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INVESTIGATING THE ROLE AND LOCALIZATION OF B CELLS IN THE RED-EARED
SLIDER, *TRACHEMYS SCRIPTA*

WHITNEY N. HURST

56 Pages

The immune system of humans has been studied extensively, but very little is known about the immune response in reptiles. Our lab has been working to better understand the immune system of a pond turtle, *Trachemys scripta* (red-eared slider). Turtles are known for their longevity and their immune system should play an important role in their ability to survive. Our main interest is understanding B cell function and location in slider turtles. B cells are a type of white blood cell that play a vital role in the immune response of humans, with the majority secreting antibodies that mark invading bacterial cells to be destroyed. Some B cells can ingest pathogens directly in order to get rid of them through a process known as phagocytosis. We first confirmed that turtle B cells can survive cell sorting. Next, we investigated the phagocytic properties of turtle B cells. The phagocytic cells were sorted on a flow cytometer. This showed that phagocytic B cells could also survive being sorted. The sorted cells were used to complete an ELISpot assay to see if B cells could produce antibodies after phagocytosis. The results show that the B cells do produce antibodies after phagocytosis. Lastly, we wanted to look at the location of B cells in the intestines of turtle hatchlings. We were able to identify B cell clusters within hatchling intestines by using immunohistochemistry. Knowing the location of B cells helps us understand how reptiles use this tissue to defend against pathogens.

KEYWORDS: Turtle, immunity, B cells, phagocytosis, flow cytometry, immunohistochemistry

INVESTIGATING THE ROLE AND LOCALIZATION OF B CELLS IN THE RED-EARED
SLIDER, *TRACHEMYS SCRIPTA*

WHITNEY N. HURST

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

2021

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INVESTIGATING THE ROLE AND LOCALIZATION OF B CELLS IN THE RED-EARED
SLIDER, *TRACHEMYS SCRIPTA*

WHITNEY N. HURST

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ACKNOWLEDGMENTS

I would like to start off by thanking my co PIs, Dr. Laura Vogel and Dr. Rachel Bowden for giving me the opportunity to work on this research project. This work would not have been possible without their support and guidance. They were always more than willing to be of assistance when it came to my research or writing. I would like to thank my lab members Anthony Breitenbach and Rosario Marroquin-Flores for their help in the lab and out in the field. I greatly appreciate the training and patience you provided while setting traps. I would also like to thank all of the undergraduates that helped in the lab and trapping in the field with this project; Hailey Fitzsimmons, Paige Farchmin, Ceara Abbott, Allison Mool, Hanna Paton, and Julia Godsey. All of your assistance made a positive impact on this project. Finally, I would like to thank my husband, Dylan, for his constant support and encouragement throughout this journey. Thank you for never letting me give up and consistently reminding me that I was capable to complete this degree. This work was supported by National Science Foundation grant Grant/Award Number 1725199 to LA Vogel and RM Bowden, National Institutes of Health Award 1R15AI140118 – 01 to LA Vogel and RM Bowden and National Science Foundation grant Grant/Award Number DBI-1828136 to KA Edwards.

W.N.H.

CONTENTS

	Page
ACKNOWLEDGMENTS	i
TABLES	iv
FIGURES	v
CHAPTER I: AN OVERVIEW OF THE IMMUNE SYSTEM OF REPTILES	1
Overview	1
Model organism	2
Innate Immunity	3
Adaptive Immunity	5
GALT	10
CHAPTER II: MATERIALS AND METHODS	14
Hatchling/adult collection and tissue preparation	14
HL673 mAB preparation	15
Lymphocyte collection and preparation for flow cytometry	15
ELISpot Assay	18
Immunohistochemistry with whole mount tissue	19
H&E and Immunohistochemistry with tissue slides	20
CHAPTER III: RESULTS AND DISCUSSION	23
B cells are identified in whole blood	23
Titration of SA-PE-Cy7 to determine best dilution	24
B cells identified in hatchling spleen and liver tissue	25
B cells identified in hatchling distal and proximal intestinal tissue	26

Unable to distinguish intestinal B cells in adult intestinal tissue using whole mount immunohistochemistry	27
H&E and Immunohistochemistry staining of adult turtle spleen and intestinal tissue	28
B cells can produce antibodies after phagocytosis	30
CHAPTER IV: CONCLUSION	33
REFERENCES	34
APPENDIX: TABLES AND FIGURES	40

TABLES

1. Hematoxylin and eosin staining procedure	22
2. B cells that first ingested beads in the phagocytic assay were still able to secrete antibodies	57

FIGURES

1. Schematic representation of fluorochrome use in flow cytometry	41
2. Example of flow cytometry analysis	42
3. Streptavidin-coupled fluorochromes can be used to identify turtle B cells labeled with biotinylated anti-light chain monoclonal antibody	43
4. Titration of SA-PE-Cy7	44
5. B cells are present in hatchling spleen tissue	45
6. Dissected cells may be present in hatchling liver tissue	46
7. B cells are present in hatchling distal intestinal tissue	47
8. B cells are present in hatchling proximal intestinal tissue	48
9. B cells are not able to be detected in adult distal intestinal tissue	49
10. B cells are not able to be detected in adult proximal intestinal tissue	50
11. Lymphocytes are present in periarteriolar regions of adult turtle spleen tissue	51
12. Staining of adult turtle intestinal tissue reveals many lymphocyte-like cells distributed in the lamina propria	52
13. HL673 staining failed to identify B cells in adult turtle spleen tissue	53
14. Adult splenic B cells are able to phagocytose beads	54
15. Flow cytometry analysis of adult turtle blood following a phagocytic assay	55
16. B cells that had first phagocytosed beads are still able to secrete antibodies	56

CHAPTER I: AN OVERVIEW OF THE IMMUNE SYSTEM OF REPTILES

Overview

Some microbes that are found within an organism's environment can negatively impact fitness and survival. Over evolutionary time, organisms have developed different and complex immune responses to help combat these pathogenic microbes. While all living organisms have some basic type of immunity, my research focuses on long-lived vertebrates. All vertebrates have an immune system composed of two branches: the innate and adaptive immune systems. The innate immune system acts rapidly after a pathogen or toxin enters the organism and is the initial host response. A number of molecules and cells act to try and prevent the spread of the infection and activate the second branch of the immune system [1]. The adaptive immune system is composed of white blood cells called lymphocytes that have specificity for individual pathogens or toxins and serve to eliminate the infection as well as prevent future infection (immunological memory). Mice and humans have primarily been the model organisms to study the immune system, and there is little known about the immune system of reptiles. By studying a non-model organism, it allows us to have a different perspective on how other immune systems are working and could lead to discoveries that are beneficial to the human immune system and help improve efforts to conserve endangered reptile species.

Our lab has been working to better understand the adaptive immune system of the red-eared slider turtle, *Trachemys scripta*. We are particularly interested in a type of lymphocyte called a B cell that serves as a critical role in the protection from infection. It has been found that turtle peripheral blood B cells are capable of both the production of antibodies and can use the process of phagocytosis to engulf and digest pathogens, which is surprising and different from how most human B cells behave [2, 3]. First, I will give an overview of the immune system,

specifically focusing on the B cell function in reptilian immunity. Next, I will discuss experiments that were conducted with *T. scripta* including looking at the phagocytic capabilities B cells by using whole blood samples and looking at antibody secretion using an ELIspot assay. Lastly, the localization of B cells in the red-eared slider was examined by live whole mount histochemistry of intestine and fixed tissue section of intestinal, spleen, and thymus tissue.

