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Use of imaging flow cytometry to assess the hematology of eastern massasauga rattlesnakes (*Sistrurus catenatus*)

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DEDICATION

This thesis is dedicated to my parents, Stephen and Margret Kovach, who helped foster my interest in the natural world, through camping, hiking, and exploring the globe, and for letting me fill my childhood pockets with interesting shells and stones. I also want to dedicate this thesis to my grandparents, Mary Jane and Ronald Slesinski for all of the encouragement and inspiration they have provided me.

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ABSTRACT

The federally threatened eastern massasauga rattlesnake is faced with many threats, including the newly recognized snake fungal disease. Unfortunately, the immune response to the disease is unknown. Leukocyte profiles are used as health assessment tools for many species, and imaging flow cytometry can be used as an application to characterize leukocyte profiles. Our goal was to use imaging flow cytometry to identify leukocyte profiles of eastern massasauga rattlesnakes and determine differences in profiles between sexes, reproductive status, ages, location, and individuals with clinical signs of snake fungal disease. Blood samples from 201 individuals were collected from ten locations throughout the Lower Peninsula of Michigan from May through August in 2018 and 2019. We captured 29 individuals with clinical snake fungal disease signs over the sampling period. We used imaging flow cytometry to enumerate and sort leukocytes, generating leukocyte profiles. Our eastern massasauga leukocyte profiles contained lymphocytes most abundantly, followed by heterophils, then eosinophils, basophils, azurophils, and least abundantly monocytes. We found that adult eastern massasauga rattlesnakes have lower mean total leukocytes compared to juvenile and young snakes. Gravid female snakes have lower basophil counts compared to non-gravid females. We also observed a trend of higher leukocyte counts associated with severe clinical signs of SFD. Our study furthers the understanding of leukocyte profiles of eastern massasaugas.

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ABBREVIATIONS

Allophycocyanin – APC

Bois Blanc Island – BBI

Centimeters – cm

Cluster of Differentiation – CD

Corticosterone - CORT

Data Analysis File – DAF

Degrees Celsius – °C

Degrees Fahrenheit – °F

Flourescein isothiocyanate – FITC

Grams – g

Global Positioning System – GPS

Hours – h

Imaging Flow Cytometry – IFCM

Incorporated – Inc.

International Union for the Conservation of Nature – IUCN

Megawatt – mW

Microliter – µl

Milliliter – mL

Nanometer – nm

Passive Integrated Transponder – PIT

Phycoerythrin – PE

Phosphate Buffered Saline – PBS

Principal Component Analysis – PCA

Quantitative Polymerase Chain Reaction – qPCR

Snout-Vent-Length – SVL

Snake Fungal Disease – SFD

Trademark – TM

CHAPTER I

INTRODUCTION

Across the globe, vertebrate wildlife populations are shrinking. Every year, more species become threatened or completely go extinct (Hoffmann et al. 2010). The International Union for the Conservation of Nature's (IUCN) Red List currently lists 382 reptile species threatened with extinction, including 97 species of snakes (Hoffmann et al. 2010). Snake species face many stressors including invasive species, changes in land use and loss of habitat, climate change, unsustainable natural resource use, pollution, purposeful eradication and persecution, road mortality, and diseases (Hoffmann et al. 2010). Individuals have few defenses against the compiling stressors, but in response to some stressors, like disease, species have a biological defense, their immune systems (Cooper and Alder, 2006).

Eastern Massasauga Rattlesnake

The focal species of this study, the eastern massasauga (*Sistrurus catenatus*), is a small, thick-bodied venomous reptile. Eastern massasaugas are gray or light brown with a dark colored saddle pattern running down their back and a heart shaped head. Typically, adult males are approximately 61.2 cm and females are approximately 52.3 cm in length (Szymanski, 1998). Females are ovoviviparous, carrying approximately 5-20 young and typically give birth biennially. Eastern massasaugas take two to three years to reach sexual maturity and can live up to 14 years (Szymanski, 1998).

The eastern massasauga is found throughout the Great Lakes region with populations found in Wisconsin, Illinois, Indiana, Iowa, Michigan, Ohio, Pennsylvania, New York, and Ontario, Canada (Szymanski, 1998). Unfortunately, the status (extant or extinct)

of many of these populations is currently unknown as data is lacking. The greatest stronghold of the species is located in Michigan, with many populations scattered throughout the lower peninsula of the state. Eastern massasaugas have very specific habitat requirements, varying by season, using connected wetlands during the spring and fall and seeking-out upland habitats to bask and give birth during the summer (Weatherhead and Prior, 1992). Eastern massasaugas will brumate during the winter in wet environments to prevent desiccation in crayfish and mammal burrows, rock crevices and old root systems (Szymanski, 1998).

The eastern massasauga is a fundamental species in its environment. The rattlesnake acts as both a predator and prey species, connecting an intricate food web (Moore and Gillingham, 2006). Acting as a predator, the eastern massasauga helps to control rodent species that are commonly considered pests to humans including mice, shrews, and moles. By eating these pest species, eastern massasaugas are also helping to control the spread of tick borne diseases, as these rodents commonly act as a primary vessel for tick movement. Eastern massasaugas also play the role of a principal indicator species of undisturbed healthy environments because they have such specific habitat requirements including open sunlit areas intermixed with shaded areas, surface water, and variable elevation (Szymanski 1998). These specific habitat requirements can best be met in an undisturbed healthy environment without invasive species, changes in land use, or pollution.

As a result of a steady decline in populations, the eastern massasauga rattlesnake was listed as a threatened species under the Endangered Species Act in September of 2016 (Szymanski 1998). Eastern massasauga rattlesnake populations have been rapidly

dwindling and are at risk of extinction as a result of stress from many threats. Threats include habitat loss, vegetative succession, road mortality, hydrologic alteration, collection, purposeful eradication and persecution, and isolated populations (Smith et al. 2018; Bailey et al. 2011). The newest threat to the species is an emergent disease, snake fungal disease (Allender et al. 2013).

Snake Fungal Disease

A severe skin infection was first observed in a timber rattlesnake population in New Hampshire in 2006 (Lorch et al. 2016). In 2008 similar infections were observed in an eastern massasauga rattlesnake population located in Illinois (Lorch et al. 2016). Since the first appearance of SFD in 2006, an additional 37 snake species have been identified with the severe skin infection, now termed 'snake fungal disease' or SFD (Guzman-Vargas et al. 2020); SFD is also sometimes referred to as Ophidiomycosis in published literature.

The disease is caused by the fungus *Ophidiomyces ophiodiiocola* and attacks the keratin rich scales of the epidermis of a snake (Allender et al. 2016b). SFD causes lesions, lacerations, and scarring that can result in disfiguration. The skin is the body's first line of defense against foreign pathogens and SFD directly attacks this biological defense, leaving an individual in a compromised state. Snake fungal disease has been classified as a chronic disease, as individuals who become infected typically die from the physiological effects the disease causes rather than the fungus itself (Lorch et al. 2016). The lacerations and scarring caused by the disease can lead to difficulty in infrared sensing, vision, olfaction, movement, and reproduction. Impacts of the disease lead to a poor quality of life and ultimately death is likely. Detection of the presence of SFD has been formerly determined through quantitative polymerase chain reaction (qPCR) testing on scale clippings or swabs from

symptomatic areas (e.g. lesions). However this method has proved unreliable and therefore a visual assessment of the presence of SFD signs may be a more reliable method to determine the presence of *Ophidiomyces ophiddiocola* (Hileman et al. 2018).

To overcome the disease is difficult as there are still many unknowns concerning SFD. It is unknown where the disease originated, how long it has been impacting species, or how it is transmitted between individuals and between populations. It is also unknown if SFD causes an immune response in individuals (Allender et al. 2016b).

Hematology

Hematology is defined as the study of blood components, and is an important tool used in diagnosis of diseases and studying immune systems. Blood can contain red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes) in different abundances with different morphologies based on species (Quadrini et al. 2018). Immune systems function to recognize and fight foreign pathogens like viruses, bacteria, fungi, and parasites (Cooper and Alder, 2006). Hematology can be used to determine the health of an individual by examining, for instance, leukocyte profiles (Bell and Gregory, 2014). A leukocyte profile is a characterization and classification of different types of leukocytes (i.e. lymphocytes, monocytes, basophils, etc.) (Bell and Gregory, 2014; Brown and Wittwer, 2000). In snake species, leukocyte types include lymphocytes, azurophils, basophils, heterophils, monocytes, and eosinophils (Stacy et al. 2011). The occurrence of eosinophils has been debated in some species of snakes because their appearance, size, and function are very similar to snake heterophils (Zimmerman et al. 2010). Azurophils are leukocytes that are found only in reptiles (Davis et al. 2008; Jenkins-Perez, 2012). Leukocytes include two categories of cells, granulocytes and agranulocytes. Granulocytes

include acidophils and basophils. Acidophils can be further broken-down into heterophils and eosinophils. Agranulocytes include lymphocytes, monocytes, and azurophils (Campbell et al. 1996; Rowley and Ratcliffe, 1988). Each type of leukocyte performs a specialized immunological function. For instance, these functions can include wound healing, inflammatory response, and fighting infections (viral, parasitic, bacterial, or fungal) (Rowley and Ratcliffe, 1988).

The morphology and abundance of cell types can differ between taxonomic group and even species. Morphology is the size, shape, and structure of cells. For example, mammals have a type of white blood cell called neutrophils, while reptiles have heterophils. Within the same taxonomic family, abundance of blood cells can differ between sexes, ages, response to disease, reproductive status, and even locations. Unfortunately, reptile studies looking at these differences are limited (Nardini et al. 2013, Stacy et al. 2011; Zimmerman et al. 2010). Previous studies have used hematology to assess the leukocytes of reptiles including green sea turtles (*Chelonia mydas*) (Muñoz et al. 2014; Wood and Ebanks, 1984), logger head turtles (*Caretta caretta*) (Kakizoe et al. 2007), mugger crocodiles (*Crocodylus palustris*) (Stacy and Whitaker, 2000), western fence lizards (*Sceloporus occidentalis*) (Motz et al. 2014), garter snakes (*Thamnophis sirtalis*) (Bell and Gregory, 2014; Gangloff et al. 2016; Palacios and Bronikowski, 2017), neotropical snakes (Carvalho et al. 2016; de Carvalho et al. 2017), amazon tree boas (*Corallus hortulana*) (Quadrini et al. 2018), and eastern massasaugas (Allender et al. 2016b).

Imaging Flow Cytometry

An imaging flow cytometer (IFCM) is a specialized instrument that combines microscopy with flow cytometry, a measure of the chemical and physical characteristics of

cells. An IFCM works by passing one cell at a time through a laser, which reflects light at different wavelengths to capture a detailed microscopic image of each cell. The size, shape, texture, florescence, and internal components can be determined through the image produced by INSPIRE™ software (Benoist and Hacohen, 2011). Approximately five thousand images can be captured per minute (Amnis Technologies, Millipore Sigma). Using IFCM software, different populations of cells can be characterized based on morphology or staining. After identifying different cell populations, tables, histograms, dot plots, and other figures can be created through the software (Benoist and Hacohen, 2011). Using IDEAS™ software, a data analysis file (DAF) can also be created with gated populations that can be used as a template to characterize all subsequent blood samples for comparisons.

PURPOSE

The results from this study can help us to better understand mean leukocyte counts for eastern massasauga rattlesnakes in Michigan's Lower Peninsula and how they vary based on impact of disease, sex, reproductive status, age, and location. Specifically, we are interested if the disease, SFD, will have any impact on leukocyte counts of eastern massasaugas. Our results can be compared to the results of prior studies examining the impacts of disease, sex, reproductive status, age, and location through leukocyte counts of varied reptile species. Further understanding of mean values of each type of leukocyte (lymphocyte, heterophil, monocyte, etc.) may allow us to understand the impacts of disease, because each type of cell has a specialized function. Mean leukocyte values may also lead to a new diagnostic tool to detect infected individuals or individuals mounting an immune response to disease, such as SFD.

SCOPE

The study of reptile hematology is still in its infancy, with many details about the functions, abundances, and morphologies of reptile leukocyte cells undetermined. Imaging flow cytometry combines the powers of microscopic analysis with flow cytometry and has never been used, to our knowledge, to analyze snake leukocyte counts. The last known remaining stronghold for the eastern massasauga is located in Michigan's Lower Peninsula, where this study takes place. Snake fungal disease threatens the eastern massasauga, but its impact on the immune system of a snake is unknown. Our study encompasses the use of IFCM to study the hematology of the eastern massasauga rattlesnake, a species at risk from SFD. The valuable data from our research will be an important resource for herpetologists, hematologists, animal epidemiologists, and biologists wanting to better understand leukocyte profiles of eastern massasaugas and how they are influenced by disease, sex, age, reproductive status, and location in Michigan.

