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Illinois State University, mashford142@yahoo.com

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OUT OF THE SHELL: EXPLORING THE PHAGOCYTIC CAPABILITIES OF THE B CELL AND ISOLATED LYMPHOID FOLLICLE-LIKE STRUCTURES IN THE RED-EARED SLIDER, *TRACHEMYS SCRIPTA*

MARC A. ASHFORD JR.

88 Pages

Management of immunity is crucial for survival in all living organisms. While there is a large amount of research for established model organisms, such as the mouse model, much less is known about reptiles. When compared to immunity in mammals, we discover many differences such as susceptibility to certain pathogens, alteration of immune processes due to environmental temperature, the total absence of certain lymphoid organs contained in mammals, among many others. Despite this, the reptile has shown in many studies that they are worthy systems to study immunity in an evolutionary perspective and recent studies may show that they may have therapeutic use as well. Of particular interest to our lab is the management of gut mucosal immunity and phagocytic B cells in *Trachmys scripta*, commonly known as the red-eared slider. The thesis work reported here focuses on projects examining both of these. Reptiles lack many lymphoid structures within the gut cavity that are crucial for maintaining homeostasis and survival in mammals. Preliminary work examining lymphoid aggregates in hatchling *T. scripta*, suggested that they may contain lymphoid structures similar to those known as isolated lymphoid follicles in mammals. Our investigation determined that these structures, like the isolated lymphoid follicle, are composed of B cell aggregates, able to respond to enteric bacteria colonization, and increase in quantity as you progress distally within

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the small intestine. It is important to note that is the first time these B cell structures have been shown in a reptile. We also sought to understand the phagocytic capabilities of B cell in the red-eared slider. We hoped to learn if these cells possessed the ability to recognized different pathogens (Gram (-/+) and fungal particles), determine the sizes limitations of consumed particles, and also whether they had regulatory qualities. Unfortunately, we encountered many technical difficulties coupled with issues in the bioparticles particles we chose, which resulted in the absence of any productive data collection. It is our hope to pursue this study in the future. Overall, these studies aid in the understanding of the reptile immune system.

KEYWORDS: B cell; Reptile; Mucosal Immunity; Turtle; Immunology; Phagocytosis

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MARC A. ASHFORD JR.

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Fulfillment of the Requirements
for the Degree of

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MARC A. ASHFORD JR.

COMMITTEE MEMBERS:

Rachel M. Bowden, Co-Chair

Laura A. Vogel, Co-Chair

Ben M. Sadd

Nathan T. Mortimer

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M. A. A.

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CONTENTS

Page

ACKNOWLEDGMENTS i

FIGURES vi

CHAPTER I: REPTILIAN IMMUNITY: THERE IS MORE THAN MEETS THE SCALE 1

1 | OVERVIEW 1

2 | STUDY SYSTEM 2

3 | INNATE IMMUNITY 5

4 | ADAPTIVE IMMUNITY 8

4.1 | Cell-Mediated Immunity 9

4.2 | Humoral Immunity 11

5 | GALT IMMUNITY 15

6 | CONCLUSION 17

REFERENCES 20

CHAPTER II: *TRACHEMYS SCRIPTA* HATCHLING EXPOSURE TO SALMONELLA ALTERS PRESENCE OF INTESTINAL LYMPHOID AGGREGATIONS 37

ABSTRACT 37

1 | INTRODUCTION 38

1.1 | Management of Gut Mucosal Immunity in Mammals 38

1.2 | Reptilian Gut Mucosal Immunity 40

2 | MATERIALS AND METHODS 42

2.1 | Egg and Hatchling Care 42

المنارة للاستشارات
www.manaraa.com

2.2 | *Salmonella* Exposure 43

2.3 |Small Intestine Histology 44

2.4 | Whole-Mount Immunohistochemistry 44

2.5 |Spot Proportion Determination 45

2.6 |Statistical Analysis 46

3 | RESULTS 47

3.1 |Intestinal Histology Yielded ILF-like Structures 47

3.2 |ILF-Like Structures are more Abundant in the Distal Portion of the Intestine 47