Model organism

T. scripta are medium to large freshwater pond turtles. They are native to Illinois and in the U.S., range from southeastern Virginia south to Florida and west to New Mexico. They prefer quiet waters with soft bottoms and a variety of aquatic plants [4]. Due to their ectothermic nature, they prefer areas that allow them to bask in the sun. Female *T. scripta* have a nesting season from May-July roughly. Their nests can be located on unshaded land near water. Typically, females will lay 1-30 eggs with a mean of 10.5 per nest and will lay 1-5 clutches per year. Once the eggs are laid, they will incubate on average 61.1-108.0 days [4]. When the turtle hatches, it will immediately be exposed to many microbes in the nest environment which their immune system will encounter. The red-eared slider hatchlings will stay in this nest environment over winter throughout much of their range. Once winter ends, hatchlings leave the nest and will then migrate to the water.

While they are young, their diet consists of various vegetative species, crayfish, insect larvae, tadpoles, and many other aquatic animals found in their environment. During adulthood their diet consists mainly of plant life [6]. Occasionally, red-eared slider turtles will consume the flesh of dead animals [7] Red-eared slider turtles enter brumation when temperatures drop below 10°C. Brumation is similar to hibernation in mammals [8], but the animal is awake and still able

to move and eat. Slider turtles can stay in brumation during the winter for up to 180 days [4]. Longevity is another known trait of turtles. Captive *T. scripta* can live on average up to 41.3 years, while the average wild lifespan is 30 years. Giant tortoises have been found to live an excess of 100 years [9]. The marshes and streams that slider turtles are commonly found in are often poor quality with high microbial loads. The ability for this animal to live for a long period of time in pathogen-rich environments makes it a great model organism to study immune functions.

Innate Immunity

The innate immune system is considered the first line of defense against pathogens. This system plays a critical role in preventing infection and maintaining homeostasis. Physical barriers are the primary mechanisms of innate immunity in preventing infection and the spread of microbes. While all living vertebrates have skin as their main protective barrier, reptiles form a thick epidermis with layers of α - and β - keratin layers [10]. If a pathogen can overcome these physical barriers, elements of the innate immune system are encountered. Innate immunity in reptiles acts quickly in response to pathogens by utilizing lysozymes, the complement system, antimicrobial peptides, and non-specific leukocytes [2].

Lysozymes compromise the bacterial cell wall which leads to the lysis of the bacteria [2]. These proteins have been identified from some species of turtles, including the soft shelled turtle (*Trionyx sinensis*), the Asiatic soft shelled turtle (*Amyda cartilaginea*) and the green sea turtle (*Chelonia mydas*) [11]. In vertebrates, defensins are one of the major classes of antimicrobial peptides. They play a critical role in innate immunity in most animals. These antimicrobial peptides are often expressed after a bacterial or viral infection. β -defensins are commonly

expressed in epithelial cells, but the first β -defensin was identified in turtle leukocytes [11].

Defensins have α - helix characteristics, but predominantly have β - sheets. No known genes for α -defensins have been found in reptiles, but in some species, such as the red-eared slider turtle β -defensin genes and peptides have been identified [11].

Macrophages, monocytes, neutrophils, basophils, and eosinophils are leukocytes of the innate immune system found in both mammals and reptiles. These cells use surface receptors to recognize broad categories of microbial products and tend to be specific for a type of microbe (virus vs bacteria for example) rather than a particular species. Macrophages and monocytes release cytokines and are phagocytic cells that process and present antigens [12]. Neutrophils are granule-containing phagocytes that are present in mammalian species. Reptiles have cells that function similarly to neutrophils called heterophils. These cells have nuclei with more rounded appearance [13]. Basophils in turtles have been shown to be analogous to mammalian basophils [14]. Although they are typically low in number, eosinophils in reptiles also function similarly to mammalian eosinophils [15]. Basophils and eosinophils are thought to play a key role in protection against parasitic infections.

As part of the innate immune system, the complement system enhances the ability of antibodies and phagocytic cells to clear pathogens. The complement system has three main functions that have been documented: opsonization, chemotaxis, and lysis. It functions as a cascade of proteases that activate each other in an enzymatic fashion and is comprised of several membrane-bound regulators and receptors. The classical pathway, lectin pathway, and alternative pathway can all activate the complement cascade by different recognition molecules [16]. One of the most important roles of the complement system is to help activate the adaptive immune response. Both the alternative and classical pathways have been shown to exist in

reptiles [17]. It is suggested that the lectin pathway is present in reptiles, but this pathway has not been extensively studied in all species [17]. A recent study has shown that the common snapping turtle (*Chelydra serpentina*) and alligator snapping turtle (*Macrochelys temminckii*) exhibit different primary complement defense mechanisms. *C. serpentina* utilized the alternative pathway as its primary defense mechanism while *M. temminckii* relied heavily on the lectin-mediated pathway [18].

This information shows us that there are many similarities and differences in the innate immune system among mammals and reptiles. Overall, innate immune response is vital for the survival of an organism. Without this response pathogens could quickly infect an organism and potentially be detrimental to the organism's health. This response has evolved over time to become extremely efficient in all vertebrates.

Adaptive Immunity

In contrast to the innate immune system, the adaptive immune system is pathogen-specific and depends on the generation of cells that each express different antigen receptors. Cell-surface receptors are utilized by specialized cells called lymphocytes to recognize antigenic epitopes of specific pathogens. The cells respond to the antigen by clonal amplification and cellular differentiation [19]. After the initial pathogen is encountered, memory cells are formed that can be present in the host for life. This provides immunological memory and allows a rapid response if an organism is re-exposed to the same pathogen. T lymphocytes mature in the thymus and B lymphocytes are antibody producing cells produced from the bone marrow. Both cells play a vital role in the adaptive immune response and are highly mobile, circulating throughout

the body [20]. The adaptive immune system is made up of cell mediated and humoral adaptive immunity mediated by these two different cell types.

Cell Mediated Immunity: During the innate response, phagocytes are digesting and engulfing pathogens. The pathogen is broken down into small peptide fragments that bind to the Major Histocompatibility Complex (MHC) and are transported to the cell surface to be displayed. In mammals, antigen presenting cells will then migrate to lymph nodes to interact with T lymphocyte cells and initiate adaptive immunity [21]. T cells are the main class of lymphocytes involved in cell mediated immunity. Development of T cells occurs in the thymus from lymphoid progenitors that come from the bone marrow or fetal liver [20]. Specialized receptors are displayed on the surface of T cells, called T cell receptors (TCRs). TCRs can bind with great specificity to the foreign peptides displayed on MHC-peptide complexes. When a T cell recognizes the specific antigen for its TCR it becomes activated [21]. Once a T cell is activated it can differentiate into helper T cell (Th) or cytotoxic T cell (TC) [22]. Helper T cells stimulate antibody production through B cells and cytotoxic T cells attack and kill infected cells [21]. Many helper T cell differentiation pathways have been described in mammals (for example, TH1, TH2, TH17, etc.) and the subtype of helper cell can drastically change the immune response. [22] Regulatory T cells play a pertinent role in regulating the immune network. There have been at least three types of regulatory T cells identified in mammals. CD4⁺ and CD25⁺ are regulatory T cells that are naturally generated in the thymus and inhibit the differentiation and function of both Th1 and Th2 cells [23].

Although not a lot is known about the exact nature of T cells in reptiles, they do possess a thymus and there have been functional T cell studies done in snakes, lizards, turtles, and tuatara [24]. During this study, researchers were able to successfully stimulate mitosis among cultures of

whole blood from tuatara and measure cell proliferation of mononuclear cells that were isolated from the blood. They were able to quantify T cell proliferation by a colorimetric assay in response to chemicals known to stimulate T cells in mammals, although T cells themselves were not directly observed [24]. In other studies, CD4 and MCHII genes were identified in the western painted turtle genome [25], and in the Chinese crocodile lizard, *Shinisaurus crocodulurus*, sequences of T cell receptors β chain (TBRC-2) were detected from the reconstruction of the liver transcriptome [26], suggesting these subsets may exist again without directly observing the cells. The presence of splenic lymphocytes was shown during a study looking at the role of the thymus in the immune response of the oriental garden lizard, *Calotes versicolor* [27]. Researchers used histology to show the peri-arteriolar region was populated by lymphocytes that disappeared when the animal was thymectomized. No specific stains were used to determine if the cells observed were indeed T cells or which subset. One study which did directly observe T cells identified CD3+ T lymphocytes in the green turtle *Chelonia mydas* [28]. This reagent bound all T cells and didn't differentiate between CD4 and CD8 subpopulations. Given that turtles have a slower and less robust adaptive immune response compared to mammals their lack of lymph nodes, the question remains as to exact function of helper T cells in these animals. Clearly further studies need to be done to visualize and determine T cell subsets and distribution.