ASSUMPTIONS

1) We assume that captured eastern massasaugas accurately represent snake populations found throughout Michigan's Lower Peninsula.

2) We assume that the blood samples collected for this study were not biased and were representative of Michigan eastern massasauga populations.

3) We assume that assessments of clinical signs of snake fungal disease (i.e. scarring, lesions, swelling) are adequate to assign accurate SFD scores.

4) We assume that each leukocyte type was properly identified with use of all available literature based on cell size and morphology.

OBJECTIVES

Our objectives are to (1) use IFCM to identify the leukocyte profile of the eastern massasauga rattlesnake. (2) To compare the leukocyte profiles of a snakes with clinical signs of SFD compared to individuals with no clinical signs of SFD. (3) Determine how sex influences leukocyte profiles. (4) Understand how approximate age of snakes affects leukocyte profiles. (5) To determine how reproductive status influences leukocyte profiles. (6) Understand if location influences leukocyte profiles of eastern massasaugas.

SIGNIFICANCE

The future of the eastern massasauga rattlesnake is uncertain, the species is declining because of the many threats these rattlesnakes are facing, and without aid, the species may soon become extinct. Eastern massasauga rattlesnakes suffer from tremendous stressors including habitat loss, purposeful eradication, road mortality, and having isolated populations, impacts of which are being exacerbated by SFD. It is unknown if SFD will cause immune response in an individual. It is also undetermined what the mean leukocyte profiles of the eastern massasauga are, or how sex, reproductive status, age, or location cause these profiles to differ.

The eastern massasauga is found sporadically throughout several states, though populations of eastern massasaugas are often found far apart because of the very specific habitat requirements they require, and due to habitat loss. Fortunately, Michigan remains

as one of the last refuges for this dwindling species (Moore and Gillingham, 2006). It is important to protect and ensure the future of the eastern massasauga rattlesnake because of the vital role they play in the environment, acting as an indicator species of a healthy and undisturbed environment. Eastern massasaugas also act as both predator and a prey within their ecosystem, and are an important part of a delicate food web.

This study demonstrates, for the first time, the use of IFCM to assess the leukocytes of the eastern massasauga. This study also demonstrates the identification of a leukocyte profile for the eastern massasauga, for the first time, and how disease, sex, reproductive status, age, and location influence leukocyte counts. This study is vitally important because the eastern massasauga is not only a federally threatened snake species, but it is also at risk from SFD.

DEFINITIONS

Brumation – a state of torpor exhibited by reptiles to cope with low temperatures in winter months.

Caudal vein – the largest vein in a vertebrates tail.

Gravid – term used to describe a reptile whom is carrying eggs internally.

Leukocyte profile – a characterization and classification of types of white blood cells.

Ovoviviparous – young are produced by eggs which hatch within the mother's body and she delivers them through a live birth.

Scutes – the belly scales on a snake.

CHAPTER II

Use of imaging flow cytometry to assess the hematology of eastern massasauga rattlesnakes (*Sistrurus catenatus*)

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ABSTRACT

The federally threatened eastern massasauga rattlesnake is faced with many threats, including the newly recognized snake fungal disease. Unfortunately, the immune response to the disease is unknown. Leukocyte profiles are used as health assessment tools for many species, and imaging flow cytometry can be used as an application to characterize leukocyte profiles. Our goal was to use imaging flow cytometry to identify leukocyte profiles of eastern massasauga rattlesnakes and determine differences in profiles between sexes, reproductive status, ages, location, and individuals with clinical signs of snake fungal disease. Blood samples from 201 individuals were collected from ten locations throughout the Lower Peninsula of Michigan from May through August in 2018 and 2019. We captured 29 individuals with clinical snake fungal disease signs over the sampling period. We used imaging flow cytometry to enumerate and sort leukocytes, generating leukocyte profiles. Our eastern massasauga leukocyte profiles contained lymphocytes most abundantly, followed by heterophils, then eosinophils, basophils, azurophils, and least abundantly monocytes. We found that adult eastern massasauga rattlesnakes have lower mean total leukocytes compared to juvenile and young snakes. Gravid female snakes have lower basophil counts compared to non-gravid females. We also observed a trend of higher leukocyte counts associated with severe clinical signs of SFD. Our study furthers the understanding of leukocyte profiles of eastern massasaugas.

INTRODUCTION

Wild vertebrate populations are decreasing and reptiles are facing exceptionally great declines, with approximately one in five species at risk of extinction (Böhm et al. 2013). Threats include invasive species, changes in land use, loss of habitat, climate change, unsustainable natural resource use, isolated populations, pollution, purposeful eradication and persecution, road mortality, and diseases (Hoffmann et al. 2010). The focal species of this study, the eastern massasauga rattlesnake (*Sistrurus catenatus,* formerly *Sistrurus catenatus catenatus)*, is classified as threatened or endangered throughout its entire range (Smith et al. 2018).

Eastern massasaugas are a small, thick bodied, cryptic, venomous snake species found in wetlands and adjacent connected uplands. This elusive snake species is found throughout the Great Lakes region, though the most numerous populations of the species can be found in Michigan (Smith et al. 2018). Eastern massasaugas are considered keystone species as they play a vital role in their ecosystems, acting both as a predator and prey. The presence of the species can also indicate environmental health (Bailey et al. 2011; Smith et al. 2018, Moore and Gillingham, 2006). Eastern massasaugas, however, face multiple threats including purposeful eradication, habitat destruction, road mortality, and isolated remnant populations (Smith et al. 2018; Szymanski et al. 1998), but the newest threat to this at-risk species is snake fungal disease (Lorch et al. 2016). Snake fungal disease (SFD) was first identified in Michigan on an eastern massasauga in Grayling, MI in 2013 (Tetzlaff et al. 2015). This was the second location identified with SFD infected eastern massasaugas in the United States, as previously SFD was found on an eastern massasauga in Illinois in 2008 (Allender et al. 2016a). Since its initial discovery, SFD has been documented in at

least two additional populations of eastern massasaugas in Michigan's Lower Peninsula (Allender et al. 2016a; Hileman et al. 2018).

Snake fungal disease is caused by the fungus *Ophidiomyces ophiodiicola,* which attacks snakes keratin-rich scales, causing cutaneous lesions, abscesses, and scarring (Allender et al. 2013; Lorch et al. 2016). These injuries have chronic impacts on an individual and can lead to difficulty eating, breathing, moving, reproducing, and may even lead to death (Lorch et al. 2016). Detection of SFD infected snakes has been determined through scale clippings or swabbing of symptomatic areas and using quantitative polymerase chain reaction (qPCR) testing. However, this detection method often leads to false negatives and false positives and is considered unreliable. Hileman et al. (2018) show that swabbing with a single swab had a false positive rate of 73%. Therefore, in our study, we instead used a visual assessment of clinical SFD signs on snakes. McKenzie et al. (2018) state that a visual assessment is a reliable indicator of *Ophidiomyces ophidiiocola* occurrence compared to swabbing or scale clippings.

It is hypothesized that, like most diseases, SFD causes an immunological response in infected snakes. Immune systems recognize and fight foreign pathogens like viruses, bacteria, fungi, and parasites (Cooper and Alder, 2006). Hematology, the study of blood components, is an important tool used in diagnosing diseases and studying immune systems. Hematology can be used to determine the health of a snake by examining leukocyte (i.e. immune) cells such as lymphocytes, azurophils, basophils, heterophils, monocytes, and eosinophils (Bell and Gregory, 2014; Brown and Wittwer, 2000). Each type of leukocyte performs a specialized immunological function in the body. Assessing the

abundance of each type of leukocyte can reveal the immunological response of individuals to a particular disease (Quadrini et al. 2018; Zimmerman et al. 2010).

The role of each immune cell has not been extensively studied in reptiles; however, some limited information is known. Lymphocytes (comprising B cells, T cells, and natural killer cells) play a variety of roles, including modulating the immune response, immunoglobulin production, wound healing, and fighting parasitic and viral infections (Rowley and Ratcliffe, 1988, Stacy et al. 2011). The azurophil plays a role in inflammatory and infectious disease response. Basophils are not well studied, but in turtle species they are associated with viral and parasitic infections (Rowley and Ratcliffe, 1988; Stacy et al. 2011). The heterophil plays a role in inflammatory disease, phagocytizing foreign pathogens and bacteria, and is functionally similar to mammalian neutrophils, fighting fungal infections (Stacy et al. 2011; Zimmerman et al. 2010). Monocytes are typically suggestive of inflammatory, parasitic, and bacterial diseases. Eosinophils are not well studied but are believed to fight parasitic infections (Stacy et al. 2011).

While we have extensive knowledge of mammalian immune systems, little is known about reptilian immune system components and functions (Zimmerman et al. 2010). Leukocyte abundance, morphology, and staining patterns vary greatly depending on species (Stacy et al. 2011). Within a species, leukocyte abundance can vary based on sex, reproductive status, age, location, and response to disease; hematology can also be used to assess these differences. In mammals, females tend to have more productive immune responses than males (Shames, 2002). Male mugger crocodiles (*Crocodylus palustris*) have higher heterophil counts; while female crocodiles have higher lymphocyte counts (Stacy and Whitaker, 2000). Green sea turtles (*Chelonia mydas*) show no sex dependent

differences in leukocyte counts (Wood and Ebanks, 1984). In female garter snakes (*Thamnophis sirtalis*), gravidity lowers immune function of an individual (Palacios and Bronikowski, 2017). When considering how age can influence leukocyte abundance, different reptile species show the same trend, with adults having lower leukocyte counts compared to juveniles, such as in mugger crocodiles (Stacy and Whitaker, 2000) and loggerhead sea turtles (*Caretta caretta*) (Kakizoe et al. 2007). Garter snakes have different abundances of leukocytes depending on their geographic locations (Gangloff et al. 2016). In western fence lizards (*Sceloporus occidentalis*), disease caused a greater immune response in female lizards compared to males (Motz et al. 2014). Allender et al. (2016b) found no immune response in eastern massasaugas to SFD. Leukocyte abundance and immune response can also vary based on each unique individual.

Over the past two decades, the use of imaging flow cytometry (IFCM) has grown significantly in studies of hematology in clinical laboratory settings (Brown and Wittwer, 2000). Imaging flow cytometry can provide a rapid analysis of multiple characteristics of cells in a sample including size, shape, texture, internal components, and fluorescence (Benoist and Hacohen, 2011; Roussel et al. 2010; Brown and Wittwer, 2000). Imaging flow cytometry works by passing individually suspended cells through a laser, which reflects the light at different wavelengths and captures a detailed image of each cell for analysis (Benoist and Hacohen, 2011; Roussel et al. 2010; Brown and Wittwer, 2000). The application of IFCM is best utilized with florescent labels, such as fluorescently labeled antibodies, that can tag specific leukocyte cell types for identification (Cherie, 2004). However, because of the limited research in reptile hematology, reptile antibodies have not yet been commercially produced. Fortunately, many antibodies created for one specific

species (e.g. mouse), are cross-reactive with several other species (e.g. fish, chickens, and humans). Therefore, using a highly cross-reactive antibody may allow for identification of leukocytes in reptile blood.

Our study aims to use IFCM to identify the leukocyte profiles of the eastern massasauga rattlesnake. We compared the leukocyte profiles of snakes with clinical signs of SFD to individuals with no clinical signs of SFD. We also determined how sex, age, reproductive status, and location influenced leukocyte profiles. This study is the first demonstration of how IFCM can be used to study the leukocytes of a federally threatened snake species, the eastern massasauga rattlesnake, at risk from SFD.

METHODOLOGY

Study Sites and Snake Capture

Fieldwork was conducted from May-July in 2018 and May-August 2019 at ten locations, nine distributed throughout Michigan's Lower Peninsula, and one on Bois Blanc Island (Figure 1). All sites were located on state game areas, state parks, state forest areas, or national forests that contained intact habitat and eastern massasauga populations. Study sites were selected based on historic records of eastern massasauga sightings, and surveying took place on days with appropriate weather conditions (no rain and > 59 °F). Each site was surveyed multiple days by at least two surveyors for approximately 1-3 hours beginning at approximately 0900h. Surveyors meandered unsystematically through sites attempting to encounter snakes. Eastern massasaugas were captured opportunistically with the use of snake tongs and placed in a cloth bag, which was safely secured in a bucket until surveying was complete for the day. Upon capture, environmental

data were recorded including cloud cover, shaded air and substrate temperature. Snake positional data at capture was also recorded including if the snake was in full sun, part sun, or full shade, also if the snake was fully exposed, partially covered, or completely covered by vegetation. A Global Positioning System (GPS) was used to mark the exact capture location (latitude, longitude) of each snake so that they could be returned to their capture location following sample and data collection.