3.3 |Prevalence of *Salmonella* Pre-inoculation 48

3.4 |*Salmonella* Exposure Increases ILF-like Aggregations 48

4 | DISCUSSION 49

5 | CONCLUSION 52

REFERENCES 60

CHAPTER III: CHARACTERIZATION OF THE PHAGOCYTIC CAPABILITIES OF THE *T. SCRIPTA* B CELL 67

ABSTRACT 67

1 | INTRODUCTION 67

1.1 | Phagocytic B Cells In A Reptile 67

2 |MATERIALS AND METHODS 70

2.1 | Lymphocyte Collection and Preparation 70

2.2 |Phagocytic B cell Assay 71

2.3 |Bioparticles and Beads 72

المنارة للاستشارات
www.manaraa.com

2.4 | Microscopy 72

3 | RESULTS 73

3.1 | Bioparticles were too Bright for the BD FACSCalibur 73

3.2 | Determination of Phagocytosis by Fluorescent and Confocal Microscopy 74

4 | DISCUSSION 75

5 | CONCLUSIONS 77

REFERENCES 84

المنارة للاستشارات
www.manaraa.com

FIGURES

Figure Page

1. A graphical representation of the experimental design using hatchling *T. Scripta* in our study 53

2. 7µm sliced histological sections of hatchling small intestines stained with H&E. 54

3. Oil immersion (100X) of intestinal section containing lymphoid aggregates 55

4. Clusters of B cells are present throughout hatchling intestines 56

5. Frequency of B cell aggregates is increased in distal sections 57

6. Prevalence of *Salmonella* in tested water samples before and after inoculation 58

7. A higher proportion of spots were observed in hatchlings that were exposed to *Salmonella* 59

8. Sample flow cytometry analysis showing gating of cell/bead populations 78

9. Turtle blood cells were able to phagocytose bioparticles, but fluorescence bleed over prohibited identification of B cells 79

10. Trypan blue failed to quench fluorescence bleed over 80

11. Bioparticles quenched without cells still prove to be too bright 81

12. Fluorescent microscopy failed to detect stained lymphocytes. 82

13. Bioparticles Show False Positive in GFP and RFP Filters 83

المنارة للاستشارات

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CHAPTER I: REPTILIAN IMMUNITY: THERE IS MORE THAN MEETS THE SCALE

1 | OVERVIEW

Immunity is a mechanism that allows an organism to protect itself from potential pathogens. In vertebrates, immune function has been well defined in mammals, but for non-model organisms such as reptiles, much less is known. It has been noted several times in the literature that characterizing immunity in non-model organisms can provide value to our overall understanding of immunity. For instance, studies on the variable lymphocyte receptor (VLR) genes of lamprey and hagfish, two jawless fishes, has revealed important clues about the evolution of antigen recognition within jawed and jawless vertebrates (Boehm et al., 2012), and the fruit fly, *Drosophila melanogaster*, now serves as a popular model organism to study immune regulation at the level of a whole organism. Beyond this established role, *D. melanogaster* also played an important role in understanding innate immunity with the discovery of the Toll receptor/pathway, which would later provide important insight for Toll-like receptors (TLRs) in mammals (Buchon, Silverman, & Cherry, 2014). Here I will review the current knowledge of reptilian immunity, with special attention to our study system, the Red-eared slider turtle (*Trachemys scripta*), and other chelonians. I also aim to provide relevant examples of their contribution to the larger understanding of immunity and potential future directions for reptilian immunity-oriented research.