Humoral Immunity: While cell mediated immunity involves T lymphocytes, humoral immunity involves B lymphocytes. In mammals, B cells originate from hemopoietic stem cells in the bone marrow where they go through developmental stages acquiring antigen specificity. They will next exit the bone marrow and complete development to the mature or naïve stage in the spleen [20] where they will differentiate to subsets. Naïve B cells are generally divided into

three subsets, B-1 B cells, follicular B cells and marginal zone (MZ) B cells [29]. Marginal-zone B cells respond without priming to blood-borne pathogens and follicular B cells mediate adaptive immunity [29]. Follicular B cells are termed B-2 cells. B-1 cells are a separate population of B cells that are found in the spleen, intestine, and peritoneal and pleural cavities [30]. These are the first B cells to appear during development and appear to have a more primitive function than other B cells. Both kinds of B cells are replenished throughout the life of an adult animal, but they accomplish this in different ways. B-1 cells differ in the fact that they are produced mainly in the fetus before birth and repopulate in the peripheral areas in which they reside. B-2 cells are produced constitutively in the bone marrow throughout life [31]. Unlike self-renewing B-1 cells, B-2 cells are replenished throughout life by progenitors in the bone marrow. In adults, no newly differentiated B-1 cells enter the peripheral pool, but undifferentiated progenitors in the bone marrow continually give rise to B-2 cells. Only a few percent of total B cells present in the blood and spleen of mammals are B-1 cells, while the majority are B-2 cells [32]. Antibodies produced by B-1 cells tend to be of lower affinity, typically only IgM isotype and lack immunological memory [32].

B cells are activated by the binding of an antigen to the B-cell receptor (BCR). This initiates a cascade of intracellular signaling that leads to the internalization of the antigen for processing and presentation to T cells. Two types of BCR isotypes are expressed on mature B-cells, IgM and IgD [33]. During an immune response, B cells also undergo immunoglobulin (Ig) isotype switching which results in a change from IgM and IgD expression by naïve B cells to the expression of one of the downstream isotypes such as IgG, IgA or IgE [34]. The antibody isotypes in poikilothermic animals also appears to be much less diverse than mammals [35]. Initially, antibodies similar to mammalian IgM were found in tuatara (*Sphenodon punctatum*)

serum. Four heavy chain classes; IgM, IgD, IgY, and IgA have been found in several species of reptiles. In turtles it has been found that IgM and IgD are detectable 7-10 days post fertilization. IgY (which is equivalent to mammalian IgG) is only measurable at ~90 days post-hatching. This suggests that the expression of IgY may be T cell dependent [36]. Ig switch regions have been found in reptiles as well and are believed to undergo Ig isotype switching similar to mammals [37]. Importantly, turtles are missing IgA antibodies which serve a critical function in mammals to protect mucosal surfaces.

B cell subsets have not been identified in reptiles, but functionally it appears they may be most similar to B-1 cells in mammals. Due to their less robust antibody response reptiles may rely on the production of non-specific antibodies in the form of natural antibodies (NAbs) which are produced without any previous infection or foreign antigen exposure [38]. In the absence of external antigenic stimulation, natural antibodies are constitutively produced by mammalian B-1 cells and are predominately IgM. NAbs provide early protection against pathogens as these antibodies tend to be polyreactive and bind to multiple antigenic targets [39]. Poikilothermic animals make antibodies, but the response differs compared to mammals [35]. Some B cells are directed to differentiate into plasma cells during an immune response under the direction of signals received from T cells [20]. Plasma cells produce large quantities of antibodies and are mostly short lived. Reptile antibodies tend to be of lower affinity and do not increase in affinity with time, unlike what is seen in mammalian B-2 cells. Reptile antibodies do not increase in titer in a secondary response either, again in contrast to what is observed in mammalian B-2 cells [2]. These characteristics make the reptile antibody response more similar to the mammalian B-1 cell response.

Overall reptile humoral responses tend to differ in many ways compared to mammals. It is unknown if reptiles have different subsets of B cells. B cells have mostly been studied functionally through measuring antibody response and very few studies have visualized B cells or B cell distribution directly. The reptilian B cell response tends to be slower and can fail to produce antibodies that have an increase in binding affinity. Also, an increase in titer upon a second exposure does not always occur [2]. In the painted turtle, *Chrysemys picta*, no significant immune response was seen following a second injection of antigen when compared to the titer peak observed after the first injection [40]. Thus, more study of B cell function and distribution in reptiles is needed.

GALT

In mammals, mucosal surfaces and their associated lymphoid structures are part of the mucosa-associated lymphoid tissue (MALT). The gut is the largest lymphoid organ and the gut associated lymphoid tissue (GALT) plays a major role in the immune system as a part of the MALT. GALT has more than 10^{12} lymphocytes and more antibodies than any other part of the body in mammals [41]. Although the gut microbiome consists of a substantial number of bacteria, viruses, and fungi, the intestinal mucosal barrier (consisting of the mucus layer, epithelial cells, and immune cells) prevents these microorganisms from reaching systemic sites [42]. Intestinal bacteria are constantly being tested by dendritic cells in the lamina propria and will transport bacterial derived antigens to lymph nodes. Pathogen-associated molecular patterns are highly conserved structural motifs common to many microbes and can be recognized by pattern recognition receptors of innate immune cells present in the GALT. Red-eared sliders are specifically exposed to *Salmonella* on a regular basis, but it does not appear that the *Salmonella*

affects these them like it would mammals [43]. These turtles seem to be showing tolerance to certain bacteria. Recognizing these bacterial products is extremely important and leads to the initiation of humoral mucosal immune responses [44].

Adaptive immunity in the mammalian gut can be found in organized structures such as Peyer's patches and mesenteric lymph nodes as well as throughout the intestinal epithelium and lamina propria [42]. Germinal center B cells can switch the isotype of their immunoglobulin gene from IgM to other antibody isotypes when encountering T helper cells. Generally, this isotype switching will result in IgA and have a high affinity for antigens. When B cells are activated within Peyer's patches, they will also generate IgA-producing plasma cells. These plasma cells are utilized in T cell-dependent and T cell-independent responses in the gut [45] and exist in generally exist in the lamina propria [46].

IgA in mammals can also be induced in smaller structures called isolated lymphoid follicles (ILFs) [47]. ILF's of mice develop within the lamina propria after birth during the bacterial colonization of the gut. In humans, ILF's are present at birth [48]. Studies have shown that ILF's act similarly to Peyer's patches and have the ability to activate a mucosal immune response [49].

Red-eared slider turtles live most of their lives ingesting water containing many bacteria, viruses, and fungi and overall, these turtles tend to have relatively good health. Thus, it is intriguing how their immune system is functioning and allowing these turtles to live for long periods of time while remaining ultimately healthy. Unlike mammals, reptiles lack secondary lymphoid tissue such lymph nodes, germinal centers, and Peyer's patches which play a key role in the interaction between T and B lymphocytes [35]. Since turtle's lack Peyer's patches and IgA, it is unclear how their gut immunity works. One experiment done with mice showed that

Peyer's patches were not necessary for mucosal antibody production to occur [50]. The importance of Peyer's patches was discussed, but immunity occurring without them could mean that some animals could rely on other processes during immunity such as ILF-like B cells [50]. Evidence from our lab has suggested that young red-eared slider turtles have ILF-like B cell aggregates in their intestines [43]. Understanding the role of these ILF-like B cells in the slider immune system could help us better understand how their immune system as a whole is working. Not having an identical GALT immunity to mammals does not mean these animals lack immune protection. Turtles appear to maintain their health throughout their lifespan with little evidence of a decline in their immune function.