Data and Sample Collection

Snakes were processed individually. All equipment used was either single-use and disposable, or was sterilized with a 10% bleach solution after each snake was processed and gloves were changed to avoid potential SFD spread through contact. Weight and snout-to-vent length (SVL) were recorded for each snake. Based on SVL, age class was assigned as adult (≥ 45 cm for females and ≥ 43 cm for males), juvenile (44.9 - 30 cm for females and 42.9 - 30 cm for males), or young (29.9 – 21 cm for both females and males) (Allender et al. 2016b; Bradke et al. 2018; Jellen, et al. 2007). Sex was determined through cloacal probing for the presence of hemipenes, and if female, reproductive status was determined by gently palpating to determine number of young present. Reproductive status was determined as gravid (pregnant) or not gravid. Individual snakes were each marked by injecting a subdermal Passive Integrated Transponder (PIT) tag with an individual identification number. PIT tags were used for snake identification in the event of future recaptures. Snakes were thoroughly examined for clinical signs of SFD such as facial swelling, crusts, bumps along the body, cutaneous lesions, ulceration, discolored scales, and opaque eyes (Lorch et al. 2016). Based on the severity of SFD signs snakes were given a “SFD score” from 0-3, with (0) signifying no SFD signs present, (1) indicating fewer than five lesions on

the ventral or dorsal surface, (2) signifying more than five lesions on dorsal and ventral surfaces including the tail, and (3) indicating multiple severe lesions on the face and cloaca as well as the dorsal and ventral surfaces and tail (Figure 2) (McCoy et al. 2017). A blood sample was also collected for IFCM analysis. We collected approximately 100 µl (two to three drops) of blood drawn from the caudal vein and placed it in a 1.5 mL Eppendorf microcentrifuge tube with 100 µl Strek Cell Preservative™ (STREK™). Blood samples were kept on ice or refrigerated until they could be analyzed with IFCM. After all data was collected, snakes were returned to their exact capture location on the same day.

Imaging Flow Cytometry Analysis – INSPIRE™

To prepare for IFCM analysis, blood samples were first subsampled by vortexing and pipetting 10 µl of the blood sample into a 1.5 mL microcentrifuge tube with 10 µl of phosphate buffered saline (PBS). Any samples that coagulated, had hemolysis, or were lymph contaminated were not assessed. The subsample was vortexed again and placed in the Amnis Imagestream X Mark II imaging flow cytometer (Amnis Technologies, Millipore Sigma) with dual excitation lasers (488 nm and 642 nm) at 0.5 mW. Low flow speed was operated at 40 X magnification. For each sample, 20,000 events (images) were captured using INSPIRE™ software based on methods of Roussel et al. (2010). Events could then be evaluated with a data analysis file (DAF) through IDEAS™ analysis software (Amnis Technologies, Millipore Sigma).

Imaging Flow Cytometry Analysis – IDEAS™

To create the DAF, a one-step Percoll gradient was used to isolate leukocytes by adapting the methods from Carvalho et al. (2016). One mL of fresh blood mixed with 2 mL of RPMI medium was carefully overlaid on 5 mL of a 57% Percoll solution in a 15 mL

conical tube (Carvalho et al. 2016). The solution was centrifuged at 1,280 g for 15 min at 18 °C with acceleration brakes at zero. The leukocyte layer was then carefully pipetted from between the layers of plasma and Percoll and washed once in 10 mL PBS, centrifuging at 1,500 rpm for 5 min (Carvalho et al. 2016). The resulting leukocyte pellet was re-suspended in 1 mL of PBS and used to create a leukocyte DAF. Using IDEAS™ software, first, out-of-focus images and images with more than one cell were removed from analysis. Then a dot plot cytogram was used to establish collection gates based on area (size) and intensity (shape and external complexity) of cells. Collection gates for erythrocytes, heterophils, basophils, lymphocytes, eosinophils, monocytes, and azurophils were established based on cell morphology (size, shape, and appearance) and a DAF was created (Figure 3). Proper identification of each leukocyte type was based on sources of available literature containing snake leukocyte images (Bell and Gregory, 2014; Chamut and Arce, 2018; Giori et al. 2020; Salakij et al. 2002b; Salakij et al. 2002c). Debris, lysed cells, and platelets were not gated and not included in the analysis. The DAF containing gates for all leukocyte types and red blood cells was used to analyze each individual snake blood sample. The number and percent gated of red blood cells, lymphocytes, basophils, heterophils, eosinophils, azurophils, and monocytes were recorded for each sample to use for statistical analysis.

Antibody Application

Commercially available antibodies developed specifically for reptiles are rare, so cross reactivity with bird species or mammals with heterophils were selected for use. Seven antibodies were tested: PE anti-mouse CD45 clone 30-F11 (hereafter identified as CD45), PE anti-human/mouse CD11b clone M1/70 (CD11b), F4/80 PE monoclonal

antibody clone BM8 (CDF4/80), CD19 PE monoclonal antibody clone eBio1D3 (CD19), APC anti-mouse Ly-6G clone RB6-8C5 (LY-6G), APC anti-human/mouse CD45R clone RA3-6B2 (CD45R), and CD3e FITC monoclonal antibody clone CD3-12 (CD3e). To establish if antibodies worked on snake leukocytes, control samples of mouse murine cells were analyzed using a procedure adapted from Gjurich et al. (2015) and stained with florescent antibodies, and compared with stained samples of snake blood (Figure 4). Antibodies were added to samples and incubated for 30-60 min at 4 °C while protected from the light, then washed, and analyzed in the IFCM instrument, as described above. We also tested to determine if our cell preservative used on our snake blood samples (Strek Cell Preservative™) had any influence on the ability of antibodies to adhere to leukocyte cell surface by creating and analyzing Strek™ preserved mouse cell samples (STREK™).

Statistical Analysis

Prior to data analysis, any samples that contained fewer than 20,000 images were removed. Any samples that were missing information or contained only platelets and debris were also removed from analysis. The R archive network (version 3.6.2) software was used for all statistical analyses and Vegan package was applied. Data was standardized with z-score and square-root transformed. A numerical environmental score was created for analysis based on: percent cloud cover, shaded air temperature, and substrate temperature at the time of snake capture. A numerical position score was also created based on how the snake was found: completely covered by foliage, partially covered, or fully exposed and if the snake was in direct sunlight, partial sunlight, or in fully shade. We used a Principal Component Analysis (PCA) to visualize the relationship between data variables (location, year, sex, age class, SFD score, capture type, reproductive status, SVL,

mass, month, day, season, environmental score, position score, red blood cell count, leukocyte count, lymphocyte count, monocyte count, heterophil count, azurophil count, basophil count, and eosinophil count) and each individual snake from both 2018 and 2019 in a preliminary exploratory data analysis. A PCA biplot allowed for visualization of approximate Euclidian distances among objects in multidimensional space. For the PCA, if snakes were captured in both 2018 and 2019, the sample from 2019 was removed from analysis. Mean and standard error was calculated for each type of leukocyte based on sex and age class using the 20,000 captured images for each individual. Snakes with signs of SFD (SFD score > 0) or recaptured individuals were removed from these mean leukocyte calculations. Student's *t*-tests were used to determine significance between sex, reproductive status, and age classes. Analysis of variance (ANOVA) with Tukey post hoc test was used to determine if leukocyte counts differed by location, and if leukocyte counts differed between groups assigned different SFD scores (1-3). Student's *t*-test and ANOVA analyses included recapture individuals and snakes with signs of SFD. Results were considered statistically significant at $p \leq 0.05$.

RESULTS

A total of 209 blood samples were collected from 201 unique individuals, with more samples from 2019 (n=120) than 2018 (n=89). Some snakes were captured in both 2018 and 2019 (n=7), or were captured multiple times within the same year (n=1), and a blood sample was taken at each capture. We captured fewer males (n=83) than females (n=118), less than half of whom (n=41) were gravid. Captures were predominantly adult snakes (n=137) with fewer juveniles (n=38) and young snakes (n=26). Most snakes were captured

at Site C (n=67) and Site J (n=58) (Figure 1). The fewest snakes were captured at Site I (n=3), Site A (n=5), and Site D (n=6).

Imaging Flow Cytometry Analyses

Dot plot cytograms were created to establish collection gates for different cell types (Figure 3). Eastern massasauga leukocytes were identified through IFCM including lymphocytes, azurophils, basophils, heterophils, monocytes, and eosinophils (Figure 5). Antibodies (CD45, CD45R, CD11b, CDF4/80, CD19, LY-6G, CD3e) all tested negatively, showing no florescence when compared to mouse controls (Figure 4). We determined that Strek Cell Preservative™ did not have any influence on the ability for antibodies to attach to cellular surfaces (STREK™).

Sex, Age Class, Reproductive Status, and Location

A PCA was used to assess correlation of all data variables to each unique individual (n = 201). The first three PCAs explain 50% of the cumulative variance (eosinophil counts, azurophil counts, and basophil counts), and the first six PCAs explained 73% of the cumulative variance (eosinophil counts, azurophil counts, basophil counts, leukocytes total, heterophil counts, and age class). PC1 explains 23% variance on the x-axis (Figure 6) and PC2 explained 15% variance on the y-axis. Vectors close to the origin are weakly correlated, such as monocyte, capture type, position score, and lymphocytes. SFD score and leukocyte totals are not correlated. SFD score and date are strongly correlated. Leukocyte totals are strongly correlated with heterophils, azurophils, basophils, and eosinophils.

Means and standard errors for total leukocytes, heterophils, basophils, lymphocytes, eosinophils, monocytes, and azurophils were calculated for 175 individuals comparing males, females, and gravid females across age classes (Table 1). Adult snakes contained

most abundantly lymphocytes (443.48 ± 37.79 S.E.), secondly heterophils (85.07 ± 12.36 S.E.), eosinophils (66.94 ± 7.86 S.E.) and basophils (67.57 ± 6.39 S.E.), azurophils (55.15 ± 7.26 S.E.), and least abundantly monocytes (16.71 ± 4.01 S.E.) (Table 1). Juvenile and young snakes follow a similar pattern of most abundant and least abundant leukocytes with increased abundances overall.

Average total leukocyte counts did not differ by sex between males (n = 42) and females (n = 69) (p = 0.373). Reproductive status also showed no significant difference between gravid (n = 35) and not gravid (n = 34) adult females (p = 0.495). Average total leukocyte counts of adults (n = 111) are significantly lower than juvenile (n = 37) snakes (p = 0.015) and young (n = 25) snakes (p = 0.004). Average total leukocyte counts of juvenile and young snakes were not statistically different (p = 0.680) (Figure 7). Average leukocyte counts did not differ between site locations where more than ten individuals were captured including sites B, C, E, G, and J (p = 0.376).

SFD Presence and Prevalence

A total of 29 individuals were captured with clinical signs of snake fungal disease, receiving a SFD score greater than zero. Most of the snakes captured with clinical signs of SFD were initial captures (n = 18). More females were identified with clinical signs of SFD (n = 18) than males (n = 11); and most were adults (n = 27). The proportion of individuals with SFD was relatively consistent across 2018 (13%) and 2019 (14%). Only one snake was assigned an SFD score of (3), nine snakes were assigned an SFD score of (2), and 19 were assigned an SFD score of (1). Average leukocyte total cell counts for each SFD score were not significantly different from each other (p = 0.112) (Figure 8).

One adult female individual (#027-307-004) was captured in 2018 and given a SFD score of (0), and then captured again in 2019 and given a SFD score of (3). In 2018, when this individual was captured she was gravid with 8 embryos. This individual showed a considerable increase in leukocyte counts in 2019 compared to 2018 (Figure 9). Two types of leukocytes showed a drastic increase in abundance in 2019 compared to 2018 when no SFD signs were present. Heterophils tripled from (52) 2018 to (169) 2019. Azurophils also nearly tripled from (37) 2018 to (96) 2019. However, monocytes decreased in 2019 (23) compared to 2018 (30) (Figure 9).

DISCUSSION

We used IFCM to analyze the leukocytes of the eastern massasauga rattlesnake and compare leukocyte counts between sexes, reproductive status, age class, and location. We also investigated leukocyte counts of snakes with clinical signs of SFD compared to snakes that did not have SFD signs. We found significantly lower average leukocyte counts in adult eastern massasaugas compared to juvenile and young snakes, but average leukocyte counts did not differ by sex, reproductive status, or across locations. Overall, lymphocytes were the most abundant cell type followed by heterophils, then eosinophils, basophils, azurophils, and least abundantly monocytes. We also found a trend of increasing leukocyte abundance associated with an increase in SFD score based on clinical signs of SFD.

