavel et al. 2021). Amphibians were removed from remote wetlands in Ontario, Canada, and placed into outdoor mesocosms with various pesticide treatments to determine the effects on blood cell profiles and CORT. White blood cell profiles were around 93-96% lymphocytes, 1-3% neutrophils, 1-2% eosinophils, and 1% monocytes and they did find a change in leukocytes based on neonicotinoid (pesticide) exposure. Unfortunately, they do not report the CORT levels, but they did not find any significant relationship between them and experimental groups (Gavel et al. 2021). Assessment of pesticide impacts on Northern Leopard frog immune function is necessary, but understanding how environmental characteristics influence white blood cell profiles and CORT can add to conservation plans.

While all of this previous research has contributed greatly to our knowledge of amphibians, there are still some areas that require further assessment. Amphibians across North Dakota have not been properly assessed to understand which habitat characteristics are required from the small amount of public land available. North Dakota is mostly comprised of prairie pothole habitat with a wetland loss of 49% from the 1780s to the 1980s (Dahl 1990; Yager 1996;

Euliss and Mushet 1999; Gleason et al. 2008). Due to the loss of wetland habitat across the state, it is extremely important to understand what is required of the habitat to allow for persistence of anuran species. In order to understand this, traditional methods such as trapping and visual encounters are useful, but novel methods to assess correlations of habitat characteristics to CORT levels and leukocyte profiles can add to our overall assessments. Goals of this study are to determine baseline stress levels in this region, while using linear regressions to see if microenvironmental variables are correlated with either one or both measures of immune function.

2.3. Methods

This study was conducted across 24 wildlife management areas (WMAs) in eastern North Dakota. Wetlands within these WMAs were selected based on the ability of the researchers to access the land, and the proximity to other sites, to conserve time for surveys. Each of the sites were sampled at least once a month during the breeding season (April to August) in 2018, 2019, and 2020. Sampling consisted of collecting microenvironmental data in addition to larval trapping to assess presence.

Larval sampling consisted of placing at least 6 minnow traps and 2 activity traps at each site and were checked after 24 to 48 hours. In traps, if there were more than five larval Northern Leopard frogs, blood samples were collected (< 20 µL). These samples were assessed through blood smear slides; we only collected from the Northern Leopard Frog (*Lithobates pipiens*) at four life stages at the WMAs. Larval individuals had approximate Gosner stage recorded and were grouped by that stage. The Gosner stages that the larval samples were grouped by are 20-25, 25-35, 36-41, and 42-46 (Gosner 1960). Water-based CORT sampling occurred by placing individuals into a beaker for ~30 minutes (Tapley et al. 2011). Blood was collected from larval

individuals from the caudal vein for the blood smear slide. For all of the blood samples, a venous puncture using a 25-27-gauge needle with a 70µL heparinized micropipette was used, as it has been previously assessed to cause minimal trauma during collection. After CORT and blood samples were collected tadpoles were released back into the wetland. Blood smear slides were created in the field, brought back to the lab, and stained at later dates using Hema 3™ Manual Staining System.

Microenvironmental data can be divided into two groups: physio-chemical water variables and area of habitat types with 2km buffers around the WMA. Water characteristics assessed included: phosphate, nitrate, nitrite, ammonia, lead, copper, iron, dissolved oxygen, pH, and conductivity. Oakton Instruments© DO 450 meter, Hanna© Instruments HI 9811-5 meter, and API© aquarium water test kits were used to measure the water variables. In ArcGIS 10.7, two km buffers were placed around the study site, and polygons were created based on habitat type. The categories of habitat types included: wetland, open water, prairie/grassland, developed, wooded/forest, and agriculture. Total number of polygons that make up the buffer were used as a measure of heterogeneity and recorded for each site. Generalized additive models were created using the mcgv package in R 4.0.3, using a principal component analysis to determine which microenvironmental variables could predict larval presence. The variables which contributed more than 10% to the principal component, which was near significance, were selected for linear regressions. These variables include variance in phosphate, and ammonia, in addition to area of prairie/grassland, developed, and open water within a two km buffer of the WMA. All of this is further explained in Chapter 1.

Stained blood smear slide images were taken using the Leica DM500 Biological Microscope at 400X magnification. White blood cells were counted and categorized up to 100

total leukocytes and the frame was finished. The method used was differential leukocyte counts where images were taken at the top, once cells were no longer touching and went down in a snake like pattern until the 100 cells were counted or 40 images were saved. Since amphibian leukocyte counts are not a perfect science, an average from three different counters were taken for each blood slide. Examples of the five cell types can see found in Figure 6.

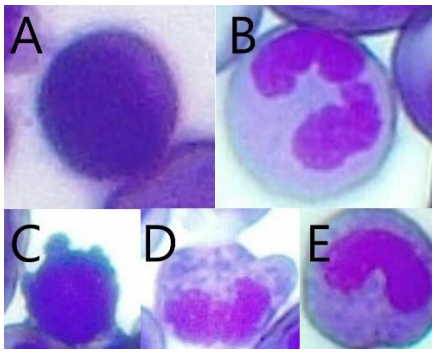

Figure 6: White blood cell examples. A: Lymphocyte, B: Neutrophil, C: Basophil, D: Eosinophil, E: Monocyte.

In order to ensure survival, we did not use blood CORT for these individuals due to the large quantity needed (Teixeira et al. 2012). For larval and adult anurans, we used a water-borne CORT sampling method since too much blood is required to run a plasma CORT assay for the small body mass. The water-borne test consisted of placing the larval individuals in a sterilized beaker filled with ~40 ml of pond water from the site where they are collected, for 30 minutes, to collect water-borne hormones. It has been established that there is not an increase or decrease in CORT levels between 30-120 minutes, so 30 minutes was selected to minimize stress to the individuals (Gabor et al. 2013). Time of day when each sample was collected is recorded and limited to minimize the effects of circadian variation. The individual weights of each tadpole in the sample were recorded. Then, they were released, and the sample was labeled. Additionally, a sample of the site water was taken to account for circulating water-borne hormones, not specific to the individuals (Gabor et al. 2013).