For this current project, we extended our work on B cells in the red-eared slider. First, I developed the complex techniques need to complete these studies including trapping and field work, blood collection and sample preparation, setting up and running the flow cytometer, phagocytic assay and ELISpot assays, whole mount and histochemistry techniques. Next, I evaluated different fluorochromes for use in multicolor flow cytometry assays and tested whether our new batch of mAb was active and appropriately biotinylated. To look at the appearance and distribution of B cells in development, we examined some hatchling tissues that we predicted should contain B cells. Hatchling spleens and livers were tested for B cells by flow cytometry and intestines tested by whole mount immunohistochemistry. Liver is thought to be an early place of B cell development that is gradually replaced by the bone marrow and other tissues with age. To look at adults, blood, spleen, and intestines were examined using flow cytometry, whole mount immunohistochemistry and tissue sectioning.

Next, I investigated more about the function of the B cells in adult blood and spleen. I tested whether or not turtle B cells were able to survive the sorting process, and also if

phagocytic B cells were able to survive the sorting process. We investigated whether phagocytosis was a terminal differentiation step for B cells by taking B cells that had ingested fluorescent beads, sorting them, then testing them in an ELISpot assay for antibody secretion. These experiments furthered our understanding of B cell distribution and function in reptiles.

CHAPTER II: MATERIALS AND METHODS

All experiments were done under IACUC approval.

Hatchling/adult collection and tissue preparation

During the 2019 and 2020 nesting season, clutches of eggs were collected from Banner Marsh (Canton, IL) from nests or gravid females. Gravid females were brought back to the lab and oviposition was induced by an oxytocin injection [51]. Eggs collected were incubated in vermiculate until hatched. Approximately six weeks after hatchlings emerged from their eggs, the animals were euthanized with an intraperitoneal injection of a euthanasia agent. A yolk sac is still present in the hatchling turtles at this age and no other food has been ingested. The yolk sac is attached to the intestine and serve as a landmark to differentiate proximal (above the yolk sac) and distal (below the yolk sac) portions of the intestine. To see if there was a difference in portions of intestinal tissue exposed to food or not, we collected distal and proximal portions of intestinal tissue from the hatchlings. The intestinal tissue was immediately flushed with cold 1x PBS, pH 7.4 to clean it out. The tissue was cut open longitudinally under a dissecting microscope. These sections were pinned into 6 well plates containing mounting wax with the lumen side up and kept hydrated with room temperature 1x PBS. The entire spleen tissue was removed and kept hydrated with room temperature 1x PBS.

Adult turtles were also collected from Banner Marsh. Baited traps were set in the marsh and within 24 hours the traps were pulled, and any adult red-eared slider turtles were kept. All turtles were marked by drilling holes in the scute of the shell. Each scute corresponded to a number system. If a turtle was already marked, the logs were checked to make sure the turtle had not been bled within the last year. Some turtles had their blood drawn in the field and some

turtles were taken back and housed in the lab. Turtles that were taken back to the lab and housed in IACUC approved water tanks until blood samples were taken. Approximately one milliliter of blood was drawn from the caudal vein of each turtle with a coated ethylenediaminetetraacetic acid (EDTA) sterile syringe, to prevent clotting. Immediately following collection, blood samples were diluted 1:1 with Ringer's solution. Diluted blood samples were then kept on ice. Turtles were returned within 48 hours of capture. Some turtles were also euthanized to collect intestinal, liver and spleen tissue for histology or flow cytometry.

HL673 mAB preparation

HL673 mAB is a mouse anti-turtle light chain protein reagent previously developed and commercially available [53]. Culture supernatant was obtained from the University of Florida Hybridoma Facility and purified by others in our lab (data not shown). Briefly, culture supernatant was subjected to Protein A affinity chromatography and eluted with acidic conditions. Fractions containing eluted proteins were pooled, dialyzed and tested for activity by ELISA (binding to turtle Ig). Some of the HL673 was conjugated to biotin by others in the lab. Briefly, HL673 was coupled to biotin using standard methods and unbound biotin removed by dialysis. The reagent was tested by ELISA to ensure it was biotinylated and active (bound turtle Ig).

Lymphocyte collection and preparation for flow cytometry

Percoll gradients were prepared by adding 20.25ml of stock sterile percoll (MP Biomedicals) to 2.25ml of 10x sterile saline solution to make a working solution. The working solution was then diluted 1:2 with saline. Whole blood samples (~1 ml) were collected and added

to a tube containing 1ml Ringer's solution and 50 ul of 0.1M ethylenediaminetetraacetic acid (EDTA). Diluted blood was carefully layered on top of a percoll gradient at a 1:1 volume ratio in a round bottom polystyrene tube. The tubes were centrifuged for 5 minutes at 400 xg at 4°C without an airbrake. The buffy coat layer containing leukocytes was removed using a Pasteur pipet and transferred to a new 15ml tube. Multiple samples were pooled then the tube was filled with Ringer's. This tube was centrifuged at 1500 rpm at 4°C for 5 minutes with a break applied. All the supernatant was decanted, and the remaining pellet was resuspended in Ringer's at a volume of 1ml for each individual turtle from which blood was collected. Using a hemocytometer chamber and trypan blue, the live cells were counted. Typically, a 1:5 dilution was used, with 10 ul of cell solution and 40 ul of trypan blue. Cell concentration and total number of cells collected was determined.

To prepare the liver and spleen tissue samples, single cell suspensions were prepared. Two frosted glass slides were used to smash the tissue pieces in petri dishes filled with Ringer's solution. Cells were pipetted up and down until mostly disrupted and large particles were allowed to settle on the bottom of the dish. The top layer containing single cell suspension was removed and transferred into 15ml tubes. The samples were centrifuged (1500 rpm for 5 min at 4°C), supernatant decanted and the cells resuspended in Ringer's and counted.

For flow cytometry, cells (5×10^5) were resuspended in 50 μ l Ringers-BSA. Next, 5 μ l of normal rat serum was added to each tube to block non-specific binding. For some tubes, HL673-biotin was added, and tubes incubated on ice for 15 minutes. After the incubation was complete, the tubes were washed with 2ml of buffer and centrifuged. The liquid was decanted, and some tubes had secondary reagents added at various concentrations as indicated in the individual experiments. The secondaries used included Streptavidin (SA)-PE-Cy7, SA-PE or SA-PE-Cy5

(all from Southern Biotechnologies). Figure 1 shows a representation of these fluorochromes, with PE having the shortest emission wavelength and Cy7 having the longest. All the tubes were incubated on ice while covered with foil for 15 minutes. They were washed with 2ml of buffer and centrifuged again. Cells were resuspended in 350 μ l of buffer for analysis. The samples were analyzed using a BD FACSMelody instrument and FACSCorus software. Files were downloaded and reanalyzed using the free FCSalyzer software version 0.9.22alpha (<https://sourceforge.net/projects/fcsalyzer/>). Live cells were gated, and the fluorescence was analyzed.