Imaging flow cytometry was used to provide qualitative descriptions of eastern massasauga leukocyte morphology (Figure 5). Eastern massasauga lymphocytes are the smallest leukocyte, approximately 6μm, and were round with a smooth appearance. Basophils are 8μm, they are small and spherical and have a granular appearance.

Eosinophils are 10μm, round and granular. Heterophils are 12μm and had a bumpy, granular appearance with a commonly visible nucleus. Azurophils are also 12μm, and can be round or monocytoid with pale cytoplasm and visible nucleoli, they are similar in appearance to heterophils. Monocytes are the largest leukocyte at 17μm, and are round or slightly oblong, and smooth in appearance.

We also used IFCM to quantify eastern massasauga leukocytes (Table 1). Lymphocytes were the most abundant cell type (60%), then heterophils (12%), followed by eosinophils (9%), basophils (9%), azurophils (8%), and least abundantly monocytes (2%). Using traditional blood smears Allender et al. (2006) found that eastern massasauga blood contained lymphocytes most abundantly (32%), then azurophils (40% in males and 11% in females), heterophils (14%), monocytes (10%), eosinophils (1%), and least abundantly basophils (< 1%).

Lymphocytes usually compose 50-80% of leukocytes (Stacy et al. 2011). In our samples, lymphocytes make up 60% of leukocytes. Our results are similar to observations for garter snakes (56%) (Bell and Gregory, 2014), king cobras (*Ophiophagus hannah*) (61%) (Salakij et al. 2002a), puff-faced water snakes (*Homalopsis buccata*) (41%) (Salakij et al. 2002c), Malayan krait (*Bungarus candidus*) (81%) (Vasaruchapong et al. 2012), black rat snakes (*Pantherophis obsoletus*) (52%) (Belić et al. 2020), eastern diamondback rattlesnakes (*Crotalus adamanteus*) (78%) (Alleman et al. 1999), monocellate cobras (*Naja kaouthia*) (67%), Siamese spitting cobras (*Naja siamensis*) (71%), and golden spitting cobras (*Naja sumatrana*) (69%) (Salakij et al. 2002b). Our results are also similar to Allender et al. (2006) who found lymphocytes to be the most abundant cell in eastern massasauga leukocyte counts.

Heterophils normally compose 8-30% of leukocytes (Duguy et al. 1970; Stacy et al. 2011). Our heterophil counts (12%) are similar to eastern massasauga heterophil counts (14%) found by Allender et al. (2006). Our results are also similar to king cobras (18%) (Salakij et al. 2002a), black rat snakes (19%) (Belić et al. 2020), and puff-faced water snakes (15%) (Salakij et al. 2002c). Eosinophils commonly represent 5-15% of leukocytes in healthy individuals (Duguy et al. 1970). Eosinophils make up 9% of leukocytes in our eastern massasauga samples, similar to puff-faced water snakes (7%) (Salakij et al. 2002c). Basophils often represent 9-26% of leukocytes (Duguy et al. 1970). Our samples contained 9% basophils, similar to garter snakes (13%) (Bell and Gregory, 2014).

Azurophils commonly represent 16-49% of leukocytes in snake blood, however this was not found in our samples of eastern massasaugas (8%) (Duguy et al. 1970). Our results are similar to Allender et al. (2006) for azurophil counts (11%) of female eastern massasaugas, but not males (40%). Our results are also similar to eastern diamondback rattlesnakes (15%) (Alleman et al. 1999), Siamese spitting cobras (10%), king cobras (14%), and Malayan kraits (14%) (Vasaruchapong et al. 2012). Azurophils are very similar in appearance to heterophils, therefore some azurophils may have been inaccurately gated as heterophils in our samples, resulting in the lack of azurophils characterized (Stacy et al. 2011).

Monocytes commonly compose 0-10% of leukocytes (Stacy et al. 2011). Our samples contained 2% monocytes. Our results are similar to garter snakes (2%) (Bell and Gregory, 2014), king cobras (0%) (Salakij et al. 2002a), puff-faced water snakes (1%) (Salakij et al. 2002c), Malayan krait (0%) (Vasaruchapong et al. 2012), black rat snakes

(3%) (Belić et al. 2020), monocellate cobras (1%), Siamese spitting cobras (1%), and golden spitting cobras (0%) (Salakij et al. 2002b).

The presence of eosinophils has been debated in some snake species, but based on cellular morphology we believe we have identified and characterized these immune cells in eastern massasauga blood (Zimmerman et al. 2010). Allender et al. (2006) also identified eosinophils in eastern massasauga blood through a blood smear. Therefore, while some species like eastern diamondback rattlesnakes do not appear to have eosinophils, we are confident that eastern massasaugas do have eosinophils (Alleman et al. 1999; Allender et al. 2013; Bell and Gregory 2014).

Leukocyte response to inflammatory components has previously been shown to depend on year and body condition index (i.e. weight and SVL) of eastern massasauga rattlesnakes (Allender et al. 2016b). However, in our two-year study, we could not conclude that leukocyte profiles vary annually. Similarly, our PCA results did not show that leukocyte total cell counts were correlated with season, month, or day (Figure 6). SVL and mass were also uncorrelated with leukocyte total cell counts based on our PCA. However, results from our PCA did show that SFD scores were highly correlated with season, month, and day (i.e. date). We surveyed May-August, but the most frequent time to observe snakes with clinical signs of SFD was mid-May through mid-June in 2018 (May 23rd – June 13th) and 2019 (May 13th – June 19th). Previously, it has been found that the severity of SFD increases in cool winter months and SFD is commonly associated with brumation and low energetic states (Lorch et al. 2016; McCoy et al. 2017). Michigan eastern massasaugas commonly come out of brumation in April or May depending on weather conditions and temperature. Our PCA shows SFD scores corresponding with date (mid-May through mid-

June), immediately after the species comes out of brumation and SFD signs are likely at their most severe.

We did not find any difference in mean leukocyte counts between sexes. Similarly, green sea turtles show no sex dependent differences in leukocyte counts (Wood and Ebanks, 1984). Allender et al. (2006) used blood smears to determine that male eastern massasaugas have higher azurophil counts compared to females. We did not find any significant difference in azurophils counts between males and females ($p = 0.712$). Male mugger crocodiles have higher heterophil counts than females, and female mugger crocodiles have higher lymphocyte counts than males (Stacy and Whitaker, 2000). We did not find any significant difference between sex for lymphocytes ($p = 0.164$) or heterophils ($p = 0.746$). Additionally, basophils ($p = 0.554$), eosinophils ($p = 0.622$), and monocytes ($p = 0.838$) were also not significantly different between males and females in eastern massasaugas (Table 1).

We did not find any difference between the mean total leukocytes of gravid females compared to not gravid females. Gravid garter snakes show decreased lymphocytes, specifically T-cells, when compared to not gravid snakes (Palacios and Bronikowski, 2017). No significant differences were observed in lymphocyte counts between females and gravid females ($p = 0.868$), though gravid females (58.49 ± 5.04) did have significantly lower basophil counts than non-gravid females (73.12 ± 5.87) ($p = 0.0314$) (Table 1). Yielding offspring has a biological impact to an individual, causing an inflammatory response in the body (Itonaga et al. 2011). While the role of basophils has not been widely studied in reptiles, it is speculated that they play a role in inflammatory response to disease and

parasites (Stacy et al. 2011). Gravid eastern massasaugas appear to have an immunological cost of basophils, likely associated with an inflammatory response to gravidity.

Adult eastern massasaugas have lower mean leukocyte counts compared to juvenile and young snakes (Figure 7). Mugger crocodiles also show a similar difference, with adults having lower leukocyte counts than juveniles and sub-adults (Stacy and Whitaker, 2000). Comparably, adult logger head sea turtles also have lower leukocyte counts than juvenile sea turtles (Kakizoe et al. 2007). Our results suggest that as individuals age, immune system capability decreases with decreased mean leukocyte counts; this is also commonly observed in aging mammals (Shames, 2002).

Site locations were not different in mean leukocyte counts for eastern massasaugas. Gangloff et al. (2016) found that blood cell abundances of garter snakes were significantly different at 12 different locations. Gangloff et al. (2016) determined differences were based on local conditions and snakes stress levels due to food availability by measuring corticosterone (CORT) and glucose, and comparing heterophil:lymphocyte ratios. We did not measure glucose or CORT in our study, and heterophil:lymphocyte ratios were not significant across our locations.

Leukocyte mean counts were used to assess differences between different SFD scores based on the presence of clinical SFD signs to understand the impact that suspected SFD has on snake leukocyte abundances. One individual snake (#027-307-004) was captured in 2018 and was given a SFD score of (0), having no clinical signs of the disease, however the following year the same individual was recaptured but this time it had severe signs of SFD and was given a SFD score of (3). This was the only snake to receive a score of (3). Blood samples were taken at both capture events and leukocyte counts were

compared, showing an increase in leukocytes from 2018 to 2019 (Figure 9). Azurophils, eosinophils, lymphocytes, basophils, and heterophils also showed an increase in 2019, when the individual had severe SFD signs. Heterophils tripled from (52) 2018 to (169) 2019. Azurophils also nearly tripled from (37) 2018 to (96) 2019. Azurophils are assumed to be involved in inflammatory and infectious disease response in snake species, while heterophils are assumed to act similar to mammalian neutrophils, fighting fungal infections (Stacy et al. 2011; Zimmerman et al. 2010). The considerable increase in counts of these two leukocytes suggests that the immune system of this individual was responding to a fungal infection with an inflammatory response (Rowley and Ratcliffe, 1988). Allender et al. (2016b) concluded that massasaugas do not appear to mount an immune response to SFD. However, the results from our study show that exhibiting clinical signs of SFD did result in increased mean leukocyte counts of one individual (Figure 9). Similarly, observing the SFD scores assigned to all snakes captured also shows an increase in leukocyte counts with increasing SFD score (Figure 8). Motz et al. (2014) comparably found that disease increases mean leukocyte counts in western fence lizards infected with malaria compared to healthy lizards. Motz et al. (2014) also found that females have greater immune responses than males. Individual #027-307-004 was an adult female eastern massasauga. Our results suggest that individual eastern massasaugas do mount immune responses to SFD signs, and that increased severity of clinical signs of SFD can cause an increased immunological reaction.

The mean leukocyte counts found in this study can be used as a health-assessment tool to determine if snakes are mounting an immune response to a disease such as SFD (Table 1). Our results show that exhibiting clinical signs of SFD correlates with increased

leukocyte counts. Previously, researchers have analyzed the presence of *Ophidiomyces ophiodiocola* by swabbing visible lesions and using qPCR, but results of this methodology are unreliable, commonly resulting in false positives and negatives (Hileman et al. 2018; McKenzie et al. 2018). Some snakes also test positively for *Ophidiomyces ophiodiocola* without showing the outward clinical signs of SFD. Assessing leukocyte counts, rather than the presence of *Ophidiomyces ophiodiocola,* could therefore be a better health-assessment tool than simply detecting the presence of the fungus through qPCR. Potentially, by using this health analysis tool (Table 1), researchers may even be able to determine if a snake is infected with SFD before external signs are apparent.

Through IFCM, we determined that the tested antibodies (CD45, CD45R, CD11b, CDF4/80, CD19, LY-6G, CD3e) failed to bind to the antibody specific antigens on snake immune cell surfaces and were therefore, ineffective (Figure 4). While numerous commercial antibodies have been created for use on mammal leukocytes, no commercially produced antibodies have been created to stain snake leukocytes. While the required antibodies could be produced through a third-party lab, this option is costly and time consuming and was not a viable option for this study. Using fluorescently labeled antibodies on a blood sample enhances the ability of an IFCM technician to accurately differentiate between different leukocyte types, by targeting specific cell antigens. Through using fluorescently labeled antibodies, identification of leukocyte cells can be more precise, identifying even misshapen or immature leukocyte cells. Though the antibodies we tried were unsuccessful, this result provides knowledge previously undiscovered. We hope that the information learned through this research may be implemented in future attempts to select and use antibodies to stain snake leukocytes.