All water samples were randomly assigned a number and recorded by site and the individual's approximate age and kept in a freezer in the lab until the CORT assay was completed following protocols established by Gabor (Gabor et al. 2018). The samples (~40ml) were thawed and pumped through a pre-filter to remove and debris and then through a Sep-Pak C18 cartridge to absorb the CORT from the sample. Next the cartridge was eluted with methanol to extract the CORT and dried under a stream of nitrogen gas. This was then reconstituted overnight in 0.175 ml of Elisa Buffer and in the morning diluted 1:32 with UltraPure water. Finally, Cayman Chemical Elisa Corticosterone kits were used and provided protocols followed. The complete protocol can be found in Appendix C. The exact volume of water from each sample was recorded for the calculation of CORT. Any samples where the intra-assay coefficient of variation was over 10% were removed. Two sites during the summer of 2020 have a subset of larval individuals that were captured with hand nets to ensure that the traps are not skewing the CORT results.

Multiple linear regressions were conducted to look for a relationship between the average neutrophil/lymphocyte ratio or CORT and the microenvironmental variables or principal component 3 from Chapter 1 (reaching significance for tadpoles).

All procedures in these methods followed protocol #A18024 and was approved by the North Dakota State University Animal Care and Use Committee. No animals underwent anesthetic or euthanasia during this study.

2.4. Results

Average neutrophil, lymphocyte, eosinophil, basophil, and monocyte counts are 22, 57, 4, 7, and 5, respectively, across all WMAs and stages (Table 8). With the average neutrophil/lymphocyte ratio being 0.4.

Table 8: Mean ± standard deviation of the white blood cell profiles at each WMA that Northern Leopard Frog tadpoles were captured.

WMA	N	Stage	Frames	Leuko	Neutro	Lympho	Eosino	Baso	Mono	Avg N/L
Black Swan	4	3	14±2	102±1	11±3	55±8	21±11	12±4	3±2	0.20
Brewer B	19	2	23±6	103±2	19±10	82±11	0.1±0.5	0.9±0.8	0.8±0.9	0.23
Camp Grafton	8	3	35±7	101±2	34±5	14±3	12±2	30±4	10±4	2.43
Camp Grafton	4	4	29±3	100±1	38±6	19±7	10±5	23±5	9±4	2.00
Eldon Hillman	4	3	25±4	108±17	53±2	17±2	6±3	14±1	9±2	3.12
Grand Forks	11	3	29±8	85±17	10±6	55±13	3±9	4±8	8±7	0.18
Grand Forks (H-C)	7	3	31±8	89±16	8±4	57±14	2±2	10±5	13±4	0.14
Knox Slough A (H-C)	19	2	31±3	95±11	37±13	26±9	4±3	18±4	10±3	1.42
Knox Slough A	3	3	27±3	100±1	37±7	34±13	3±2	15±5	11±3	1.09
Knox Slough A	11	4	31±5	100±1	41±8	28±8	7±4	16±5	8±3	1.46
Knox Slough B	4	1	33±2	69±16	8±9	45±5	2±0	7±4	6±3	0.18
Knox Slough B	17	2	33±9	66±17	10±6	35±11	4±3	7±5	10±5	0.29
Knox Slough B	8	3	29±4	71±16	10±5	35±16	6±6	4±4	16±6	0.29
Magnolia A	4	2	21±8	105±3	17±4	85±5	0.3±0.4	0.7±0.5	2±1	0.20
Maple River A	15	2	21±7	97±14	14±8	80±15	0.3±0.6	0.7±1	1±1	0.18
Maple River A	3	3	23±6	96±6	9±8	84±14	1±1	0.7±0.3	2±3	0.11
Maple River B	3	2	6±5	66±6	21±6	66±22	0±0	0.3±0.3	2±0.4	0.32
Maple River B	4	3	20±1	103±2	11±2	88±3	2±0.7	0.8±0.7	1±1	0.13
Mirror Pool B	4	1	15±32	105±4	16±6	88±6	0±0	0.3±0.5	0.4±0.8	0.18
Mirror Pool B	14	2	16±6	100±8	29±16	70±15	0.1±0.3	0.1±0.1	2±1	0.41
Mirror Pool C	4	2	24±9	94±9	37±12	54±11	0.2±0.3	0±0	2±2	0.69
Tewaukon A	15	2	14±6	104±4	32±7	70±6	0±0	0.2±0.4	1±1	0.46
Tewaukon B	20	2	14±5	103±3	19±9	83±9	0.1±0.2	0.1±0.3	1±1	0.23
Tewaukon B	15	3	12±2	104±2	24±11	79±11	0±0	0.02±0.0	0.7±0.5	0.30

N is the total number individuals sampled. Stage is the Gosner grouping. Frames refer to the number of frame shifts needed to get to the total of 100 leukocytes (mean ± standard deviation). The rest of the columns are as follows, leukocytes (total WBC), neutrophils, lymphocytes, eosinophils, basophils, and monocytes. H-C refers to individuals which were hand-caught with a net instead of through trapping.

A scatterplot matrix showing how each of the microenvironmental variables selected interact with the two measures of stress (corrected CORT and average N/L ratios) and the habitat suitability index score from the habitat suitability model in Chapter 1, graphically shows the different linear regressions conducted (Appendix B, Figure B4). The average CORT release rates that were included after subtracting the circulating CORT from the wetland water without tadpoles, correcting for resuspension volume (0.175 ml) and number of individuals (5), are 37 ± 33 picograms per minute (Table 9). All samples with an intra-assay %CV of greater than 10 were removed (4 samples removed). The inter-assay %CV was 16%.

Table 9: Average Corticosterone released in picograms per minute (mean ± standard deviation) for all sites. N is number of samples and stage is developmental group.