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2 | STUDY SYSTEM

Turtles are long-lived ectotherms that can survive in a variety of environmental conditions. *T. scripta* is a species of pond turtle that is native from Illinois to the Gulf of Mexico. While they are widely distributed, they all inhabit quiet freshwater areas with aquatic vegetation and areas where they are able to bask (Ernst, Barbour, & Lovich, 2009). Females typically lay eggs during the nesting season (May-September) by digging nests in dry unshaded areas near water (Cagle, 1946). Female sliders have been observed to lay 1-30 eggs (10.5 average) which have an incubation period of 61.1-108 days (Ernst et al., 2009). As the female leaves the nest after laying eggs, providing no post-oviposition parental care, these eggs are often subject to heavy predation with a 10.5% survivorship rate from egg to hatchlings aged 1 year in the wild (Frazier, Nat, Gibbons, Greene, & Frazer, 1990). Survivorship rates increase from 10.5% to 53.9% at age 1-2 and over 80% at all ages after that (Frazier et al., 1990). The incubation temperature of the eggs is of particular importance as this species, like many other turtles, has temperature-dependent sex determination (Janzen & Paukstis, 1991; Janzen & Phillips, 2006; Zimmerman, Vogel, & Bowden, 2010), with warmer temperatures producing females. Upon hatching, they are exposed to the microbe-rich soil. In more northerly populations, they overwinter in the nest until early spring when warmer temperatures are common (Ultsch, 2006). After emerging in the spring, the hatchlings disperse to the water and development continues.

Adult sliders typically spend a majority of their time submerged, emerging to bask periodically, but have been observed to be able to remain underwater for extended periods of time during winter hibernation (Ernst et al., 2009; Ultsch, 1989). Their aquatic habitat can range from

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microbe-rich filled marsh-like environments with large to slow moving streams. Adults have been recorded to live past the age of 35 years old in the wild, but with ideal conditions, the slider may be able to live even longer (Ernst et al., 2009). This ability to be long-lived in the wild is not confined to just one species, but common amongst many in the chelonian order (Henry, 2003). In the three-toed box turtle (*Terrapene carolina triunguis*), gravid females aged over 60 years were observed to have negligible difference in physiological processes when compared to much younger turtles (Miller, 2001). Furthermore, there have been multiple tortoises being recorded to live over 150 years in age, such as Jonathan, an Aldabra giant tortoise (*Aldabrachelys gigantea*), estimated to be over 180 years in age (Hollins, 2012; Kettle, 2014). Turtles are a valuable group to study due to their unique physiology and capacity to be long-lived in wild while seeming to be unaffected by typical effects of senescence such as decreased reproductive frequency with age and maintained in conjunction with a type 3 survivorship curve for most species (Congdon, Nagle, Kinney, & Van Loben Sels, 2001; Henry, 2003; Iverson, 1991; Miller, 2001). It has also been implicated in studies that the immune system of the turtle may be unaffected by senescence as they seem to have little alteration in humoral immune responses as they age. This was demonstrated through B cell function and antibody production in 2013 study and also in a 2010 study which observed that temperature highly affects humoral immune response, but age differences were not present (Zimmerman, Clairardin, et al., 2013; Zimmerman, Carter, Bowden, & Vogel, 2017).

The reptilian immune system is commonly categorized into two main parts, the innate component, and the adaptive component as seen in other vertebrates such as mammals. It is

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important to note that these components are not isolated, as they need one another to function properly to produce an immune response. Pathogen clearance in the body, for instance, typically starts when macrophages of innate immunity engulf foreign materials but then present these internalized proteins to circulating T cells in order to mount adaptive immune responses (Cannon & Swanson, 1992). In this way, the macrophage demonstrates interconnectivity of the two components of the immune system. Reptilian species possess many of the same lymphoid organs as mammalian species, such as a functioning thymus and spleen, but lack structures such as lymph nodes and Peyer's patches commonly seen in mammals (Zimmerman, Vogel, & Bowden, 2010). Currently, six types of leukocytes have been identified by morphology within turtles, these include basophils, eosinophils, lymphocytes, monocytes, azurophils, and heterophils (Stacy, Alleman, & Sayler, 2011). Despite there being much known about the physiology of turtles, and *T. scripta* in particular (Frazier, 1990), many aspects of their immune system are not well defined. Within the last decade, however, there have been several studies in *T. scripta* that have given insight into both their adaptive and innate immunity (Palackdharry, Sadd, Vogel, & Bowden, 2017; Zimmerman, Clairardin, et al., 2013; Zimmerman, Bowden, & Vogel, 2013; Zimmerman, Vogel, Edwards, & Bowden, 2010). It has been reported that turtles, including *T. scripta*, contain populations of B cells with phagocytic potential, not unlike that of the B-1 cell in mammals (Muñoz, Franco-Noguez, Gonzalez-Ballesteros, Negrete-Philippe, & Flores-Romo, 2014; Zimmerman, Vogel, Edwards, et al., 2010). Given that turtles appear to share some, but not all, immune functions in common with other vertebrates, they provide an interesting group for further study.