The results of this work demonstrate that IFCM can be used to quantify and qualify the blood cells of snakes. While flow cytometry has been used to characterize the blood cells of three neotropical snakes (Carvalho et al. 2016; de Carvalho et al. 2017), IFCM, which combines the powers of microscopy with flow cytometry, was used for the first time, to our knowledge, to examine snake leukocytes. Imaging flow cytometry offers the advantage of microscopic visualization of cells (Erdbrügger et al. 2014); this is essential when the morphology of the study cells have not been previously described in known literature, and no fluorescently labeled antibodies can be applied to a sample. Imaging flow cytometry is an underutilized instrument in hematological studies, yet this research exhibits the instruments capabilities in analyzing eastern massasauga leukocytes.

Our results provide important advancements, as research in reptile hematology is in its infancy, and there is still much to discover. No other studies, to our knowledge, have used IFCM to analyze leukocyte profiles for eastern massasaugas. Our study was also the first to attempt to use fluorescently labeled antibodies with IFCM on eastern massasauga blood samples. We provided a robust spatiotemporal analysis of patterns of massasauga hematology, in relation to disease. We found that adult eastern massasaugas have lower mean leukocyte counts compared to juvenile and young snakes. Gravid female snakes have lower basophil counts than not gravid females. Lymphocytes are the most abundant leukocyte, while monocytes are the least abundant. We also observed that eastern massasaugas with clinical signs of SFD appeared to exhibit an immune response to the disease. The results of this study can provide a framework for future diagnostic health-assessments to identify snakes mounting an immune response. Results from this study help to build a foundation for future researchers in understanding the responses of eastern

massasaugas leukocytes to the magnitude of stressors they face and future enhancement in conservation measures for this imperiled species.

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TABLES

Table 2.1: Leukocyte counts from 175 eastern massasauga rattlesnake blood samples based on 20,000 cells analyzed through imaging flow cytometry (IFCM) for each individual. Means and standard errors for total leukocytes, heterophils, basophils, lymphocytes, eosinophils, monocytes, and azurophils comparing males, females, and gravid females across three age classes (adult, juvenile, and young).

		Total Leukocytes	Heterophils	Basophils	Lymphocytes	Eosinophils	Monocytes	Azurophils
Adult	**Male**	862.07 ± 52.94	88.47 ± 13.36	71.09 ± 8.26	400.63 ± 37.30	70.4 ± 9.45	16 ± 4.58	57.58 ± 8.94
	Female	789.44 ± 72.95	81.79 ± 11.26	73.12 ± 5.87	481.94 ± 38.68	69.53 ± 6.89	16.88 ± 3.36	56.29 ± 6.58
	Gravid Female	806.34 ± 71.17	84.94 ± 12.45	58.49 ± 5.04	447.86 ± 37.39	60.89 ± 7.28	17.26 ± 4.08	51.57 ± 6.26
Juvenile	**Male**	816.56 ± 106.37	128.81 ± 16	87.12 ± 10.78	438.25 ± 104.05	93.94 ± 14.52	30.62 ± 13.53	72.75 ± 10.36
	Female	779.48 ± 103.77	107.43 ± 17.21	95.76 ± 11.86	534.48 ± 62.86	98.67 ± 15.91	17.48 ± 3.55	79.52 ± 15.57
Young	**Male**	996.18 ± 113.41	189.18 ± 30.93	128.09 ± 19.01	465.82 ± 56.16	155.91 ± 20.66	13.36 ± 3.09	131.45 ± 15.82
	Female	748.47 ± 146.10	164.93 ± 32.42	95.13 ± 11.03	394.53 ± 66.44	110.93 ± 17.06	13.07± 2.26	99.8 ± 16.31
Total		828.36 ± 95.24	120.79 ± 19.09	86.97 ± 10.26	451.93 ± 57.55	88.61 ± 13.11	17.81 ± 4.92	78.4 ± 11.41

FIGURES

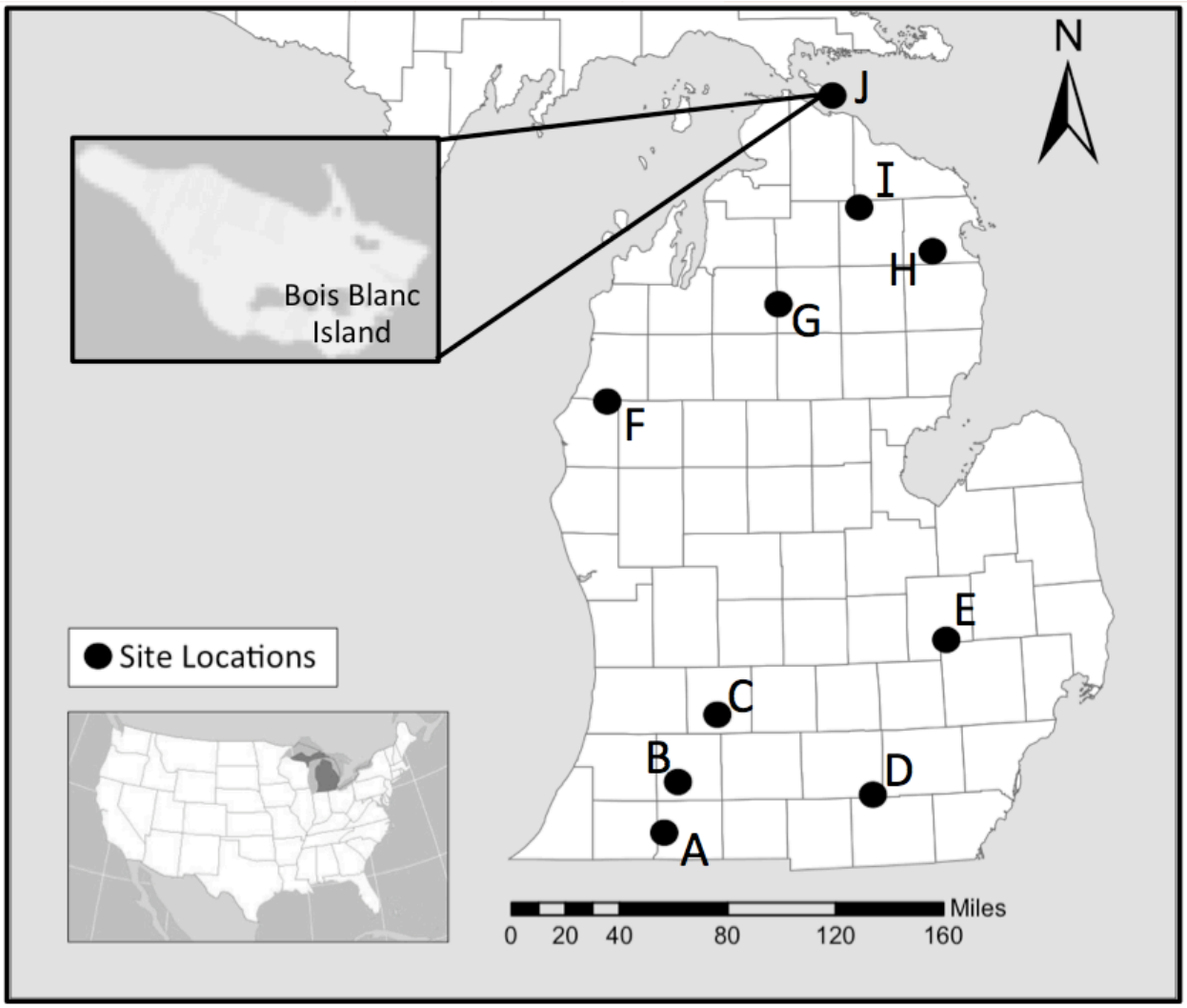

Figure 2.1: Approximate locations of ten survey sites and sampling locations throughout the Lower Peninsula of Michigan, USA in 2018 and 2019 where eastern massasaugas were captured and blood samples were taken.

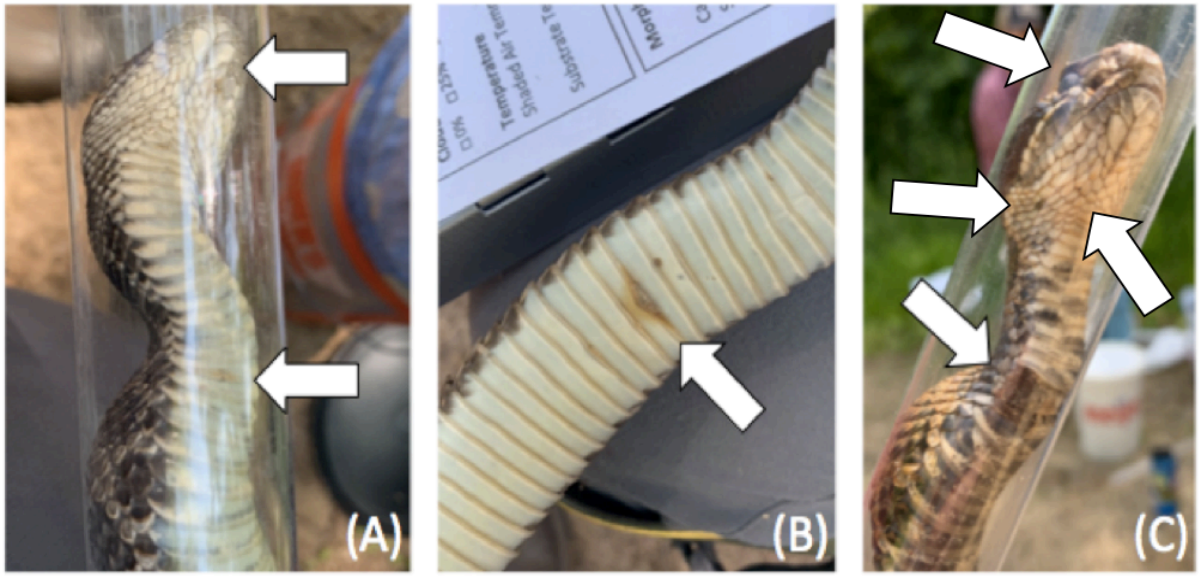

Figure 2.2: Visualization of snake fungal disease (SFD) scoring based on presence and severity of clinical SFD signs on eastern massasauga rattlesnakes indicated with white arrows. (A) Individual with an SFD score of (1), crusts located on chin and neck. (B) Individual with an SFD score of (2), crusts located on ventral scutes and along body. (C) Individual with an SFD score of (3), lesions on face, neck, and ventral side of body. See methods for SFD score determination based on clinical signs of SFD.

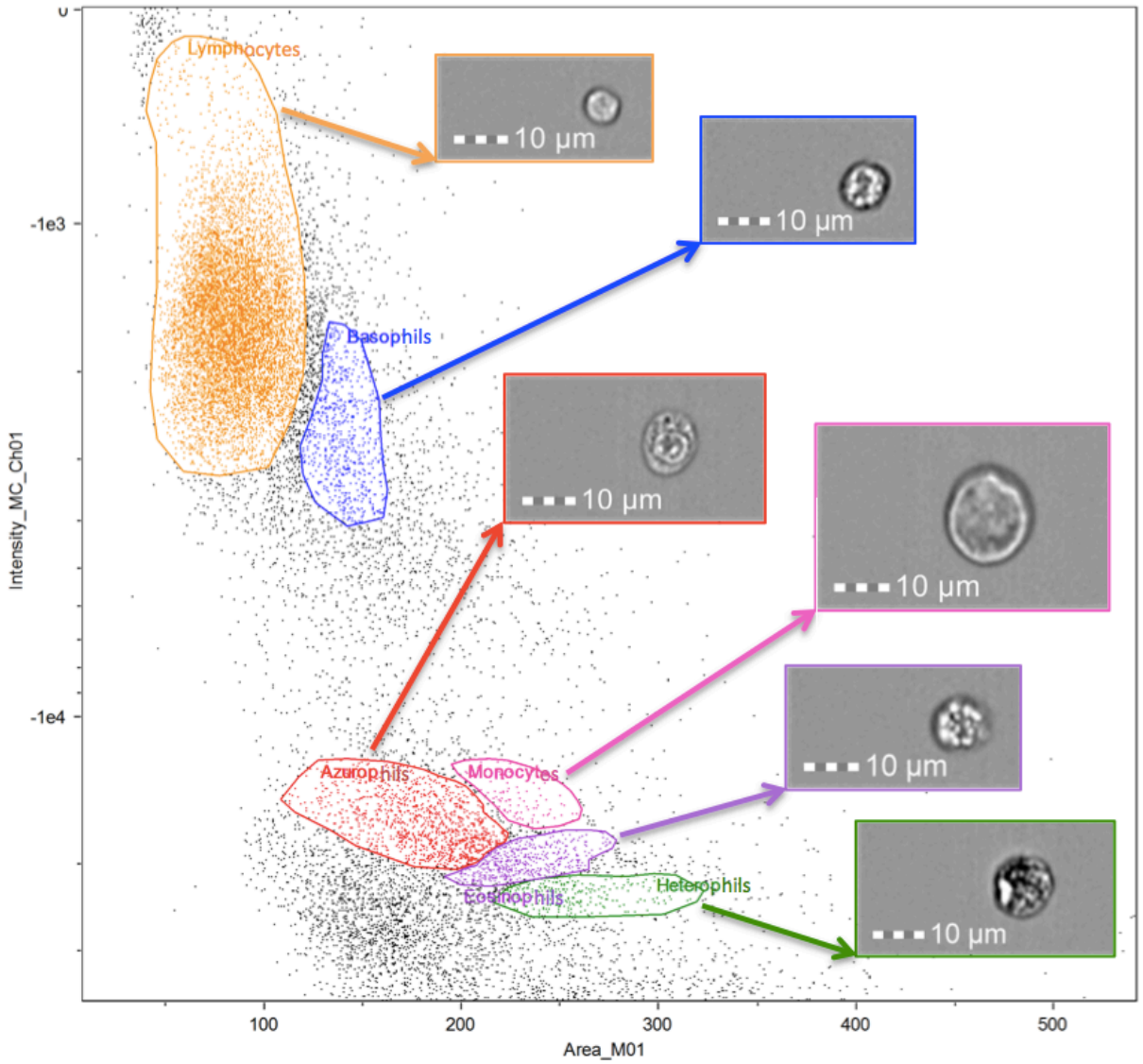

Figure 2.3: Dot plot cytogram showing imaging flow cytometry (IFCM) collection gates based on area (x-axis) and intensity (y-axis) of one eastern massasauga blood sample based on 20,000 cells total. Each dot represents one cell image. Gates drawn around each leukocyte population based on physical appearance (size and shape) of each cell type. Lymphocytes are circled in orange, basophils in blue, azurophils in red, monocytes in pink, eosinophils in purple, and heterophils in green.