WMA	N	Stage	CORT (pg/min.)
Camp Grafton	1	2	25±0
Camp Grafton	1	3	1±0
Grand Forks	5	2	28±26
Grand Forks	2	3	60±12
Knox Slough A	4	2	14±26
Knox Slough A	2	3	30±4
Knox Slough B	1	1	15±0
Knox Slough B	4	2	19±34
Knox Slough B	1	3	15±0
Maple River A	2	2	3±1
Maple River A	1	3	62±0
Maple River B	3	2	113±99
Maple River B	1	4	5±0
Mirror Pool B	4	2	2±1
Mirror Pool C	4	2	51±38
Mirror Pool C	1	3	55±0
Tewaukon A	2	2	84±49
Tewaukon A	1	4	0.1±0
Tewaukon B	3	2	91±30
Tewaukon B	3	3	61±17
Tewaukon B	1	4	4±0

Multiple linear regressions were conducted to look for a relationship between the average neutrophil/lymphocyte ratio or CORT and the microenvironmental variables, while controlling for the stage of the tadpole and the sample size. A significant regression equation for average N/L ratios and Principal Component 3 was found ($F_{(3,20)} = 15.19$, $p<0.0009$), with an R^2 of 0.3992. Another significant regression was for the average N/L ratios and surrounding area of prairie grassland with ($F_{(3,20)} = 5.69$, $p<0.039$), with an R^2 of 0.158. Instead of prairie/grassland habitat, the surrounding developed area was also significant ($F_{(3/20)} = 16.65$, $p<0.001$), with an R^2 of 0.34. Both of these indicate a slightly positive relationship between the N/L ratio to area of prairie and developed land and a negative relationship to principal component 3. All other regressions conducted were not significant, while some approached significance (Table 10).

Table 10: Multiple linear regressions conducted with the averages of the two measures of stress correlated to the microenvironmental variables of principal components of them.

Regression Equation	P-Value	R^2	Slope	Intercept
CORT ~ Avg N/L	0.1097	-0.0245	-19.27	85
CORT ~ HSI	0.126	-0.003	190.91	-120
Avg N/L ~ HSI	0.947	-0.0463	-0.1612	0.2583
Avg N/L ~ PC3	**0.001**	0.3992	-0.4067	-0.1085
Avg N/L ~ Phosphate	0.165	0.0519	-0.2215	0.0671
Avg N/L ~ Ammonia	0.418	-0.012	-8.226	0.3423
Avg N/L ~ Open water	0.668	-0.0367	-0.0000005	0.1081
Avg N/L ~ Prairie/Grassland	**0.0394**	0.158	0.0000006	-0.5277
Avg N/L ~ Developed	**0.0012**	0.33891	0.0000093	-0.0821
CORT ~ PC3	0.0782	0.0563	9.987	65
CORT ~ Phosphate	0.0877	0.0422	11.014	53
CORT ~ Ammonia	0.260	-0.093	1059.98	53
CORT ~ Open water	0.177	-0.0454	0.000057	64
CORT ~ Prairie/Grassland	0.616	-0.185	-0.000001	77
CORT ~ Developed	0.269	-0.0965	-0.00016	66

All regressions included stage of the tadpole and relevant sample size to control for those factors.

2.5. Discussion

Unfortunately, a majority of the linear regressions conducted were not significant, showing that there is no relation between CORT levels and habitat suitability or microenvironmental variables. This outcome is not entirely unexpected as CORT levels were not known to change due to other environmental stressors, such as pesticides (Gavel et al. 2021). The inter-assay %CV of 16 is high, but is typical for water-borne analyses, if not on the lower end (Gabor et al. 2013; Millikin et al. 2019). It is possible that a habitat variable, which was not assessed in this study could correlate to CORT levels. The regression including CORT that was close to significance was phosphate with a p-value=0.09 and an R^2= 0.04, showing a probable positive relationship between the two variables. Of the three significant regressions, the R^2 value of the predicted neutrophil to lymphocyte to the area of prairie/grassland is considered weak at 0.158. Additionally, the correlation with average N/L ratios to PC3 has a significant negative relationship, which shows that the principal components, nearing significance in the generalized additive models were not only important for predicting presence, but also stress. One promising outcome is from the regression including developed area.

Neutrophil to lymphocyte ratios are known to increase with CORT in other amphibian species (Falso et al. 2015). Therefore, it is consistent that exposure to a stressor, like increased surrounding developed area, can be predicted to increase N/L ratios. With an R^2 of 0.34, this correlation is moderate and is more stable than prairie/grassland. The total number of sites where blood draws were successful was only 14, not including the various stages found at those sites. It is extremely likely that increasing the sample size would result in different outcomes. As North Dakota becomes increasingly developed, it would be interesting to see the outcome of a replica of this study in the future.

When comparing the two measures of stress across sites there is high variability. Many sites which had high biodiversity such as Grand Forks, Eldon Hillman, Mirror Pool, and Tewaukon had our highest CORT and average neutrophil to lymphocyte ratios. This is interesting since this would infer that these individuals were more stressed. Some possible explanations of this include increased competition and predation. Many of these sites had aquatic and terrestrial predators present including large water beetles, and garter snakes. As a matter of fact, these sites were the best to visit if looking for a wide variety of amphibians or reptiles. Pointing out that even though a site has high biodiversity, it maybe more stressful for the larval stages of amphibian development, while providing valuable habitat to a broad range of species. In the future, it could add on to these regressions and models to add measures of biodiversity, and a measure of predator presence to create a more complete picture of the other factors. These may be contributing to higher stress levels, but without the knowledge of this gained from the field experience it was not accurately assessed.

Additionally, the CORT release rates, and the average N/L ratios were not significantly correlated. While this is not entirely surprising as this can be highly variable throughout the literature, it is worth noting. Measuring both of these metrics of stress provide more information than just one, as previous research into stress tends to focus on CORT levels. Considering that the only correlations that were significant included the average N/L ratios and not the CORT levels, conclusions can be drawn, which otherwise would not have been observed. Providing both acute (CORT) and long-term measures (N/L) of stress should be the new standard at least in amphibian stress research as CORT is somewhat unreliable and variable. In the future it would also be interesting to see if it could be possible to add measures of stress into habitat suitability models to create more robust habitat suitability models, similar to the model built in Chapter 1.