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3 | INNATE IMMUNITY

Innate immunity serves as the first line of defense against potential pathogens. Because of this, innate immune responses are typically quick and non-specific. Innate immunity utilizes physical/chemical barriers (e.g., epithelial skin layer), anti-microbial peptides (AMPs) (e.g., α-defensins and β-defensins), the complement cascade, innate immune cell responses, and signaling proteins (e.g., cytokines) until it clears the threat, or provides enough time for an adaptive response to occur. The innate branch of the immune system is thought to have evolved first between the two major branches, with the adaptive branch developing much later. Researchers determined that innate components such as first TLR gene in multicellular eukaryotes date back to roughly a billion years ago (Ausubel, 2005; Buchmann, 2014; Kvennefors et al., 2010; Temperley, Berlin, Paton, Griffin, & Burt, 2008). While components of adaptive immunity were first being observed roughly 500 million years ago in invertebrates (Delvaeye & Conway, 2009; Iwanaga, 2002) and in ancient jawed fish around 430 million years ago (Bayne, 2003).

The complement system consists of three main pathways: classical, alternative, and lectin, which all utilize a suite of specific proteolytic enzymes to modify the abundant plasma protein, C3. Modification of C3 results in fragmentation of the protein, where one fragment acts as an opsonin, while the other fragment functions as an inflammation mediator (Merle, Church, Fremeaux-Bacchi, & Roumenina, 2015). It is also important for B and T cell regulation within immune responses (Dunkelberger & Song, 2010). In studies of immunodeficient patients and mice with genetic knockouts, it's been observed that compromise of the complement cascade

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results in increased *S. pneumoniae* and *Haemophilus* influenza prevalence, but also *neisserial* infection and others as well (Dunkelberger & Song, 2010; Ross & Densen, 1984). Seeing that the complement is so important mammals, it is no surprise that its presence has also been observed in reptiles. In reptiles, it has been shown that they contain both the alternative and classical complement pathways, with the optimum effectiveness of each pathway being modulated by temperature (Koppenheffer, 1987). In a study using two different reptiles, the Western fence lizard (*Sceloporus occidentalis*) and the Southern alligator lizard (*Elgaria multicarinata*), it was observed that the bactericidal activity of the alternative complement pathway is more aggressive than sera from humans and deer mice (*Peromyscus maniculatus*), being able to kill the Lyme disease spirochete *Borrelia burgdorferi* (Kuo, Lane, & Giclas, 2000). While a study in the Common map turtle (*Graptemys geographica*) and *T. scripta* have shown that external temperatures effect on complement activity as well (Freedberg et al., 2008). A recent study in the Prairie rattlesnake (*Crotalus viridis*) found that their serum had high bactericidal effectiveness and was able to inhibit bacterial growth (Baker & Merchant, 2018).