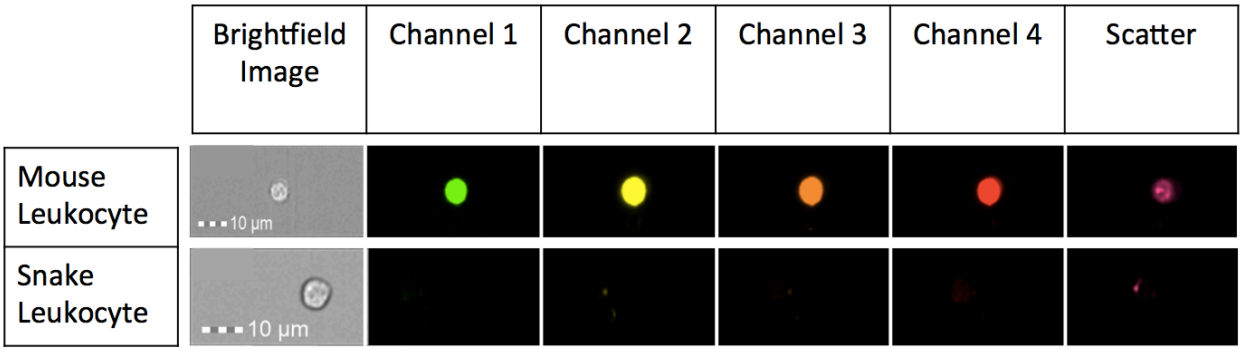

Figure 2.4: Visualization of mouse (top) and snake (bottom) leukocytes stained with one of the fluorescently labeled antibodies (CD45R) tested with the imaging flow cytometry (IFCM) analysis channels. The 'Brightfield Image' channel shows the microscopic image of each cell analyzed, channels (1-4) show florescence at different wavelengths, and the 'Scatter' channel shows particle size light reflectance. The mouse leukocyte (top) shows antibody adherence and florescence in all channels. The snake leukocyte (bottom) shows no antibody adherence and no florescence in any of the channels. Seven additional antibodies (CD45, CD11b, CDF4/80, CD19, LY-6G, CD3e) were tested, however none showed florescence with snake leukocytes.

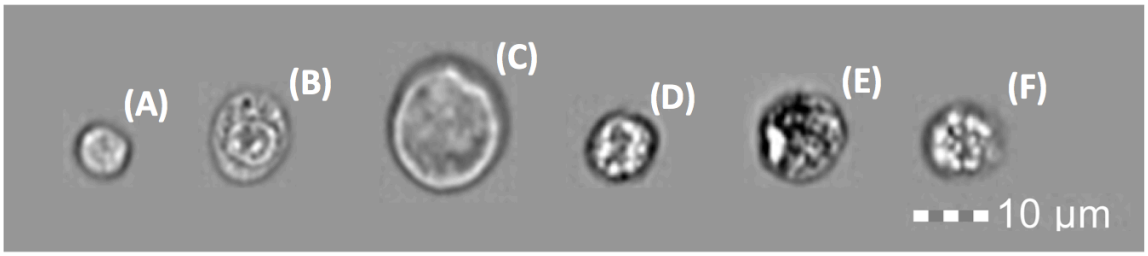

Figure 2.5: Microscopic imaging flow cytometry (IFCM) pictures of each type of white blood cell identified in eastern massasauga blood. (A) Lymphocyte. (B) Azurophil. (C) Monocyte. (D) Basophil. (E) Heterophil. (F) Eosinophil.

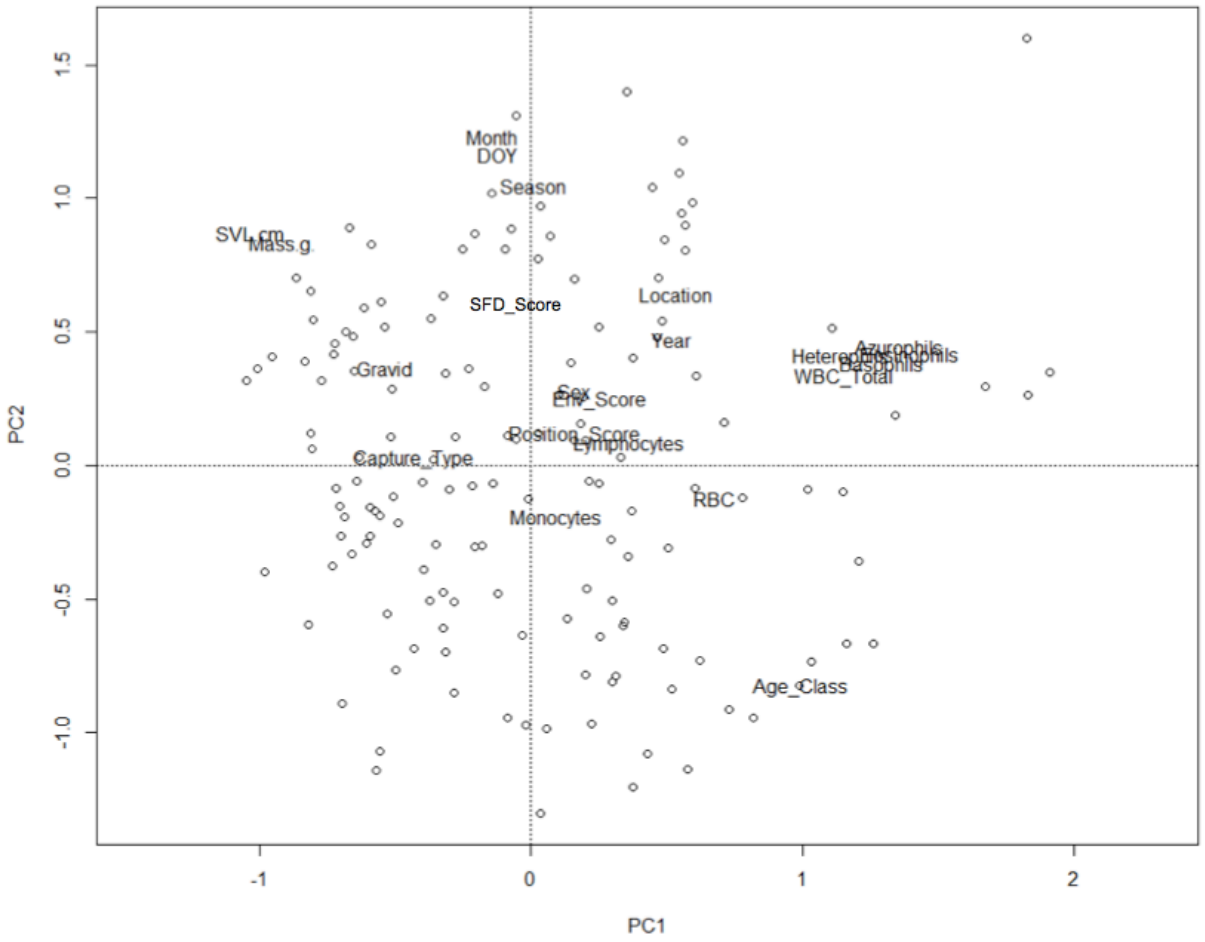

Figure 2.6: Principal Component Analysis (PCA) showing the relationship between data variables and 201 individual snakes. Each circle represents one eastern massasauga. PC1 explains 23% variance (x-axis) and PC2 explains 15% variance (y-axis). Abbreviations are as follows: RBC, red blood cells, WBC_Total, white blood cells (leukocytes) total, SVL_cm, snout vent length in centimeters, Mass_g, mass in grams, SFD, snake fungal disease, DOY, day of year, ENV_score, environmental score.

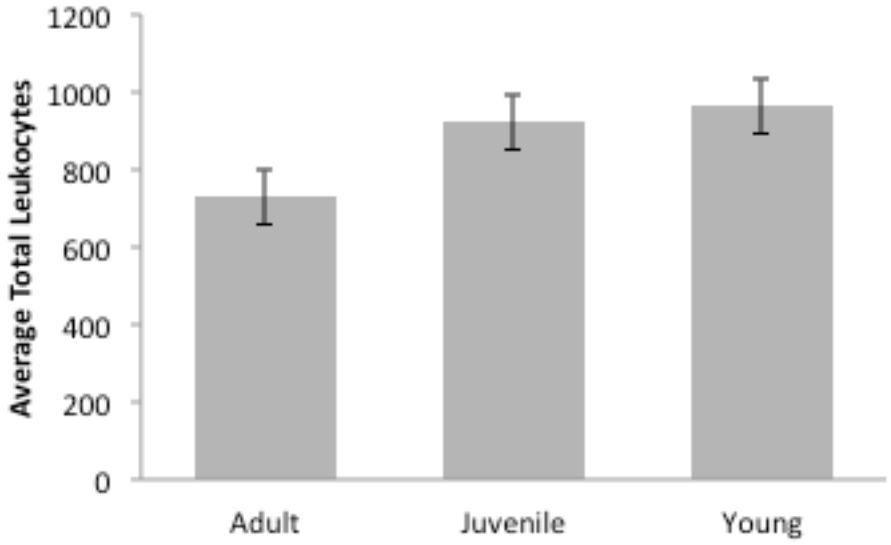

Figure 2.7: Bar graph comparing average total leukocyte counts for eastern massasaugas based on 20,000 cells across the age classes of adult (n = 111), juvenile (n = 37), and young (n = 25). Age classes assigned based on snakes length (see methods age class determination based on length). Black bars represent mean standard error. Adult counts were significantly lower than juveniles (p = 0.015), and young snakes (p = 0.004) analyzed through a Student's *t*-test.