The white blood cell counts from this study provide a baseline for Northern Leopard frog tadpoles in eastern North Dakota. It is possible for agencies to utilize the cost and energy effective method of blood smears to assess immune function of amphibians. While CORT is often the go-to for researchers, it has not been found to respond as readily to environmental stressors as shifts in leukocytes have for Northern Leopard frog tadpoles (Gavel et al. 2021). Average levels of all white blood cell types except lymphocytes are higher in this study than described by Gavel (2021). There are many possible explanations for these being high, including other factors such as disease. Eosinophils and monocytes are associated with disease/infection and, on average, are higher in amphibians than other vertebrates (Davis and Durso 2009). This could possibly explain why the CORT levels were not significant if the individuals were impacted by disease or infection. Results from this study provide a baseline for future measures of amphibian stress in North Dakota. Additionally, this study suggests that developed land surrounding reproductively successful wetlands can increase neutrophil/lymphocyte ratios in Northern Leopard frogs.

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CHAPTER 3. CONCLUSIONS

The objective of Chapter 1 was to create an updated habitat suitability model for a native amphibian to North Dakota, the Northern Leopard frog *(Lithobates pipiens).* As amphibians have been declining globally, including Northern Leopard frogs in neighboring states, it is important to understand what habitat is required for persistence. Utilizing data collected in the field in conjunction with online habitat and presence data, an ensemble suitability model was created. This shows that a majority of the state is suitable, with areas of lower suitability in the prairie pothole region of the state. In addition to the suitability model, generalized additive models were created to determine if the same microenvironmental variables influence presence in sites with adults (no breeding detected) and larval individuals. A majority of the additive models were not significant, with the exception of one of the larval model variables reaching significance. The variable was mainly associated with levels of phosphate and ammonia, in addition to the area of prairie, developed, and open water surrounding the site. Results show that there is a positive relationship between prairie and developed area with N/L ratios, but normally, one would expect there to be a negative correlation with prairie habitat. It is possible that this could end up being significant with an increased number of wetlands sampled. The results could be due to the restricted number of presence data available, but it can still be used to direct future surveys and conservation efforts.

Utilizing what was found in Chapter 1, the goal of Chapter 2 was to determine if suitable habitat and/or microenvironmental variables, which were nearing significance, could be correlated with two different measures of immune function. Water-borne corticosterone levels were measured in conjunction with leukocyte profiles to determine if there were any relationships with the 5 variables. Typically, the neutrophil to lymphocyte ratio is used as a

measure of stress: the higher the ratio, the more stressed the individual. The significant results from this chapter were with the average neutrophil to lymphocyte ratios being positively correlated to increased developed area surrounding the wetland. While the other regressions conducted were not as significant, there were a few interesting aspects to the results. It does seem that there is a possible negative relationship between corticosterone levels and neutrophil and lymphocyte ratios, but this is only nearing significance. This has been found in previous amphibian literature, but it tends to change depending on species (Davis and Maney 2017). Additionally, there was a near significant positive relationship with variance in phosphate and corticosterone levels. It would be interesting to see if this relationship were to increase with a larger sample size.

Together, these two chapters provide insight to habitat suitability and the impacts on immune function of Northern Leopard frogs in North Dakota. One interesting take-away from the results is that the generalized additive models in the first chapter did not show great significance of developed land on adults and only near significance for tadpoles. However, the linear regression of this relationship separately from the other microenvironmental data was significant. The impact of habitat characteristics on anuran presence and stress is not entirely understood, but this study provides a direction for future work to focus on the characteristics which were approaching significance.