To examine phagocytic capacity of reptilian B cells we used a slightly modified version of the method described by Zimmerman *et al.* [3]. After counting, cells were added to an appropriately sized culture plate in individual wells depending on exact experimental parameters. Polystyrene microbeads (1 μ m in size) were obtained from Polysciences. Bead to cell ratios were 10, 20, and 40. The appropriate amount of each bead was calculated from the stock vials and added to each well. Plates were incubated at 30°C for 3 hours in a CO₂ incubator (5%). One milliliter of Ringers-0.5% BSA was added to each well following incubation and the liquid pipetted up and down several times to dislodge the cells. Liquid from each well was put into individual tubes and centrifuged. The cells were then resuspended in 400 μ l of Ringer's BSA. To remove phagocytosed beads, cell suspensions were layered over a cushion of 3% BSA-4.5% dextrose-phosphate buffered saline and centrifuged at 1500 xg for 5 min at 4°C. Following supernatant removal, cells were washed and centrifuged as previously mentioned. The liquid was decanted from each tube and cells were then resuspended in 50 μ l Ringers-BSA. Next, 5 μ l of normal rat serum was added to each tube. For some tubes, HL673-biotin was added, and tubes incubated on ice for 15 minutes. After the incubation was complete, the tubes were washed with

2ml of buffer and centrifuged. The liquid was decanted, and some tubes then had 2µl of SA-PE-Cy7 (Southern Biotechnologies) at a 1:1000 dilution added. All tubes were incubated on ice covered in foil for 15 minutes. They were washed with 2ml of buffer and centrifuged again. Cells were resuspended in 350 µl of buffer for analysis. The samples were analyzed using a BD FACSMelody instrument and FACSCorus software. Files were downloaded and reanalyzed using the free FCSalyzer software version 0.9.22alpha (<https://sourceforge.net/projects/fcsalyzer/>). This experiment was repeated 3 times.

ELISpot Assay

Plates were prepared by adding 15µl of 35% ethanol to each well of a 96 well ELISpot plate (Millipore Multiscreen Immobulon-P membrane) for one minute. The ethanol was removed, and the wells were washed twice for three minutes each with 200µl of sterile PBS. Each well was coated with 100µl of unconjugated HL673, diluted 1:1000 for 0.5mg/ml stock in sterile PBS. The plate was incubated overnight at 4°C. Once the plate had incubated, the antibody/antigen solution was decanted under a sterile hood. Next, each well was washed twice for three minutes each with 200µl of sterile PBS. RPMI 1640 (Life Technologies) was supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin/ glutamine, 0.5% 2-mercaptoethanol, and 0.5% sodium pyruvate to make complete medium (cRPMI). Then 100µl of cRPMI was added to each well and the plate incubated for 1 hour to block any unbound membrane. Cells were prepared in cRPMI and 200µl was added to wells at different cell concentrations. The plates were incubated for 1-3 days at 37°C in CO₂ incubator. After the incubation period, the wells were decanted, and the plate was washed four times for three minutes each with 200µl a wash solution comprised of 1x PBS and 0.25% Tween 20. Then

100µl of HL673 + biotin diluted 1:1000 in 1x PBS-1% BSA-0.05% Tween 20 was added to each well and incubated for 1 hour at room temperature. The plate was washed three times with wash buffer and SA-HRP (Southern Biotech) was added (1:1000). After an hour incubation, the plate was washed 3 times with wash buffer and twice with PBS only. To prepare the substrate solution, 333µl of AEC stock solution was added to 10ml of 0.1M Acetate buffer solution. After 5 minutes the solution was filtered using a 0.45µm syringe filter and 5µl of 30% H2O2 was added. After the AEC substrate solution was prepared, 100µl was added to each well and sat for 10-60 minutes at room temperature. Once spots reached the desired intensity, the plate was washed three times with H₂O. The plate was allowed to air dry overnight in a dark space and the spots were counted with a microscope. Cells were plated in triplicate and this experiment was repeated 3 times.

Immunohistochemistry with whole mount tissue

Intestinal sections were dissected from hatchling turtles. A proximal and distal section was removed from the intestine; one piece following the stomach and another piece before the small intestine develops into the large intestine. Using a butterfly needle for the hatchlings, the intestinal tissue was immediately flushed with cold 1x PBS, pH 7.4 to remove fecal matter. The tissue was cut open longitudinally under a dissecting microscope. These sections were pinned into 6 well plates containing mounting wax with the lumen side up and kept hydrated with room temperature 1x PBS. The intestines were incubated two times in warm (37° C) PBS + 5mM EDTA while shaking at 600-900 rpm for 10 minutes to help remove extracellular matrix. Next, the intestines continued shaking for 10 minutes in cold 1x PBS and then fixed with 4% paraformaldehyde for 1 hour at room temperature. Once the intestines were fixed, they were

treated with 1% H₂O₂ for 15 minutes at room temperature. Following fixation, 4ml of 50mM Tris buffer supplemented with 150nM sodium chloride, 0.6% Triton X-100-0.01% bovine serum albumin (BSA) was added to each well and continued to incubate while shaking overnight. After incubating, the intestines incubated overnight again with a 1:500 dilution of HL-673 mAb conjugated to biotin in buffer solution. The intestines were then washed three times for ten minutes each with 1% BSA-1x PBS and incubated with streptavidin + horseradish peroxidase (SA-HRP) diluted 1:1000 for one hour. Three more washes occurred with 1% BSA-1x PBS for ten minutes each and the intestines were treated with diaminobenzidine peroxidase substrate (DAB/Metal Peroxidase Substrate Solution; Fisher Scientific) for 15 minutes. All sections were rinsed twice with dH₂O for five minutes each and returned to 1x PBS. The sections were viewed under the dissecting scope at 60X for any dark brown/black spots. Images were captured using a Leica SP8 System with DMI8 CS inverted microscope. We were not trying to detect any type of fluorescence, but rather get clear images of the tissue. Each experiment utilized three hatchling turtles and was repeated three times.

H&E and Immunohistochemistry with tissue slides

Intestinal and spleen tissue was dissected from three adult turtles. The spleen and sections of distal and proximal intestinal tissue were fixed in 10% Formalin Fixative (neutral buffered formalin, NBF) overnight. Once the tissue had been fixed it was processed and dehydrated. The tissue began in 70% alcohol for 20 minutes and then two changes of 95% alcohol for 20 minutes. Next, the tissue went through two changes of 100% alcohol for 20 minutes, followed by two changes of Xylene for 20 minutes each. Last, the tissue went through four changes of heated Paraffin for 20 minutes each. Once the tissue was processed and dehydrated, it was embedded

into Paraffin blocks. These Paraffin blocks were cut using a microtome at 4µm and the Paraffin ribbons were put onto blank electrically charged microscope slides.

For Immunohistochemistry staining

After the tissue slides dried, they were deparaffinized by putting the in two changes of xylene, 100% alcohol and 95% alcohol for 2 minutes each. The slides were washed in distilled water twice for 5 minutes each. Slides were submerged in diluted sodium citrate buffer in Coplin jars and heated in a microwave. Once the slides were cooled, they were washed twice in PBS for 10 minutes each. A blocking solution of 1% BSA in PBS was added to the slides and they incubated for 15 minutes. Next, 1:500 dilution of HL673-biotin in wash buffer was added as a primary antibody and incubated for 30-60 minutes at room temperature. After washing the slides twice in PBS for 10 minutes each, a 1:5000 dilution of streptavidin + HRP was added to the tissue as a secondary antibody and incubated for 30 minutes at room temperature. The slides were washed in PBS and then incubated for 5-10 minutes with Metal Enhanced DAB Substrate (Fisher Scientific) on them. Once the slides were done incubating, they were washed in running tap water for 10 minutes and counterstained with hematoxylin for 30 seconds to 2 minutes. The slides were washed again for 10 minutes in tap water and cover slipped before examining them under a light microscope. This experiment was repeated 3 times.

For Hematoxylin and Eosin staining

After drying the slides completely, they were put on a Sakura Prism automated stainer and ran through the hematoxylin and eosin staining process shown in table 1.

Table 1. Hematoxylin and eosin staining procedure.