Antimicrobial peptides are present in most living organisms and are typically less than 200 amino acids in length (Bulet, Stöcklin, & Menin, 2004). There is much diversity in the structure of these peptides with linear α-helical amphipathic peptides being observed, while cyclic and open-ended cyclic structures are also present in organisms. Of these differing structures, many families of proteins have been characterized such as the defensin and cathelicidin families (van Hoek, 2014). Most AMPs have cationic regions in the protein which allow them to interact with the anionic cell walls and phospholipids layers of pathogens (Bulet et al., 2004). AMPs have

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been identified in four orders of reptiles: *Testudines* (turtles and tortoises), *Sphenodontia* (tuataras), *Squamata* (snakes and lizards), and *Crocodilia* (crocodilians) (van Hoek, 2014). In the American alligator (*Alligator mississippiensis*), several AMPs have higher bactericidal qualities than their mammalian counterparts (Merchant, Roche, Elsey, & Prudhomme, 2003), with a recent study showing promising effectiveness against multidrug-resistant bacteria (Barksdale, Hrifko, & van Hoek, 2017). In the Komodo dragon (*Varanus komodoensis*), 48 potential novel cationic antimicrobial peptides (CAMPs) have been described, with one peptide having significant potency against *Pseudomonas aeruginosa* (Bishop et al., 2017), another multidrug-resistant bacteria (Driscoll, Brody, & Kollef, 2007). At this time, much effort is being put forth to characterize novel CAMPs effective against particularly troublesome pathogens (i.e. multidrug-resistant bacteria) against which modern medicine is losing the battle. In support of this, a recent study in the Chinese alligator (*Alligator sinensis*) identified and characterized 20 novel β-defensin genes, in an effort to assist with the investigation of their potential as an antibiotic alternative (Tang, Wang, Wan, & Fang, 2018). Many of these studies focus on the *Crocodilia* and *Squamata* clades, with few studies focusing on AMPs in turtles in the literature (Chattopadhyay et al., 2006; Lakshminarayanan et al., 2008)

Regarding innate cellular responses, many innate immune cell types have been identified in reptiles. For example, mammals contain neutrophils, which are short-lived granule-containing phagocytes, while reptilian and avian species contain cells with similar functionality called heterophils. Heterophils have nuclei with more a rounded appearance than an oval-like one, typical of neutrophils (Campbell, 2004; Montali, 1988). Both cell types retain the innate

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functions of phagocytosis and the ability to release granules, and both represent a majority of leukocytes in their respective taxa (Martins, Alevi, Azeredo-Oliveira, & Bonini-Domingos, 2016). Interestingly, heterophil numbers have been shown to vary with seasonal temperature in reptiles, with higher numbers found in the warmer seasons relative to cooler seasons, but in mammals, this seasonal trend is absent, likely due to their endothermic nature (Troiano, Gould, & Gould, 2008). In addition to heterophils, reptiles contain typical granulocytes (eosinophils, basophils) and the azurophil, which is cytochemically and functionally similar to the monocyte (Stacy et al., 2011). It has been observed in snakes however that their function is more reminiscent of the neutrophil (Campbell, 2004). Reptiles also contain typical cells of the myeloid lineage, such the monocyte, and macrophage (Campbell, 2004; Stacy et al., 2011), which are also very important in innate and adaptive immune responses. Thus, while reptilian innate immunity is functionally similar to mammalian innate immunity, differences in its effectiveness and specific cellular components have given rise to many studies that further demonstrate the need to better understand the reptile immune system.

4 | ADAPTIVE IMMUNITY

A key component of adaptive immunity is the ability for organisms to form specific immune responses to pathogens. This is accomplished by the generation of memory and non-memory lymphocytes with genetically diverse antigen receptors (Cooper & Alder, 2006). In vertebrates, adaptive immunity is composed of the humoral component and the cell-mediated component. Adaptive immune cells develop from a common pluripotent hematopoeitic stem cell, which is the precursor to all lymphoid, myeloid, and erythroid cell lines (Bryder, Rossi, & Weissman,

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2006). These stem cells are located in the red bone marrow of vertebrates, which is the primary center of production of lymphoid cells and maturation center of the B cell. In mammals, lymphoid structures such as the spleen and lymph nodes contain germinal centers which are responsible for the activation of this cell.