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APPENDIX A. R-SCRIPT

```
##HSM
# Install packages
devtools::install_github('babaknaimi/sdm')
library(sdm)
installAll()
library(dismo)
library(dplyr)
library(tidyr)
library(mapview)
library(usdm)
library(sp)
library(rgdal)
library(sf)
#Get Data for North Dakota Reference
us<-getData('GADM', country='USA', level=1)
north.dakota<- subset(us,NAME_1=="North Dakota")
proj4string(north.dakota)<- CRS("+init=epsg:4326")
north.dakota2<-spTransform(north.dakota, CRS("+init=epsg:3857"))
northdakota<- st_read("E:/DisModels/NDGISHubData/NDHUB.STATE_polygon.shp")
plot(northdakota$geometry)
# Northern Leopard Frog Presence Data
NLF2 <- read.csv("E:/DisModels/NLF2.csv", header=TRUE, fileEncoding="UTF-8-BOM")
coordinates(NLF2) <- ~ long + lat
proj4string(NLF2)<- CRS("+init=epsg:4326")
```

```
data.proj<-spTransform(NLF2, CRS("+init=epsg:3857"))
data.proj
data.proj<-crop(data.proj, north.dakota2)
plot(north.dakota2)
plot(data.proj,cex=1, pch=20, col='darkgreen', axes= TRUE, add=TRUE)
#Unbiased Absence Data
NLF2 <- read.csv("E:/DisModels/NLF2.csv", header=TRUE, fileEncoding="UTF-8-BOM")
coordinates(NLF2) <- ~long+lat
projection(NLF2) <- CRS('+proj=longlat +datum=WGS84')
# circles with a radius of 50 km
x <- circles(NLF2, d=30000, lonlat=TRUE)
pol <- polygons(x)
samp1 <- spsample(pol, 1000, type='random')
plot(pol, axes=TRUE)
points(samp1, cex=0.75, pch=20, col='darkgreen')
xy2<- as.data.frame(samp1)
xy2<- xy2%>% drop_na()
coordinates(xy2) <- ~ x + y
proj4string(xy2)<- CRS("+init=epsg:4326")
xy3<-spTransform(xy2, CRS("+init=epsg:3857"))
# Get rid of NAs
NLF<- NLF%>% drop_na()
#Load Raster Files
NDVI<-raster("C:/Users/bradl/Desktop/NDVI.tif")
NDLU<-raster("E:/DisModels/LCD.tif")
```

```
bio1<-raster("E:/DisModels/1.tif")
bio2<-raster("E:/DisModels/2.tif")
bio3<-raster("E:/DisModels/3.tif")
bio4<-raster("E:/DisModels/4.tif")
bio5<-raster("E:/DisModels/5.tif")
bio6<-raster("E:/DisModels/6.tif")
bio7<-raster("E:/DisModels/7.tif")
bio8<-raster("E:/DisModels/8.tif")
bio9<-raster("E:/DisModels/9.tif")
bio10<-raster("E:/DisModels/10.tif")
bio11<-raster("E:/DisModels/11.tif")
bio12<-raster("E:/DisModels/bio12.tif")
bio13<-raster("E:/DisModels/bio13.tif")
bio14<-raster("E:/DisModels/bio14.tif")
bio15<-raster("E:/DisModels/15.tif")
bio16<-raster("E:/DisModels/16.tif")
bio17<-raster("E:/DisModels/17.tif")
bio18<-raster("E:/DisModels/18.tif")
bio19<-raster("E:/DisModels/19.tif")
NDW<-raster("E:/DisModels/NDh2o1.tif")
#Reproject raster files to same CRS
NDW <- resample(NDW,bio2)
NDLU <- resample(NDLU,bio2)
bio1 <- resample(bio1,bio2)
bio3 <- resample(bio3,bio2)
```

```
bio4 <- resample(bio4,bio2)

bio5 <- resample(bio5,bio2)

bio6 <- resample(bio6,bio2)

bio7 <- resample(bio7,bio2)

bio8 <- resample(bio8,bio2)

bio9 <- resample(bio9,bio2)

bio10 <- resample(bio10,bio2)

bio11 <- resample(bio11,bio2)

bio121<-crop(bio12, north.dakota)

bio122<-projectRaster(bio121, bio2)

bio12 <- resample(bio122,bio2)

bio131<-crop(bio13, north.dakota)

bio132<-projectRaster(bio131, bio2)

bio13 <- resample(bio132,bio2)

bio141<-crop(bio14, north.dakota)

bio142<-projectRaster(bio141, bio2)

bio14 <- resample(bio142,bio2)

bio15 <- resample(bio15,bio2)

bio16 <- resample(bio16,bio2)

bio17 <- resample(bio17,bio2)

bio18 <- resample(bio18,bio2)

bio19 <- resample(bio19,bio2)

NDVI<- crop(NDVI, north.dakota2)

NDVIS <- projectRaster(NDVI,crs = crs(bio2))

NDVI<- resample(NDVIS, bio2)
```

```
#Create Stack of Rasters

preds<- stack(NDLU, bio1, bio2, bio3, bio4, bio5, bio6, bio7, bio8, bio9, bio10, bio11, bio12,
bio13, bio14, bio15, bio16, bio17, bio18, bio19, NDW, NDVI)

plot(preds)

#Remove NA's

rna <- reclassify(preds, cbind(NA, 0))

#Remove Highly Correlated rasters

vif(rna)

ex<- raster::extract(rna, data.proj)

v<- vifstep(ex)

v

preds2<-exclude(rna, v)

#Crop Rasters to North Dakota

plot(preds2)

preds3<-crop(preds2, north.dakota2)

#Distribution

library(sdm)

d<- sdmData(~.,train=data.proj, predictors= preds3, bg=xy3)

d

#Statistical Models

m<- sdm(~., d, methods=c('glm', 'gam', 'brt', 'rf', 'fda', 'maxent', 'SVM', 'MARS'),
replication=c('cv'), cv.folds=5, test.p=30)

m

gui(m)

roc(m,smooth=T)

#Select Best Performing Methods
```

```
m2<- sdm(~., d, methods=c('glm', 'gam', 'brt', 'rf', 'fda'), replication=c('cv'), cv.folds=5, test.p=30)

m2

gui(m2)

roc(m2, smooth=T)

#Predict Distribution

p1<- predict(m2,preds2, filename='NLF.img', overwrite=TRUE)

plot(p1)

#Ensemble Methods

en1<- ensemble(m2,p1,filename= 'NLFU.img', setting = list(method='weighted',
stat='auc',opt=2))

#Crop to North Dakota

NLFcrop<- crop(en1, north.dakota2)

#Add fun colors

fun_color_range <- colorRampPalette(c("#F8FF91", "#065A2C"))

my_colors <- fun_color_range(11)

#Project CRS

NLFS<- projectRaster(NLFcrop, crs = '+proj=longlat +datum=WGS84')

#Plot suitability

plot(NLFS, col=my_colors, main="Predicted Habitat Suitability of Northern Leopard Frog",
expression(italic("L. pipiens")), xlab="Longitude", ylab="Latitude")

#Evaluate Model

eval<-getEvaluation(m2,stat=c('TSS', 'AUC', 'Kappa'),opt=1)

getModelInfo(m2)

#Variable Importance

vi<- getVarImp(m2,id=21:25,wtest='test.dep')

getVarImp(m2,id=1,wtest='training')
```

```
plot(vi, 'auc', col=my_colors)

plot(vi,'cor')

vi <- getVarImp(m2, method='brt')

vi

plot(vi, gg = F)

#Niche Models

niche(x=preds3, h=en1, c('X1','NDVI'))

#Plot ROC

rcurve(m2)

roc(m2)

#TSS, AUC, and Kappa boxplots

EVAL<- read.csv("E:/DisModels/EVAL.csv", header=TRUE, fileEncoding="UTF-8-BOM")

boxplot(TSS~model,data=EVAL, xlab="Models", ylab="TSS", col=my_colors)

boxplot(AUC~model,data=EVAL, xlab="Models", ylab="AUC", col=my_colors)

boxplot(Kappa~model,data=EVAL, xlab="Models", ylab="Kappa", col=my_colors)

##GAMs

#ALL NLF GAM

install.packages("mgcv")

library(mgcv)

#Read in Microenvironmental Data

GAMvar<- read.csv("E:/DisModels/VarWater2.csv", header=TRUE, fileEncoding="UTF-8-
BOM")

#PCA

NLF.pca <- prcomp(GAMvar[,c(5:21)], center = TRUE,scale. = TRUE)

summary(NLF.pca)

#Add PCA to GAMvar
```

```
x.new<-cbind(GAMvar,NLF.pca$x[,1:4])
str(x.new)
#Make it a data.frame
PCAs<-cbind(x.new, GAMvar$NLF)
library(vctrs)
PCA2 = as.data.frame(PCAs)
#GAM of
log_mod <- gam(NLF~ PC1 + PC2 + s(PC3) + s(PC4) ,
               data = PCA2,
               family = binomial("logit"),
               method = "REML")
summary(log_mod)
gam.check(log_mod)
AIC(log_mod)
plot(log_mod)
#NLF Metamorphs and Tadpoles GAM
log_mod2 <- gam(NLF.Tad~ PC1 + s(PC2) + PC3+ s(PC4) ,
               data = PCA2,
               family = binomial("logit"),
               method = "REML")
summary(log_mod2)
gam.check(log_mod2)
AIC(log_mod2)
plot(log_mod2)
```