Step	Reagent	Time
1	Xylene	3:00
2	Xylene	3:00
3	100% alcohol	1:00
4	100% alcohol	1:00
5	95% alcohol	1:00
6	95% alcohol	1:00
7	Wash	1:00
8	Hematoxylin	4:00
9	Wash	1:00
10	Clarifier	0:30
11	Wash	1:30
12	Bluing reagent	0:30
13	Wash	1:30
14	95% alcohol	0:30
15	Eosin	2:00
16	95% alcohol	0:30
17	95% alcohol	0:30
18	100% alcohol	1:00
19	100% alcohol	2:00
20	Xylene	2:00
21	Xylene	2:00
22	Xylene	Exit

CHAPTER III: RESULTS AND DISCUSSION

B cells are identified in whole blood

Research previously done in our lab has shown that B cells were able to be identified using flow cytometry by staining cells with HL673-biotin and detection with SA conjugated fluorochromes [2]. The HL673 mAb was originally produced in mice against green turtle antibodies and was found to bind to light chain proteins [53]. Because it recognizes light chain proteins, it should bind to all isotypes of turtle antibodies whether secreted and found in serum or membrane bound on the surface of B cells. Because each batch of biotinylated antibody is prepared independently, it needs to be tested to ensure reactivity and proper biotinylation.

An analysis gate was drawn on the scatter plot around where live white blood cells are found in our samples [2] and a sample is shown in Figure 2. In the turtle, red blood cells are larger and more oval in shape. They will generally appear to the right of the gate shown if they have not been entirely removed by the percoll gradient centrifugation. They do not directly interfere with the analysis, but we do exclude them from the events analyzed. Dead cells will be smaller in size and appear to the left of the analysis gate. These cells are excluded from the analysis because they may contribute to autofluorescence and cause false positives. Gating was done for each flow cytometry sample.

As shown in Figure 3A, in the absence of any added stains, there is low fluorescence as expected (left panel; unstained). As a control for non-specific binding of the secondary reagent, it was added to a sample without the mAb. The middle panel of Figure 3A contains cells incubated with SA-PE only and no non-specific binding was observed, as expected. The right panel of Figure 3A shows the cells stained with both HL673-biotin and SA-PE. A second positive

population is observed that represents B cells in the sample. Cells like T cells, heterophils, etc. fall into the left peak on this plot and represent the unstained portion of the sample. Additionally, we evaluated different secondary reagents. Subsequent experiments with our turtle cells were to be performed using dual staining with fluorescein isothiocyanate (FITC) conjugated beads for our phagocytic assay. Because the beads are very brightly labeled, and our streptavidin (SA) conjugate should have an emission spectrum as far as possible from FITC (572 nm) to ensure no bleed through of the fluorochrome that would create a false positive. Thus, we tested several different streptavidin conjugates: PE (phycoerythrin) (582 nm), PE-Cy5 (697 nm) and PE-Cy7 (783 nm).

In Figure 3B and C, SA-PE-Cy5 and SA-PE-Cy7 were compared to what was observed in 3A with the SA-PE. In both cases, the middle panels with the secondary reagent only showed the staining peak slightly shifted to the right, however this slight shift did not obscure positives. This could be an indication that the solutions need to be diluted further or a slight amount of background binding has occurred. However, in the right panels of Figures 3B and C, there were clear fluorochrome conjugates and HL673-biotin together (right column). Therefore, the HL673-biotin was active and any of these fluorochromes could be used. We chose to continue with SA-PE-Cy7 due to it having the longest emission spectrum.

Titration of SA-PE-Cy7 to determine best dilution

After choosing to continue with SA-PE-Cy7, we found the optimal streptavidin-fluorochrome dilution for staining turtle B cells. White blood cells were prepared as described in methods and stained with different dilutions of SA-PE-Cy7. Samples were analyzed by flow cytometry as described and gated on live cells. In Figure 4, a sample of unstained cells is shown

as a control (top). We were able to compare the SA-PE-Cy7 dilutions to determine that 1:100 dilution (row A) was the optimal choice over a dilution of 1:500 (row B). The staining peak for cells stained with a dilution of 1:500 had no change compared to the unstained cells, whereas the staining peak of cells stained with a dilution of 1:100 shifted to the right. This indicates that the 1:500 dilution is most likely to dilute and 1:100 dilution is optimal for staining turtle B cells.

B cells identified in hatchling spleen and liver tissue

Our lab has previously studied B cells in the peripheral blood of adult red-eared sliders but hasn't published on the distribution or function of B cells at younger ages or in other tissues. Due to the availability of some hatchlings euthanized for other projects in the lab, we were able to obtain some lab-raised hatchling samples. Spleen is known to be an important secondary lymphoid organ in mammals, and we predicted in the turtle given the lack of lymph nodes, the spleen would have a significant portion of B cells. For spleen, no particular issues arose when preparing the tissue as described in methods. However, for the liver, preparing the tissues resulted in a heavy lipid layer upon density gradient centrifugation. While we attempted to physically remove the lipid layer, it is possible some of the lipids remained and mixed with our recovered cells. Figure 5 shows gated live cells obtained from hatchling spleen samples. A scatter plot for this sample is shown in the top left. FITC staining is shown on the X axis to show there is no bleed over in the absence of FITC dyes. Cells with no staining were a control and are shown in the top right. Cells stained only with SA-PE-Cy7 are seen in the bottom left. This shows that the fluorochrome alone does not show a B cell population. When stained with HL673-biotin-SA-PE-Cy7 (bottom right) a clear B cell population can be seen within the gated area. In Figure 6, hatchling liver cells are stained the same as in Figure 5. However, in this case,

the lower left panel when the cells were stained with only SA-PE-Cy7, cells were found in the gated region, indicating some non-specific binding. While the percentage of cells did increase upon HL673 staining (lower right panel), we were not able to distinguish a clear B cell population in the liver. Thus, we have shown for the first time the presence of B cell populations in the spleen of young turtles. While we also suspect there are B cells in the liver, future experiments will need to investigate other methods of preparing the cells for staining due to the heavy lipid layer seen upon density gradient centrifugation.

B cells identified in hatchling distal and proximal intestinal tissue

Previous results in our lab have shown that small B cell clusters were present in intestinal tissue of hatchlings and were more numerous in distal sections [43]. By repeating this experiment and showing similar results, I could eventually compare hatchling intestinal tissue to adult intestinal tissue. After staining with HL673-biotin followed by SA-HRP and substrate, Figure 7 shows an example of the distal portion of intestinal tissue. The top left picture was the unstained intestinal tissue used as a control. The top right picture shows intestinal tissue stained only with SA-HRP only. Consistent with previous results, no spots are seen which indicates no B cell staining and low background. When distal sections were stained with SA-HRP and HL673-biotin, numerous small spots were seen (bottom picture) showing the presence of B cells.

Proximal sections that were collected from these hatchlings are shown in Figure 8. The top left section was unstained as a control and the top right was stained only with SA-HRP. Both sections have no spots seen within the tissue. The bottom picture shows the proximal intestine section stained with SA-HRP and HL673-biotin and spots are again clearly seen within the tissue. While both distal and proximal section have B cells present, it has been shown that more

B cells are typically seen within the distal section. This is due to an increase in migrating lymphocytes being observed as they are progressing through the small intestine [43]. Previously, it has been shown that there are more spots seen in the distal portion of the intestinal tissue than in the proximal sections. While my results do not indicate whether one section has more spots than the other, we do see spots in both the distal and proximal portions of intestinal tissue.

Unable to distinguish intestinal B cells in adult intestinal tissue using whole mount immunohistochemistry

Since B cells have been identified in intestinal tissue of hatchling turtles, we wanted to see if B cells would also be present in intestinal tissue of adult turtles. Distal and proximal sections of intestinal tissue was collected and prepared for whole mount immunohistochemistry as described in methods. Three separate individuals were used for each experiment and were prepared in separate wells. Figure 9 includes sections of distal intestinal tissue. A control piece of intestinal tissue is seen in the top left picture which had no staining. The distal intestinal tissue seen in the top right picture was stained only with SA-HRP. The bottom picture shows distal intestinal tissue stained with SA-HRP and HL673-biotin. Unlike hatchling distal intestinal tissue, no spots are seen within the adult distal intestinal tissue. McDonald and Newberry [55] reported that in using this technique for mouse intestine, staining of subepithelial cellular populations without removal of the epithelium were universally unsuccessful, suggesting that antibodies cannot effectively penetrate the serosa or the intact epithelium and associated mucous layer. To circumvent these limitations, they used an EDTA-based protocol to remove the epithelial barrier while maintaining the underlying cellular components with their three-dimensional structure. It is possible that this procedure wasn't powerful enough to remove the layers in adult turtles.