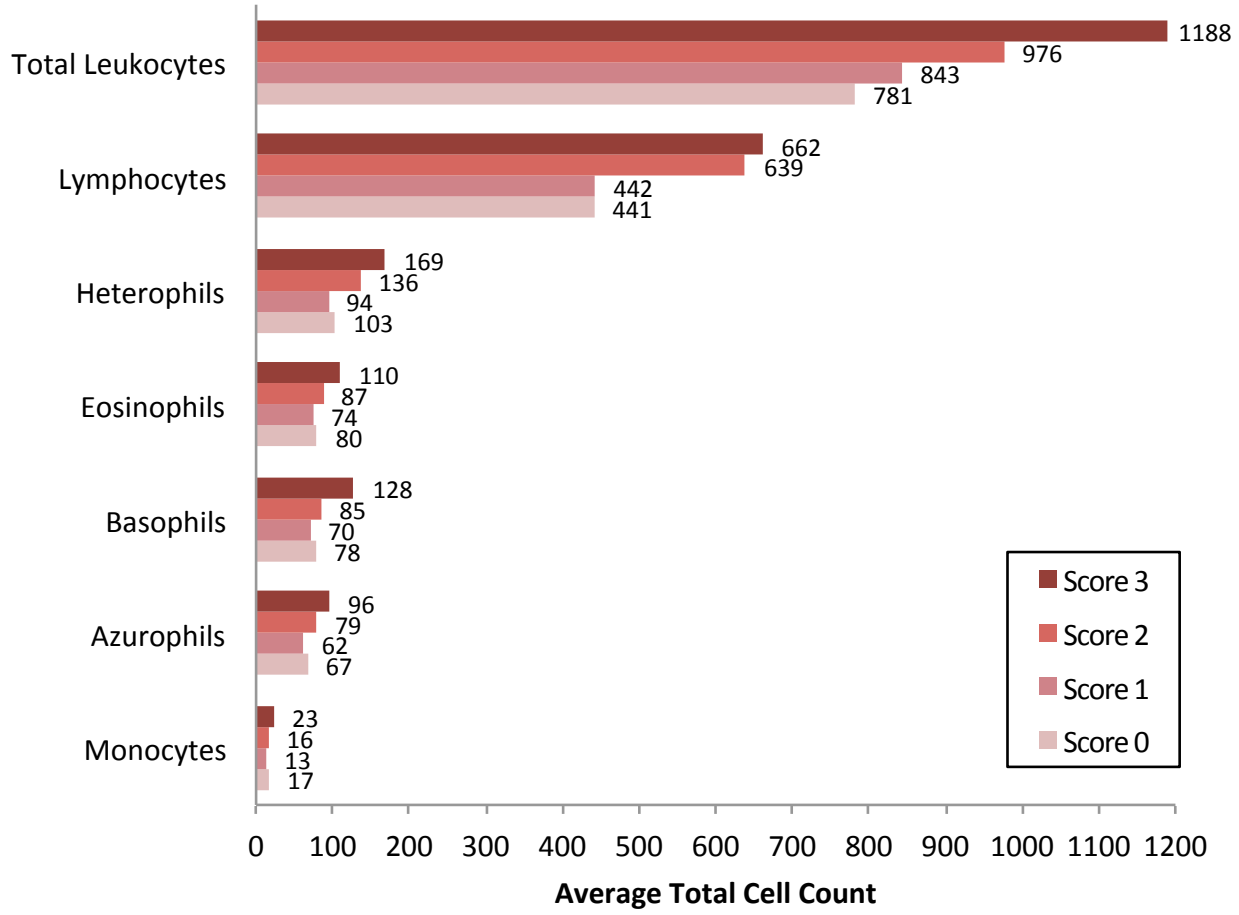

Figure 2.8: Mean leukocyte counts of individuals with different SFD scores based on the presence and severity of SFD signs based on 20,000 cell images analyzed with imaging flow cytometry (IFCM). One snake had an SFD score of (3), nine snakes had an SFD score of (2), 19 snakes had an SFD score of (1), and 172 snakes had an SFD score of (0). The darkest red bars represent the highest SFD score (3), lightest red bars represent the lowest SFD score (0), colors and SFD scores are represented sequentially. Leukocyte cell counts did not differ significantly ($p = 0.112$) based on a Student's *t*-test.

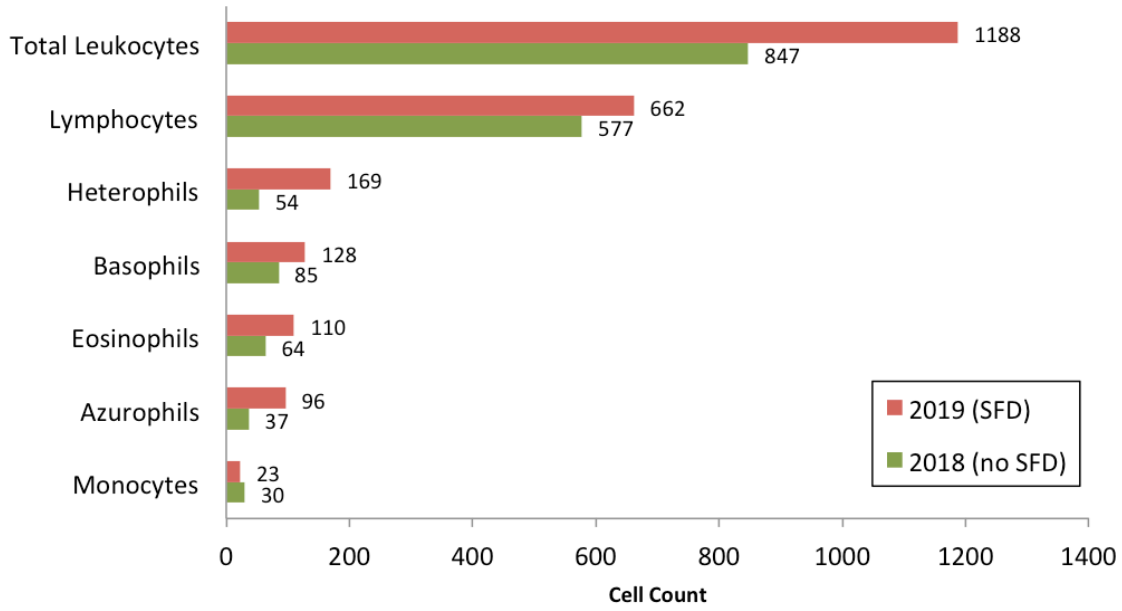

Figure 2.9: Leukocyte cell counts for snake #027-307-004, an adult female eastern massasauga rattlesnake. Leukocyte counts are based on 20,000 cells analyzed with imaging flow cytometry (IMFC). Red bars represent cell counts from 2019 when the individual received a SFD score of (3). Green bars represent counts from 2018 when the individual received an SFD score of (0).

CHAPTER III

EXTENDED LITERATURE REVIEW

Eastern Massasauga Rattlesnake

The eastern massasauga rattlesnake is a venomous reptile that is most frequently found in states including Ohio, Indiana, Pennsylvania, Wisconsin, Illinois, Minnesota, Missouri, Iowa, and in Ontario, Canada (Szymanski, 1998). In all states where this snake is found, it is listed as endangered, threatened, or a species of concern. Thus, even in states where the snake is most commonly found it is still very rare and infrequent (Szymanski, 1998). The eastern massasauga rattlesnake is also known colloquially as the eastern massasauga, prairie rattlesnake, swamp rattler, and spotted rattler (Szymanski, 1998). The scientific name of this rattlesnake is *Sistrurus catenatus,* formerly *Sistrurus catenatus catenatus*. *Sistrurus catenatus* is one of three species within the genus *Sistrurus*. The other two species in this genus are *S. edwardsii* and *S. tergeminus* (Szymanski, 1998). Massasauga is derived from an Ojibwe word meaning 'great river mouth' alluding to the habitats in which this snake lives (Szymanski, 1998).

The behaviors of this rattlesnake help to allow individuals to identify it. The eastern massasauga rattlesnake is active throughout the spring, summer, and fall, and brumates during the winter (Szymanski, 1998). It is found outside of brumation April through October, though it varies depending on temperature and food availability. While the eastern massasauga rattlesnake is venomous, it is docile and is not commonly known to strike. The eastern massasauga is more commonly known to take shelter or flee rather than strike or attack (Szymanski, 1998). This rattlesnake also shows strong fidelity within habitats, often returning to the same sunbathing locations and same brumation spots each

year, or a spot that is very nearby (Szymanski, 1998). Harvey and Weatherhead (2006) observed an unusually high mortality rate during brumation over winter, which suggested a limited number of available and proper brumation sites and extreme weather events. The eastern massasauga rattlesnake bears live young (Szymanski, 1998). A female rattlesnake may reproduce once per year, depending on resource availability, however it is more common to reproduce every other year or every three years (Szymanski, 1998). One female may have five to twenty offspring during each reproduction event. These rattlesnakes reach sexual maturity at three to four years of age (Szymanski, 1998). Lifespan in the wild is unknown, though in captivity they have a high lifespan of up to twenty years (Szymanski, 1998).

The eastern massasauga rattlesnake has several distinguishable features. The average length of an adult eastern massasauga is less than one meter, and the snakes have very thick bodies (Szymanski, 1998). The heads of these rattlesnakes are heart or triangular shaped and the eyes have cat-like diamond shaped pupils. The eastern massasauga also has the distinguishable segmented rattle at the end of its tail (Szymanski, 1998). The color of the rattlesnake ranges from a grey to a brownish-gray with dark-brown or blackish spots on its head and running down its back (Szymanski, 1998). This snake also has a few white markings on its head and sometimes running down its body that surrounds the dark spots in a stripe. The belly of this snake is also black in color with occasional white commonly looking like a scrambled checkerboard. Young eastern massasauga rattlesnakes are usually very similar in appearance but have more vivid colors and smaller bodies (Szymanski, 1998).

The habitats where eastern massasauga rattlesnakes are most commonly found are in temperate forests and wetlands. This includes swamps, prairies, farmland, bogs, marshes, meadows, and fens (Szymanski, 1998). The eastern massasauga rattlesnake also shifts locations seasonally. The eastern massasauga spends spring, winter, and fall in wetlands, and moves to higher and drier grounds in the summer. It is believed that rattlesnakes choose wet locations for hibernation to prevent desiccation (Moore and Gillingham, 2006). Switching of locations seasonally varies regionally and among different populations of rattlesnakes (Szymanski, 1998). The eastern massasauga rattlesnake can most commonly be found in locations that allow for areas of direct sunlight, used for basking, and areas of shade with rocks or logs that are used as shelter. A water source is also a necessity in the form of a stream, river, or pond (Szymanski, 1998). As common with most snakes, they are most active, eating, moving, and mating during the warmest part of the day (Szymanski, 1998).

The eastern massasauga rattlesnake feeds mainly on endothermic organisms, which it senses with the heat detecting pits on the sides of its face. However, it may also use features like sight, sensing vibrations, and smell to detect prey (Szymanski, 1998). The most common hunting strategies for the eastern massasauga rattlesnake to use are the ambush tactic, where they chose a location and wait for the food to come close to them and then strike. When the massasauga rattlesnake strikes its prey it punctures it with its hollow fangs and injects a modified saliva (venom) that rapidly kills its prey (Szymanski, 1998). The eastern massasauga rattlesnake feeds mostly on small rodents such as mice, shrews, and voles. Though it may also eat other smaller snakes, frogs, toads, and even small birds. Predators of the eastern massasauga rattlesnake include herons, hawks, foxes, skunks,

larger snakes, and raccoons (Szymanski, 1998), although the biggest threat to this rattlesnake is humans.

Snake Fungal Disease

Snake Fungal Disease has been identified on a museum specimen captured in 2000 (Allender et al. 2016b). Both wild and captive snakes have been observed with the disease, though it is considerably more common in wild snake populations. SFD is predominantly found in the eastern United States, though a few captive snakes from Europe and Australia have also been found with the disease (Franklinos et al. 2017; Lorch et al. 2016).

Clinical signs of SFD can appear anywhere on the body of a snake. However signs are most commonly present on a snakes face and ventral surface (Guzman-Vargas et al. 2020). This is because of the way a snake moves through its environment, head first, on its belly. Clinical signs include cutaneous lesions, nodules, pustules, and ulcers. Additional signs of SFD include discolored or crusty appearing scales, swollen areas and disfigurement (Guzman-Vargas et al. 2020).

The disease has been also found to cause more frequent molting and risky behaviors (Lorch et al. 2016). Risky behaviors include more frequent basking, or an earlier emergence from hibernation, in an attempt to raise body temperature to fight the disease. Unfortunately these risky behaviors can make infected individuals more vulnerable to predation or death from exposure to the elements (Lorch et al. 2016). Impacts of the disease lead to a poor quality of life and ultimately death.

Hematology

Hematology is a tool used in ecological studies for making clinical diagnoses, understanding response to diseases, and assessing the overall health of an individual.

Hematology is most often used through a blood smear, and involves identification and counting different types of blood cells including erythrocytes, leukocytes, and thrombocytes (Quadrini et al. 2018). An increasing number of ecologists are using hematology through white blood cell counts to assess the overall health of organisms, known as leukocyte profiles (Davis et al. 2008).