APPENDIX B. SUPPLEMENTAL FIGURES

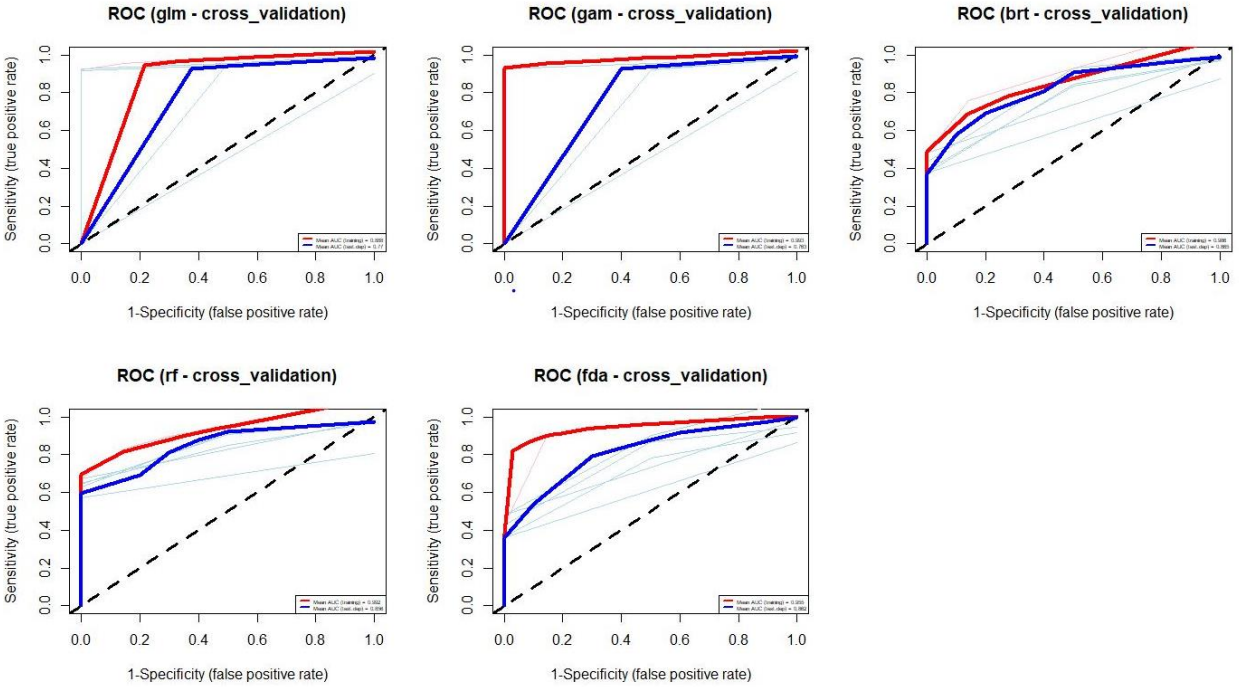

Figure B1: Average ROC curves for all of the statistical models used in the HSM.

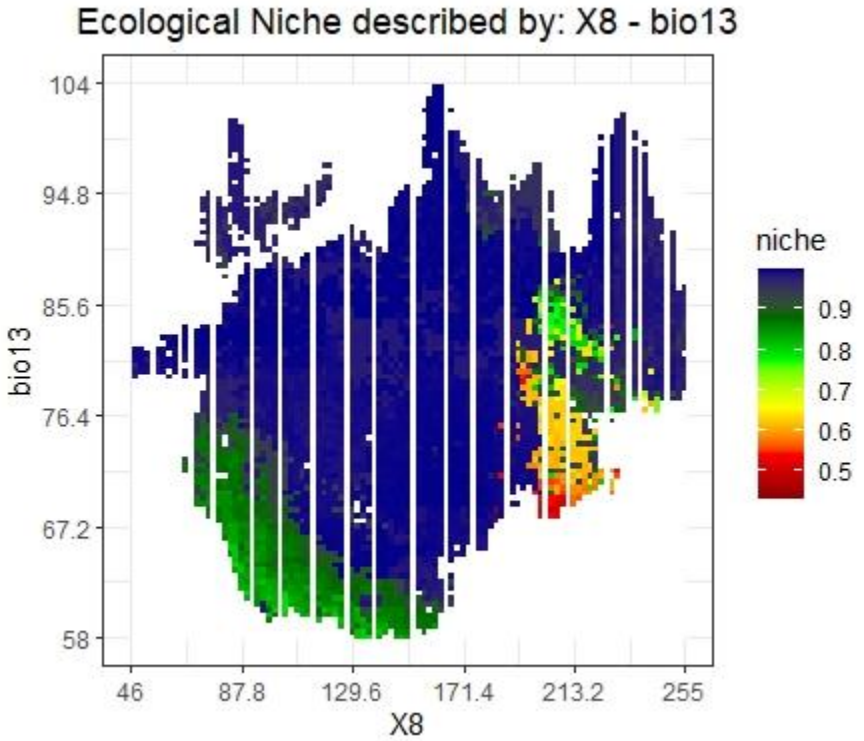

Figure B2: Ecological Niche of the two important predictive macro-variables, X8 and bio13.