In Figure 10 adult turtle intestinal tissue from the proximal location is shown. Proximal tissue as a control with no staining is seen in the top left picture. The top right picture shows proximal intestinal tissue with SA-HRP only staining. In the bottom picture proximal intestinal tissue is stained with SA-HRP and HL673-biotin. Similar to the adult distal intestinal tissue, no spots are seen within the tissue. This is different from what we have seen in hatchling intestinal tissue. While no spots were seen, we do not believe B cells are absent. The thickness of the adult intestinal tissue may be causing the staining to not work properly. This experiment should be modified and repeated to see if B cells can be identified using this method.

H&E and Immunohistochemistry staining of adult turtle spleen and intestinal tissue

In addition to the whole mount method, intestinal tissue and spleen tissues from adults were examined by sectioning. Tissue slides were prepared and stained using the hematoxylin and eosin protocol described in methods. Hematoxylin stains cell nuclei a purplish-blue color and the eosin stains the extracellular matrix and cytoplasm pink. This allows us to easily differentiate between cytoplasmic and nuclear parts of the cell [56]. Figure 11 shows a cross section of stained spleen tissue viewed using Leica SP8 System with DMI8 CS inverted microscope. Overall, the tissue looks like typical mammalian spleen tissue. The clear areas likely represent different angles of blood vessels (circled) traveling through the tissue. Surrounding these vessels and indicated by an arrow, there appears to be areas of concentrated lymphocytes, staining blue. As previously reported, lymphocytes have been found in the periarteriolar lymphatic sheath in reptilian spleen [27]. As expected, there are no germinal centers present within the tissue as has previously been reported for reptiles [35].

In Figure 12 cross sections of stained intestinal tissue are seen. Stained distal intestinal tissue is seen in the top picture while in the bottom picture, stained proximal intestinal tissue is seen. Villi extend into the lumen and lamina propria contain cells with lymphocyte like morphology. However, in both tissues it was difficult to identify areas where lymphocytes were concentrated. It does appear that the mucosa layer (stained dark purple) is much thicker in the proximal portion compared to the distal portion. The folds within the epithelium layer appears to be much different as well. In the proximal portion the folds are long and narrow, while the folds in the distal portion are short and wide. The proximal portion could need more surface area to make it easier to rid of pathogens due to being exposed to them before they enter the distal portion.

From previous work in our lab, B cells have been identified in intestinal tissue of hatchling turtles using whole mount immunohistochemistry. We wanted to see if B cells could be identified on slides of turtle spleen tissue by using an immunohistochemistry staining protocol. Tissue sections were used to create slides and stained using our immunohistochemistry protocol described in methods. In Figure 13, the top tissue section was stained only with HRP as a control. The bottom section was stained with HL673-biotin + HRP. We expected to see similar dark spots like those seen on the hatchling intestinal whole tissue in Figure 7. Ideally, there should not be any spots seen in the HRP stained tissue. Our results show some dark spots in the tissue section only stained with HRP. The tissue stained with HL673-biotin + HRP also shows dark spots and appears to have more of them. These results do not seem to give a clear answer if this stain is working properly. Many mAb no longer bind after tissues are fixed as the fixation can alter the structure of protein epitopes. No studies have previously investigated this for the

HL673 mAb. In addition, there are many different ways reported to “retrieve” the 3D structure of protein epitopes but each needs to be tested with each antibody specifically. Repeating this experiment would help resolve these technical issues and give a better understanding if this stain is working properly.

B cells can produce antibodies after phagocytosis

Our lab had previously identified phagocytic B cells, but not all B cells were found to be phagocytic. This raised the question of whether there were separate subsets of cells with different functions (antibody secretion vs phagocytosis) or perhaps one cell differentiated depending on signals received. We also didn’t know if the phagocytic cells were terminally differentiated or could also then secrete antibodies. To test if B cells could also secrete antibodies after ingesting beads, we did sequential phagocytic assays followed by antibody secretion assays called ELIspots. After performing a phagocytic assay, cells from adult spleen and blood were stained with HL673 mAb and run on a flow cytometer. All procedures were described in methods. The results were analyzed on the BD FACSMelody instrument and FACSCorus/FCSAlyzer software. Figure 14 shows the results. The top left shows the side scatter plot of the beads used during the phagocytic assay and the top right shows the fluorescence of the same beads alone. The middle-left plot is a side scatter plot of unstained cells as a control, note the cells are much larger than the beads. Cells stained with Cy7 only (no beads) are seen in the plot in the middle right. Cells stained with HL673-biotin and Cy7 (no beads) are seen in the bottom left. There is a population of B cells seen in the gated area. Cells that went through the phagocytic assay and stained with HL673-biotin and Cy7 are seen on the bottom right plot. Here we can see a clear

population of B cells in the gated area to the left and another population of B cells that have ingested beads during the phagocytic assay in the gated area to the right.

The results of blood cells are seen in Figure 15. The top left shows beads only as a control and cells stained with Cy7 only are seen in the top right plot. Cells stained with HL673-biotin and Cy7 are seen in the bottom left with a gate around the B cell population. The bottom right plot has cells that underwent the phagocytic assay and were stained with HL673-biotin and Cy7. The normal B cell population is gated on the left and the B cells that have ingested beads are gated on the right. For both tissues, B cells that had ingested beads were sorted out and used in an ELISpot assay as described in methods.

In Figure 16, representative pictures from the ELISpot assays of B cells without LPS present from spleen tissue and whole blood are shown. B-1 like cells including our reptile B cells are thought to constitutively secrete low levels of antibodies. However, in order to stimulate antibody production in some samples, we added lipopolysaccharide (LPS) to the cultures. In mammals, this is an effective way to cause B cells to secrete regardless of their specificity as the signal works through Toll like receptors. No cells were put in the well shown in the top left picture as a control. Unmanipulated white blood cells (no staining or phagocytic assay) were used in the well shown in the top right picture. B cells that ingested beads during the phagocytic assay and were sorted using the flow cytometer were used in the well shown in the bottom left picture. Sorted B cells that did not ingest beads were used in the well shown in the bottom right picture. Red spots indicate a single B cell that is producing antibodies. In this assay, the size of the spot is relative to the amount of antibody produced. As expected, no spots were seen in the well with no cells. Spots are also seen in the wells with control unmanipulated white blood cells and B cells that did not ingest beads but were sorted. Again, this is expected since B cells are

known to produce antibodies. This panel also shows that B cells were able to survive the sorting process. Wells with B cells that ingested beads during the phagocytic assay also had numerous spots seen. The spots seen were counted over multiple wells and experiments and reported in table 2. That means the cells that had phagocytosed beads were still able to secrete antibodies.

Summarizing several experiments, spots were seen in unmanipulated white blood cells with and without LPS present. On average, the same number of spots were seen, indicating that adding LPS did not enhance antibody secretion. B cells that were sorted that did not ingest beads also had spots in the presence or absence of LPS. The sorted B cells without beads that did not have LPS had only a slightly higher average number of spots seen compared to LPS being present, again indicating that LPS did not enhance the response. Spots were also seen on the wells of sorted B cells that had ingested beads. Again, the cells without LPS had a higher number of spots seen on average compared to the cells with LPS present. These results indicate that B cells in the red-eared slider can still produce antibodies after they have ingested a foreign body. These results could give us a key piece of information about their immune system that could explain how turtles are able to live such long and relatively healthy lives.