4.1 |Cell-Mediated Immunity

The cell-mediated component of the adaptive immune system is mediated by a lymphoid cell called the T cell. T cell progenitors are produced in red bone marrow and T cells undergo maturation in the thymus. In mammals, T cells are necessary for B cell activation, responsible for cell-mediated apoptosis when an intracellular infection arises, and also are shown to have many regulatory functions (Mosmann & Sad, 1996). These responses are determined primarily through the T cell receptor (TCR), major histocompatibility complex (MHC) receptor, as well as other molecules (Rossjohn et al., 2015). T cells are typically classified by the types of co-receptors expressed, which either give a “helper” function (CD4) or an effector function (CD8).

In reptiles, little is known about the function and development of T cells. Genes for CD4, CD8, CD3, TCR, and MHC I and II were identified in the genome of the Painted turtle (*Chrysemys picta*) (Bradley Shaffer et al., 2013). These receptors have also been identified in other reptiles as well (Edwards, Grahn, & Potts, 1995; H. C. Miller, Belov, & Daugherty, 2005). In a study done using the Carolina anole (*Anolis carolinensis*), the Siamese crocodile (*C. siamensis*), and *A. sinensis*, the CD1 gene, a type of antigen-presenting molecule, was located; CD1 has a significant role in T cell activation (Yang et al., 2015), providing further existence of their

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presence of T cells in reptiles. Many early studies involved observations of the size of the thymus (Borysenko & Cooper, 1972). While thymic involution is observed to occur with aging in mammalian species (Bodey et al, 1997), it's been shown that turtles and other reptiles undergo seasonal involution (Saad & Zapata, 1992). Involution in mammals leads to accumulation of memory cells and decreased ability to respond to new infections (Cicin-Sain et al., 2007; Mackall, Punt, Morgan, Farr, & Gress, 1998; Naylor et al., 2005). In the Caspian turtle (*Mauremys caspica*), it was concluded that changes in the organ structure were linked with seasonal changes (Leceta, Garrido, Torroba, & Zapata, 1989). This seasonal involution has been shown to result in higher thymic cell numbers and response to chemotaxis in the summer and decreased numbers in the other seasons (Muñoz & De la Fuente, 2001). More studies must be done to understand the total effects that thymic involution trigger in reptilian species and effects on memory cell production.

T cell functionality is thought to be very similar to that of mammalian counterparts, with observed cell proliferation and chemotaxis when exposed to mitogens such as concanavalin A (Con A) and phytohemagglutinin (PHA) in the Northern tuatara (*Sphenodon punctatus*) and *M. caspica* (Burnham, Keall, Nelson, & Daugherty, 2005; Muñoz & De la Fuente, 2001). A CD3+ T lymphocyte population has been isolated in the Green sea turtle (*Chelonia mydas*) (Muñoz et al., 2009) and work done on understanding hormonal effects on cell-mediated responses in separate experiments (Belliure, Smith, & Sorci, 2004; El Masri, Hakim Saar, Hesham Mansour, & Badir, 1995). Beyond these few studies, literature on actual cell functionality *in vitro* is sparse, and much more work needs to be done to truly understand T cell function and cell-mediated

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immunity in reptiles. With advancements in genomic studies, characterization of genes such as those in the TLR family are the becoming the targets of study (Priyam, Tripathy, Rai, & Ghorai, 2018). Hopefully, future work will yield more reagents to determine whether differing immunophenotypes exist within the reptilian system, such as separate T cell populations.

4.2 | Humoral Immunity

The humoral arm of adaptive immunity allows organisms to have prolonged, specialized immune responses to pathogens and is vital to protection at mucosal surfaces. The primary lymphoid cell responsible for this type of immunity is the B cell. As mentioned earlier, its progenitor is produced and matures in the bone marrow. The B cell's primary purpose is to produce antibodies that serve a variety of functions such as opsonization, neutralizing pathogens, and promotion of complement activation (Sebina & Pepper, 2018). In mammals, different B cell subsets exist, the main ones being the B-1 subset and the B-2 subset (Allman & Pillai, 2008; F. Martin & Kearney, 2001). These two subsets differ in their function and phenotype. B-2 cells are the main B cell population circulating in the blood and lymph of adult mammals. They produce high affinity, specific antibodies, and maintain immunological memory. In contrast, B-1 cells are located primarily in the peritoneal and pleural cavities in very low numbers (Haas, 2015; Holodick & Rothstein, 2015). They also typically produce more polyreactive, low-affinity antibodies (Griffin, Holodick, & Rothstein, 2011; F. Martin & Kearney, 2001).