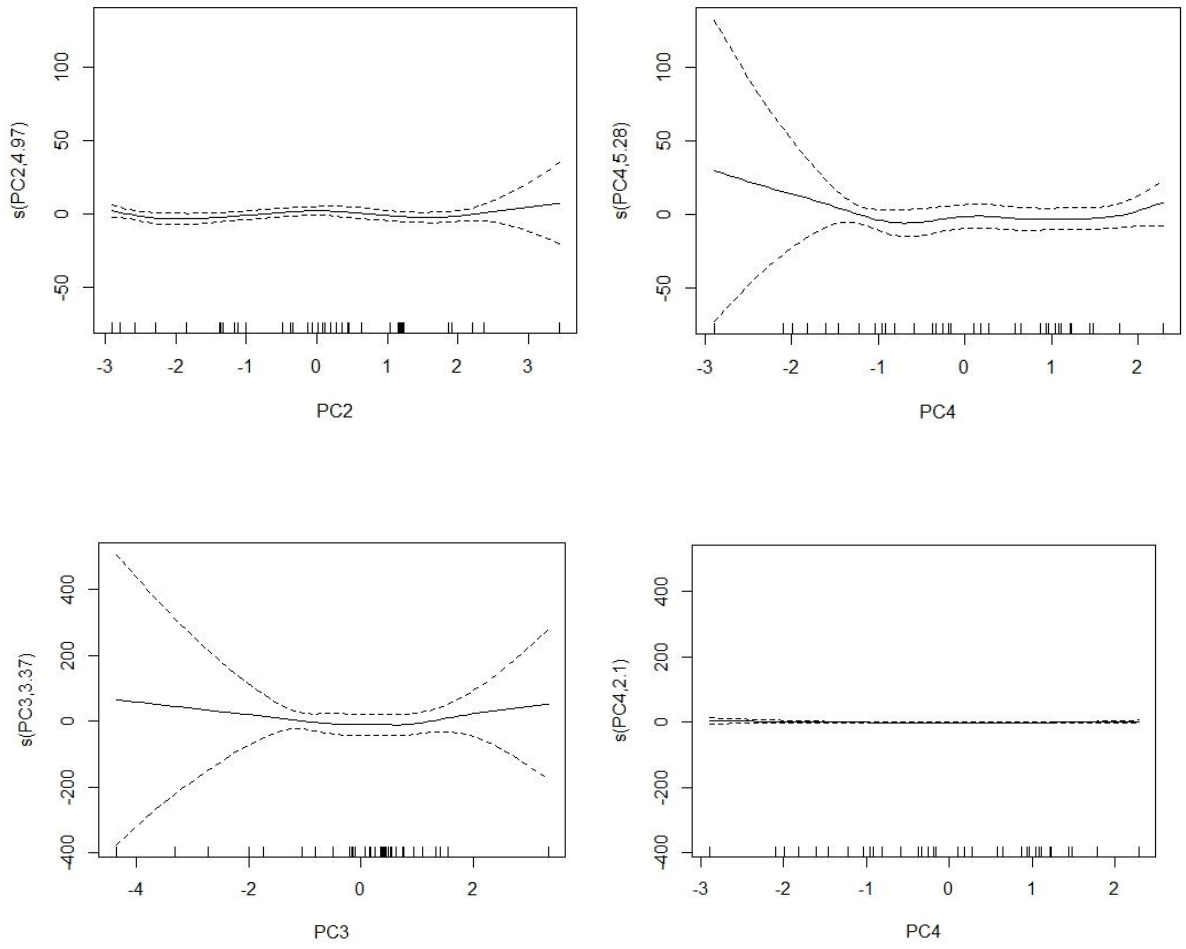

Figure B3: Plots of the smooths to PC2 and PC4 for the adult GAM. Smooths of PC3 and PC4 from the larval GAM.