The leukocytes found in the study species, eastern massasauga rattlesnake, are assumed to include lymphocytes, azurophils, basophils, heterophils, monocytes, and eosinophils based on previous studies (Allender et al. 2006; Quadrini et al. 2018). Each type of leukocyte preforms a specialized immunological function. The lymphocyte includes B cells, T cells, and natural killer cells, and plays a variety of roles including modulating the immune response, wound healing, fighting parasitic infection, viral infection, and inflammatory diseases. Snake lymphocytes can be small or large, ranging from 2-15 μm and are mononuclear with finely granular cytoplasm (Rowley and Ratcliffe, 1988). Small lymphocytes are commonly round with irregular outlines and their nuclei fill the cell. Large lymphocytes are approximately the same size as azurophils and heterophils. Lymphocytes commonly represent most (80%) of all leukocytes found in blood. The azurophil plays a role in inflammatory disease response (Rowley and Ratcliffe, 1988). Azurophils range from 10-23 μm, and can vary from round to monocytoid. Basophils are associated with viral and parasitic infections and inflammatory response (Stacy et al. 2011). Snake basophils range from 7-12 μm and are identified as small spheres with abundant granules giving the contour of the cell a cobblestone appearance. The basophils process surface immunoglobulins and release histamine on degranulation. The heterophil plays a role in inflammatory disease and phagocytosis and is functionally similar to neutrophils in

mammalian blood (Rowley and Ratcliffe, 1988; Zimmerman et al. 2010). Heterophils range from 10-23 μm though the size varies between species. Heterophils have fusiform cytoplasmic granules giving them a bumpy appearance (Rowley and Ratcliffe, 1988). The monocyte can be suggestive of inflammatory diseases such as granulomatous inflammation. Monocytes range from 8-25 μm and are commonly the largest leucocyte. Their nucleus is commonly ovoid and often bean shaped (Rowley and Ratcliffe, 1988). The eosinophil plays a role in stimulation of the immune system and fighting parasitic infections. Eosinophils are commonly associated with fighting parasitic infections. Eosinophils range from 9-20 μl (Rowley and Ratcliffe, 1988). They are large round granulocytes with cytoplasmic granules similar in appearance to heterophils. Lymphocytes, heterophils, and azurophils can be influenced by seasonal change, such as a decrease in number of cells and lowered immune response during winter (Campbell et al. 1996). The numbers and impact of immune cells is also very dependent on environmental conditions such as climate and temperature, as reptiles are ectothermic (Campbell et al. 1996).

Imaging Flow Cytometry

The tool that is used to study hematology is essential in order to deliver fast and accurate results. Previously, blood smears have been used to study hematology and determine leukocyte profiles, (Bell and Gregory, 2014; Davis et al. 2008; Motz et al. 2014) but imaging flow cytometry (IFCM) provides a faster and more accurate analysis of blood samples. Blood smears are time consuming, involving a manual count of 100-200 cells for each individual sample.

Antibodies

Fluorescently labeled antibodies can be used on blood samples to enhance the ability of an IFCM technician to differentiate different leukocyte types in a sample (Benoist and Hacohen, 2011). An antibody works by recognizing target molecules on the surface of a cell, and then binds to those target proteins. A fluorescently labeled antibody can be added to a blood sample to target one specific cell type, and when analyzed by IFCM, the target cell brightly fluoresces and is easy to distinguish (Benoist and Hacohen, 2011). Fluorescently labeled antibodies work by binding to antibody specific antigens on an immune cell's surface. An antibody's antigen-binding site, called the paratope, binds to a specific region on an antigen called the epitope that consists of 5-8 amino acids (Sompayrac, 2012). Generally, within the body of an organism, antibodies are used to tag cells for attack by the immune system, however fluorescently labeled antibodies can be used by a technician to tag leukocyte cells for identification. However, because of the limited research in reptile hematology, reptile antibodies have not yet been commercially created. Many antibodies have been created for a specific species (e.g. mouse), but are highly cross-reactive with several other species (e.g. fish, chickens, and humans). Using a highly cross-reactive antibody may allow for identify leukocyte cells in reptile blood.

EXTENDED METHODOLOGY

Study Sites and Snake Capture

Field work was conducted from May-July in 2018 and May-August 2019 at ten locations, nine distributed throughout Michigan's lower peninsula, and one on Bois Blanc Island. On Bois Blanc, several sites were surveyed, but because of close proximity they were all regarded as on study site. All sites were located on state game areas, state parks, state forest areas, or national forests that contained intact habitat and eastern massasauga populations. Study sites were selected based on historic records of eastern massasauga sightings, and surveying took place on days with appropriate weather conditions (no rain and >59 °F). Each site was surveyed multiple days by at least two surveyors for approximately 1-3 hours beginning at approximately 0900h. Surveyors meandered unsystematically through sites attempting to encounter snakes. Eastern massasaugas were captured opportunistically with the use of snake tongs and placed in a cloth bag, which was safely tied shut and placed in a bucket until surveying was complete for the day. Upon capture, environmental data were recorded including cloud cover, shaded air and substrate temperature. Snake positional data at capture was also recorded including if the snake was in full sun, part sun, or full shade, also if the snake was fully exposed, partially covered, or completely covered by vegetation. A GPS was used to mark the exact capture location (latitude, longitude) of each snake so that they could be returned to the same spot following sample and data collection.

Data and Sample Collection

Snakes were taken to a field station or improvised field lab and processed individually. Snakes were safely removed from their cloth bags and encouraged to slither

into a clear plastic tube with the assistance of a snake hook. Once their head and first-third of their body was inside of the tube, snakes could safely be picked up and handled with gloves. All equipment used was either single-use and disposable, or was sterilized with a 10 % bleach solution after each snake was processed and gloves were changed to avoid potential SFD spread through contact. Snout-to-vent length (SVL) was calculated by subtracting measured tail length from total length. Also, subcaudal scales and rattle segments were counted. Weight was determined by subtracting the weight of the empty cloth bag from the weight of the cloth bag with the snake inside. Based on SVL, age class was assigned as adult (≥ 45 cm for females and ≥ 43 cm for males), juvenile (44.9 - 30 cm for females and 42.9 -30 cm for males), or young (29.9 – 21 cm for both females and males) (Allender et al. 2016b; Bradke et al. 2018; Jellen, et al. 2007). Young snakes were classified as such if they were younger than one year of age, based on if they had no addition rattle segments other than the button rattle segment they were born with. Sex was determined through cloacal probing for the presence of hemipenes and if female, reproductive status was determined, by gently palpating females to determine number of young present. Individual snakes were marked by injecting a subdermal Passive Integrated Transponder (PIT) tag with a personal identification number. PIT tags were used for snake identification in the event of future recaptures. Rattle bases were also painted with a dull colored nail polish for easy identification of recently captured snakes in the field. Snakes were thoroughly examined for clinical signs of snake fungal disease (SFD) such as facial swelling, crusts, bumps along the body, cutaneous lesions, ulceration, discolored scales, and opaque eyes (Lorch et al. 2016). Based on the severity of SFD signs snakes were given a “SFD score” from 0-3, with (0) signifying no SFD signs present, (1) indicating fewer than five lesions on

the ventral or dorsal surface, (2) signifying more than five lesions on dorsal and ventral surfaces including the tail, and (3) indicating multiple severe lesions on the face and cloaca as well as the dorsal and ventral surfaces and tail (Figure 2) (McCoy et al. 2017). A blood sample was also collected for IFCM analysis using a hypodermic needle; 100 µl (two to three drops) of blood were drawn from the caudal vein and placed it in a 1.5 mL Eppendorf microcentrifuge tube with 100 µl Strek Cell Preservative™ (STREK™). Blood samples were kept on ice or refrigerated until they could be analyzed with IFCM. After all data was collected, snakes were returned to the exact same spot they were captured within the same day.

Imaging Flow Cytometry Analysis – INSPIRE™

To prepare for IFCM analysis, blood samples were first subsampled by vortexing and pipetting 10 µl of the blood sample into a 1.5 mL microcentrifuge tube with 10 µl of phosphate buffered saline (PBS). The subsample was vortexed again and placed in the Amnis Imagestream X Mark II Imaging flow cytometer (Amnis Technologies, Millipore Sigma) with dual excitation lasers (488 nm and 642 nm) at 0.5 mW. Low flow speed was operated at 40 X magnification. For each sample, 20,000 events (images) were captured using INSPIRE™ software based on methods of Roussel et al. (2010). Events could then be evaluated with a data analysis file (DAF) through IDEAS™ analysis software (Amnis Technologies, Millipore Sigma).

Imaging Flow Cytometry Analysis – IDEAS™

To create the DAF, a one-step Percoll gradient was used to isolate leukocytes by adapting the methods from Carvalho et al. (2016). One mL of fresh blood mixed with 2 mL of RPMI medium was carefully overlaid on 5 mL of a 57% Percoll solution in a 15 mL

conical tube (Carvalho et al. 2016). The solution was centrifuged at 1,280 g for 15 min at 18 °C with acceleration brakes at zero. The leukocyte layer was then carefully recovered from between the layers of plasma and Percoll and washed once in 10 mL PBS, centrifuging at 1,500rpm for 5 min (Carvalho et al. 2016). The resulting leukocyte pellet was re-suspended in 1 mL of PBS and used to create a leukocyte DAF. Using IDEAS™ software, first, out-of-focus images and images with more than one cell were removed from analysis. Then a dot plot cytogram was used to establish collection gates based on area (size) and intensity (shape and external complexity) of cells. Collection gates for heterophils, basophils, lymphocytes, eosinophils, monocytes, and azurophils were established and a leukocyte DAF was created. This DAF was used on a random blood sample and a collection gate for red blood cells was established. Debris, lysed cells, and platelets were left un-gated. The resulting DAF containing gates for all leukocyte types and red blood cells was used to analyze each individual snake blood samples. The number and percent gated of red blood cells, lymphocytes, basophils, heterophils, eosinophils, azurophils, and monocytes were recorded for each sample to use for statistical analysis.

Antibody Selection and Application

Antibodies were selected based on application (IFCM), excitation lasers (488 nm and 642 nm), conjugate (APC, PE, FITC), species reactivity (mouse), and target ability (cross-reactivity). We selected for high cross reactivity with different tetrapod vertebrate classes (mammals, birds, reptiles, amphibians), with the assumption that, the more cross-reactive, the greater chance it would react with snake cells. Commercially available antibodies developed specifically for reptiles are rare, hence cross reactivity with bird species or mammals with heterophils were selected. Based on specific selection criteria,

seven mouse leukocyte antibodies were selected: PE anti-mouse CD45 clone 30-F11 (hereafter identified as CD45), PE anti-human/mouse CD11b clone M1/70 (CD11b), F4/80 PE monoclonal antibody clone BM8 (CDF4/80), CD19 PE monoclonal antibody clone eBio1D3 (CD19), APC anti-mouse Ly-6G clone RB6-8C5 (LY-6G), APC anti-human/mouse CD45R clone RA3-6B2 (CD45R), and CD3e FITC monoclonal antibody clone CD3-12 (CD3e). To establish if any of these antibodies worked on snake leukocytes, control samples of mouse murine cells were prepared by using a procedure adapted from Gjurich et al. (2015) and stained with florescent antibodies to be analyzed and compared with samples of snake blood. Antibodies were added to samples and incubated for 30-60 min at 4 °C while protected from the light, then washed, and run in the IFCM instruement, as described above. We also tested to determine if our cell preservative used on our snake blood samples (i.e. STRECK cell preservative), had any influence on antibody ability to adhere to leukocyte cell surface by creating and analyzing Strek™ preserved mouse cell samples (STREK™).

Statistical Analysis

Prior to data analysis, any samples that contained fewer than 20,000 images were removed. Any samples that were missing information or contained only platelets and debris were also removed from analysis. The R archive network (version 3.6.2) software was used for all statistical analyses and vegan package was applied. Data was standardized with z-score and square-root transformed. A numerical environmental score was created for analysis based on: percent cloud cover, shaded air temperature, and substrate temperature at the time of snake capture. A numerical position score was also created based on how the snake was found: completely covered by foliage, partially covered, or

fully exposed and if the snake was in direct sunlight, partial sunlight, or fully in the shade. We used a Principal Component Analysis (PCA) to visualize the relationship between data variables (location, year, sex, age class, SFD score, capture type, reproductive status, SVL, mass, month, day, season, environmental score, position score, red blood cell count, leukocyte count, lymphocyte count, monocyte count, heterophil count, azurophil count, basophil count, and eosinophil count) and each individual snake from both 2018 and 2019. A PCA biplot allows for visualization of approximate Euclidian distances among objects in multidimensional space. For the PCA, if snakes were captured in both 2018 and 2019, the sample from 2019 was removed from analysis. Mean and standard error was calculated for each type of leukocyte based on sex and age class using the 20,000 captured images for each individual. Snakes with signs of SFD or repeat individuals were removed from mean leukocyte calculations. Student's *t*-tests were used to determine significance between sex, reproductive status, and age classes. Analysis of variance (ANOVA) with Tukey post hoc test was used to determine if differences between location and leukocyte counts were different, and if SFD scores and leukocyte counts were different. Results were considered statistically significant at $p < 0.05$.

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