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It is unknown if B cell subsets exist in reptiles, however, many lines of evidence suggest that B cell function is more equivalent to mammalian B-1 cells. Characteristics of antibody production in reptiles more closely resemble B-1 than B-2 cells. Following immunization, there is a modest increase in antibody titer in the serum which happens slowly over time and titer does not change significantly with a second exposure (T.M Work, Balazs, Rameyer, Chang, & Berestecky, 2000; Zimmerman, Vogel, & Bowden, 2010). Antibodies are polyreactive and commonly referred to as natural antibodies (Panda & Ding, 2015), which characteristically have a low binding affinity to multiple epitopes (Middleton, Nelson, Gartrell, & La Flamme, 2015; Zimmerman, Bowden, et al., 2013). Mammalian B-1 cells have also been shown to produce primarily natural antibodies (IgM, IgG3, and IgA), but lack production of higher affinity antibodies (Ohdan et al., 2000). Genomic evidence and antibody analyses have shown that reptiles contain IgM (Sun, Wei, Li, & Zhao, 2012), IgD (Gambón-Deza & Espinel, 2008; L. Li et al., 2012; Wei et al., 2009; Xu, Wang, & Nie, 2009), two forms of IgY (L. Li et al., 2012; Wang et al., 2012) and in the Leopard gecko (*Eublepharis macularius*), the presence of an IgA-like (Gambón-Deza, Sánchez-Espinel, & Valdueza Beneitez, 2007). It is important to note that IgY has only been shown to be expressed in avian and reptilian species (Warr, Magor, & Higgins, 1995; Zhang, Calvert, Sutton, Doré, & Dor, 2017). IgY is functionally similar to IgG in mammalian organisms (Wang et al., 2012), and thought to be the evolutionary precursor to IgG and IgE (Pettinello & Dooley, 2014; Zhang et al., 2017). While one form of IgY retains the ability to bind to Fc (Fragment crystallizable) receptors for leukocytes that express it, another truncated form exists as well, with a hypothesized role in antigen neutralization without inflammation (L. Li et al., 2012; Thierry M. Work et al., 2015). Fc receptors are expressed on leukocytes and the

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different types Fc (e.g. Fc-gamma) allow antibodies to bind and help with pathogen identification which can induce cytokine release, engulfment of a pathogen, among other functions (Raghavan & Bjorkman, 1996).

B-1 cells in mammals produce mainly low-affinity antibodies and usually do not participate in germinal center reactions. B-2 cells are the cells that produce high-affinity antibodies and interact with T cells in the germinal centers of lymph nodes. Notably, turtles lack lymph nodes and germinal centers (Rios & Zimmerman, 2015). Antibodies produced by reptilian B cells are generally thought to be low affinity from an experiment done in *T. scripta.* Using LPS (Lipopolysaccharides) expressed from Salmonella, it was observed that while antibody production was increased with age and season, the overall avidity of antibody to the antigen was not altered (Zimmerman, Clairardin, et al., 2013). In a competitive binding ELISA using keyhole limpet hemocyanin, overall binding avidity was not altered even with repeated exposures to the antigen to simulate vaccination, whereas, in mammals, secondary doses of antigens typically result in significantly higher recorded binding to antigens (Zimmerman, Bowden, et al., 2013). Further, reptilian B cell populations more closely resemble mammalian B-1 cells due to their ability to undergo phagocytosis (Gao et al., 2012; Novaes e Brito et al., 2010). Following the discovery that some amphibian species and jawed fish contained B cells capable of phagocytosis (J. Li et al., 2006; Øverland, Pettersen, Rønneseth, & Wergeland, 2010), our lab was able to identify such cells in the turtle (Zimmerman, Vogel, Edwards, et al., 2010). Phagocytic B cells were also identified in the Loggerhead sea turtle (*Caretta caretta*), giving rise to the idea that these types of cells may be conserved in many other types of turtles (Rousselet

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et al., 2013). While researchers first identified phagocytic B cells in mammals in 1995 in mice (M A Borrello & Phipps, 1995), there was relatively little work done on this previously unappreciated B cell function. More recently, they have been discovered in human, non-human primates, and many other mammals (Griffin et al., 2011; Haas, 2015), and they belong to the B-1 subset. Thus, several lines of evidence suggest reptilian B cells are more similar to mammalian B-1 cells.