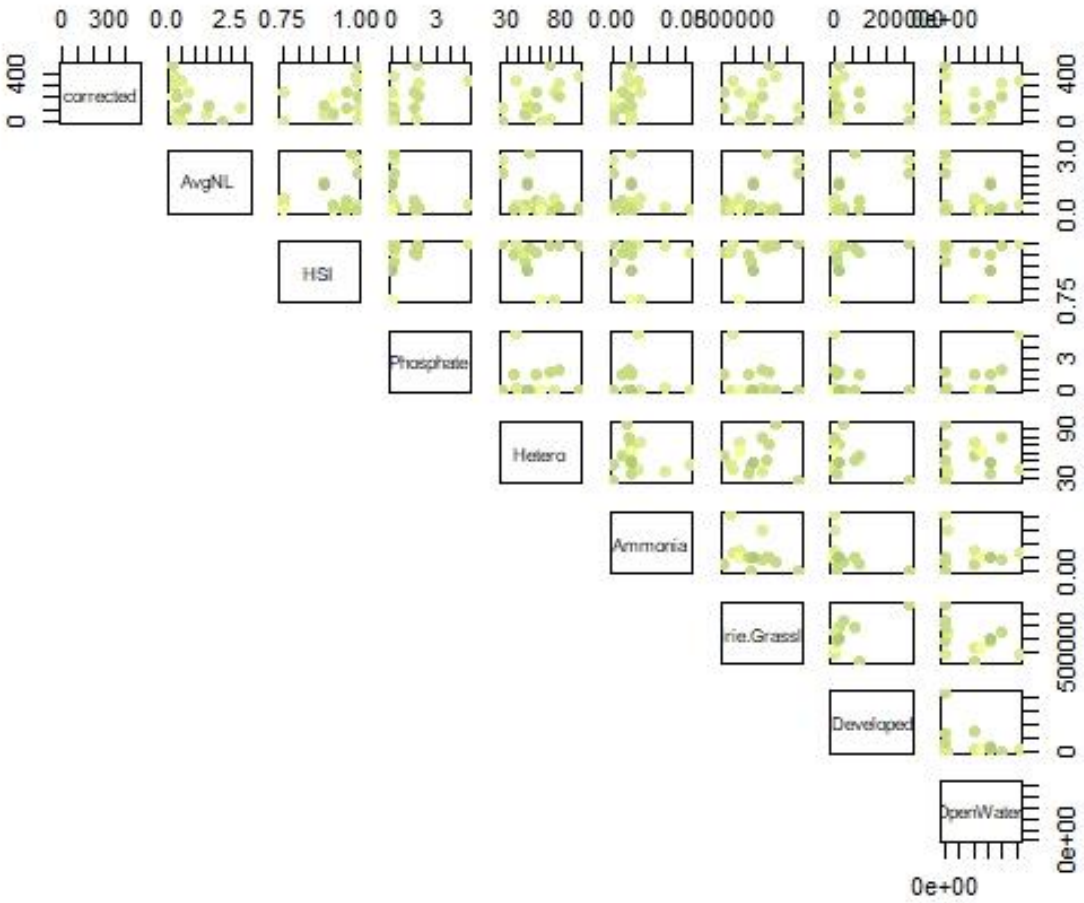

Figure B4: Scatter plot matrix of the corrected CORT levels, average neutrophil/lymphocyte ratio, habitat suitability index, variance in phosphate, heterogeneity of habitat, variance of ammonia, and areas of prairie/grassland, developed, and open water habitat types.

APPENDIX C. WATER-BORNE CORTICOSTERONE PROTOCOL

Water-borne cortisol extraction protocol:

All samples (~ 40 ml each) were collected out in the field and stored on ice until brought in to freezer. Each site has a baseline sample to subtract the circulating CORT. All samples have date, time, location, Gosner stage, mass (g) and volumes (ml) after pre-filtering recorded.

1. Extraction

All water samples are stored in a freezer at -20°C, after collected from the field.

When ready for extraction the water sample needs to be thawed then pumped at 25 ml min-1 through a pre-filter (0.45mm pore-size: Pall Life Sciences, U.K) (Ellis et al. 2004) to remove any debris.

Next the C18 cartridge needs to be primed with 4ml of HPLC-grade methanol and 4 ml distilled water. The sample is passed through an activated Sep-Pak Plus C18 cartridge under vacuum pressure. This cartridge is then washed with 5 ml of DI water and then stored on ice or frozen.

2. Elution process

The columns are then thawed or taken off the ice and eluted with 4 ml of HPLC grade methanol and placed into borosilicate vials. Eluted by placing the columns into the borosilicate vials putting the 4ml of methanol on top and centrifuging them.

Next the methanol is evaporated under a stream of nitrogen gas in a 37-45°C water bath.

Then the residue is resuspended in 175 µl of the prepared ELISA buffer, covered with parafilm, vortexed for one minute. Next they are placed into the refrigerator overnight before assay.

3. Assay

First the buffers need to be prepared:

1. ELISA Buffer: One vial of ELISA Buffer Concentrate (10ml) will be added to 90ml of UltraPure water. (make sure that the vial is rinsed to remove anything that has precipitated). Store in fridge (good for 2 months).

2. Wash Buffer: 5ml Wash Buffer Concentrate is diluted with Ultrapure water for a final volume of 2 liters (1995 ml of Ultrapure Water). 1ml of Polysorbate 20 is added (viscous liquid need may need a syringe). Store in fridge (good for 2 months).

Standard preparation:

1. Equilibrate pipette tip in standard, filling and expelling the tip multiple times.

2. 100 µl of the ELISA Standard is placed into a clean test tube and diluted with 900 µl of Ultrapure water. Store in fridge (good for 6 weeks). (BULK STANDARD)

3. With 8 labeled test tubes fill the first with 900 µl of ELISA Buffer. And the remaining 7 with 750 µl of ELISA Buffer.

4. Transfer 100 µl of the Bulk Standard (#2) into test tube #1 and mix thoroughly.

5. Transfer 500 µl from tube #1 into tube #2, mix thoroughly and continue this process through all 8 with 500 µl from the previous tube. These standards can only be stored for 24 hours.

CORT AChE Tracer:

1. Reconstitute 100 dtn of tracer in 6ml of ELISA buffer. Store in fridge (good for 4 weeks).

CORT Antiserum:

1. Reconstitute 100 dtn of antiserum in 6ml of ELISA buffer. Store in fridge (good for 4 weeks).

Plate Set-Up:

Run samples in triplicate

1. Add 100 µl of ELISA Buffer into the 2 NSB wells. Add 50 µl of ELISA Buffer into the 3 B0 wells.

2. Equilibrate pipette tip in each standard before: Add 50 µl from tube #8 into the 2 S8 wells and continue with each standard.

3. Add 50 µl of samples into the corresponding wells. Each sample diluted 1:32.

4. Add 50 µl Tracer to all wells EXCEPT the 1 TA and 2 Blk wells.

5. Add 50 µl Antiserum to all wells EXCEPT the 1 TA, 2 NSB, and the 2 Blk wells.

6. Cover each plate with plastic film shake for 10 minutes on plate shaker and place in fridge overnight.

Developing Plate:

1. Reconstitute 100 dtn Ellmans Reagent in 20 ml of Ultrapure water right before use.

2. Empty the wells and rinse five times with Wash Buffer.

3. Add 200 µl of Ellmans Reagent to each well.

4. Add 5 µl of tracer to the 1 TA well.

5. Cover plate with plastic film and place in dark to develop in 90-120 minutes.

Reading the Plate:

1. Wipe the bottom of the plate with a clean tissue.

2. Remove the plate cover and make sure to not splash the Ellman's Reagent.

3. Read the plate at a wavelength between 405 and 420 nm (412nm). Plate should be read when the absorbance of the B0 wells are in the 0.3-1.5 A.U. range. If the absorbance exceeds 2.0 wash the plate and add the reagent again.