CHAPTER IV: CONCLUSION

We were able to identify B cells in red-eared slider whole blood samples using SA-PE, SA-PE-Cy5 and SA-PE-Cy7 fluorochromes. From these fluorochromes we determined that SA-PE-Cy7 was the best to use in multicolor assay due to its long emission spectrum. We then determined that 1:100 dilution of the SA-PE-Cy7 was the most optimal dilution with the least background and highest sensitivity. Using single cell suspensions and SA-PE-Cy7, we were able to identify B cells in hatchling spleen and potentially liver tissue. Next, we were able to identify B cells in distal and proximal intestinal tissue from hatchling turtles using a whole mount immunohistochemistry protocol previously used in our lab. We attempted to distinguish B cells in adult tissue using the same protocol, but we were unsuccessful. It is likely that the thickness of the adult intestinal tissue is causing the staining procedure not to work properly. Adult spleen and intestinal tissue were stained using a hematoxylin and eosin procedure to look at the tissue at a cellular level. These tissues appear to be normal when compared to mammalian tissue, but these techniques do not allow B cells to be identified. Lymphocyte-like cells were observed around the arterioles in the spleen and distributed throughout the lamina propria in the intestine as expected. We also tried to stain tissue sections of adult spleen using a similar protocol to the whole mount immunohistochemistry staining using the HL673 mAB to identify B cells, however the results were not clear. Finally, we were able to show that B cells could still produce antibodies after phagocytosis. Thus, phagocytosis doesn't appear to be a terminal differentiation process. In the future these experiments should be repeated to gather more information and learn more about B cells in reptiles.

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APPENDIX: TABLES AND FIGURES

Figure 1. Schematic representation of fluorochrome use in flow cytometry. Target cells within a mixture of cells are fluorescently labeled with fluorescent dyes that emit different wavelengths of light. These experiments used piggy-back dyes of PE-Cy5 and PE-Cy7 in addition to PE alone.

Figure 2. Example of flow cytometry analysis. A scatter plot with forward scatter (FSC) and side scatter (SSC) shows gating of live cell populations.

Figure 3. Streptavidin-coupled fluorochromes can be used to identify turtle B cells labeled with biotinylated anti-light chain monoclonal antibody. Adult blood samples (right column) were stained with HL673-biotin which binds to turtle light chain proteins. Samples in the middle and right columns were also stained with streptavidin-fluorochrome conjugates (diluted 1:100). Row A) PE; row B) Cy5 and row C) Cy7 fluorochromes. The left column received no stains. Samples were gated on live cells as in Figure 2.

Figure 4. Titration of SA-PE-Cy7. White blood cells were prepared and stained as in Figure 3. Different dilutions of SA-PE-Cy-7 were used. Row A) 1:100 dilution and row B) 1:500 dilution. Samples were analyzed by flow cytometry as described and gated on live cells.

Figure 5. B cells are present in hatchling spleen tissue. Hatchling spleen cells were stained as in Figure 4 and gated on live cells. Top left: scatter plot of spleen sample. Top right: control with no staining. Bottom left: stained cells with only SA-PE-Cy7. Bottom right: stained cells with HL673-biotin-SA-PE-Cy7. B cell populations are indicated. A representative experiment is shown.

Figure 6. Dissected cells may be present in hatchling liver tissue. Hatchling liver cells were stained as in Figure 4 and gated on live cells. Top left: scatter plot of liver sample. Top right: control with no staining. Bottom left: stained cells with only SA-PE-Cy7. Bottom right: stained cells with HL673- biotin-SA-PE-Cy7. B cell populations are gated.

Figure 7. B cells are present in hatchling distal intestinal tissue. Top left: unstained hatchling intestinal tissue as control. Top right: intestinal tissue stained with SA-HRP only. Bottom: intestinal tissue stained with SA-HRP and HL673+biotin to show location of B cells. Representative sections viewed at 40x magnification are shown.

Figure 8. B cells are present in hatchling proximal intestinal tissue. Top left: unstained hatchling intestinal tissue as control. Top right: intestinal tissue stained with SA-HRP only. Bottom: intestinal tissue stained with SA-HRP and HL673+biotin to show location of B cells. Representative sections viewed at 40x magnification are shown.

Figure 9. B cells are not able to be detected in adult distal intestinal tissue. Top left: unstained adult intestinal tissue as control. Top right: intestinal tissue stained with SA-HRP only. Bottom: intestinal tissue stained with SA-HRP and HL673+biotin. Representative sections viewed at 40x magnification are shown.

Figure 10. B cells are not able to be detected in adult proximal intestinal tissue. Top left: unstained adult intestinal tissue as control. Top right: intestinal tissue stained with SA-HRP only. Bottom: intestinal tissue stained with SA-HRP and HL673+biotin. Representative sections viewed at 40x are shown.

Figure 11. Lymphocytes are present in periarteriolar regions of adult turtle spleen tissue. Adult turtle spleen tissue sections stained with H&E and viewed at 40x magnification. A blood vessel traveling through the tissue is circled. A representative slide viewed at 40x is shown. Concentrated lymphocytes surrounding these vessels are indicated by an arrow.

Figure 12. Staining of adult turtle intestinal tissue reveals many lymphocyte-like cells distributed in the lamina propria. Intestinal tissue sections stained with H&E and viewed at 40x magnification. Top: stained distal intestinal section. Bottom: stained proximal intestinal section.

Figure 13. HL673 staining failed to identify B cells in adult turtle spleen tissue. Adult turtle spleen sections stained and viewed at 40x magnification. Top: HRP staining only. Bottom: HL673-biotin + HRP.

Figure 14. Adult splenic B cells are able to phagocytose beads. Top left: scatter plot of beads only. Top right: beads only shown in the same plot with settings on SA-PE-Cy7 and FITC. Middle left: control with no staining. Middle right: stained cells with SA-PE-Cy7 only. Bottom left: stained cells with HL673-biotin-SA-PE-Cy7. Bottom right: stained cells with HL673-biotin-SA-PE-Cy7 from phagocytic assay. B cell populations used for sorting (left = non phagocytic and right = phagocytic) are indicated.

Figure 15. Flow cytometry analysis of adult turtle blood following a phagocytic assay. Top left: beads only shown in the same plot with settings on SA-PE-Cy7 and FITC. Top right: stained cells with SA-PE-Cy7 only. Bottom left: stained cells with HL673-biotin-SA-PE-Cy7. Bottom right: stained cells with HL673-biotin-SA-PE-Cy7 from phagocytic assay. B cell populations are gated.

Fig. 16. B cells that had first phagocytosed beads are still able to secrete antibodies. Adult turtle B cells were first used in a phagocytic assay and B cells with and without beads were sorted and cultured in an ELISpot assay. Top left: No cells. Top right: Unmanipulated blood cells. Bottom left: Sorted B cells that have ingested beads. Bottom right: Sorted B cells that have not ingested beads.

Table 2. B cells that first ingested beads in the phagocytic assay were still able to secrete antibodies. Antibody secreting cells per 100,000 cells.

With LPS*			
No cells	Unmanipulated	Beads-sorted	No Beads sorted
0	5	NC	NC
0	8	7062	91
0	17	NC	78
0	59	41	51
0	43	28	NC
0	62	39	NC
0	9	830	484
0	11	1111	120
0	21	NC	163
Average	0	1012	110

No LPS			
No cells	Unmanipulated	Beads-sorted	No Beads sorted
0	7	14106	59
0	8	0	0
0	7	7062	3
0	54	36	101
0	56	28	35
0	NC	27	55
0	18	830	887
0	25	4715	484
0	30	4434	39
Average	0	3471	185

*Cells were first used in phagocytic assay and HL673+ cells with or without beads were sorted. Sorted cells were then subject to ELISpot assay in triplicate as described in methods. Numbers of spots per well were counted. Contaminated wells indicated with "NC". Three separate experiments are shown; top using blood B cells and other two using splenic B cells.