Due to the presence of this cell in many species, many of which with less specific adaptive immunity, it is possible that this B-1 cell could represent the primordial B cell, with it gaining more effective humoral functionality as time progressed potentially through gene duplication events giving rise to the B-2 subset (Popi, 2015). Some scientist predict that due its ability to expresses cell surface protein such as F4/80 and Mac-1 when cultured in macrophage-like conditions (Melinda A. Borrello & Phipps, 1996), that this bi-potential nature may be indicative of a myeloid origin, while others have argued that due to their unique cell surface markers and ability to produce antibodies indicate a clear lymphoid origin (Griffin & Rothstein, 2012). There has also been an alternative hypothesis, the "induced-differentiation" hypothesis, states that the B-1 cell could have always existed concurrently with the B-2 cell and formed from a common ancestor (Berland & Wortis, 2002a). Similar occurrences of cells with bipotential nature such as the CD1-restricted natural killer T cell have also been subject to study (Melinda A. Borrello & Phipps, 1995, 1996). Regarding the evolution of the natural killer T cell, it is thought to be a result of CD1 genes evolving rapidly and diverging substantially from the reptiles within eutherian species (Kumar et al., 2017), which differ from both of the above

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hypothesizes of B-1 evolution. It is unknown whether a subset of these cells exist in reptiles, but there has been discovery of the CD1 gene mentioned earlier. Overall, these B-1 cell findings suggest immune subsets may represent more of a gradient of immunophenotype, as opposed to a strict subset with no plasticity and this plasticity can be altered in specialized conditions depending on the need of the organism. As it stands now, the evolutionary origin of the B cell is still unknown, but as the progression of evolution becomes more accessible in through molecular studies, a definite answer will be hopefully discovered in the future.

5 | GALT IMMUNITY

The establishment of a well-defined gut microbiota and management is well understood to be a key to management of gut microbes in humans and other organisms (R. Martin et al., 2010; Wu & Wu, 2012). Gut-associated lymphoid tissue (GALT) is the largest lymphoid organ in the body with a reported 10^{12} lymphocytes, and higher antibody production than all other non-GALT lymphoid tissues (Brandtzaeg, 1989; Mayer, 2000). GALT immunity is essential for protection against ingested pathogens, systemic pathogens circulating in the digestive tract, and maintenance of gut flora in general. GALT immunity itself is just a portion of the much larger mucosa-associated lymphoid tissues (MALT), which encompasses all ingestive tissue and excretive tissue as a whole, as well other mucosa-lined tissue (Brandtzaeg, Kiyono, Pabst, & Russell, 2008). Common types of GALT structures include the tonsils, Peyer's Patches (PP), mesenteric lymph nodes, isolated lymphoid follicles (ILFs), and other tissues. GALT immunity functions by having a mucous barrier slow or prevent antigens from passing directly into the circulating bloodstream of an organism. If the antigens breach the mucous layer and epithelial

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layer they interact with the lamina propia. The lamina propia, in most vertebrates, is loosely organized lymphoid tissue connected to gut epithelial tissues that house various immune cells (Mayer, 2000). Aggregations of lymphocytes (i.e. PPs and Mesenteric lymph nodes) are constantly monitoring antigens caught in mucus that line local gut tissues. This allows increased antibody production and proper inflammation reactions. While PP formation is driven developmentally, ILF formation has been found to be induced through enteric stimulation and diet (Colombo, Scalvenzi, Benlamara, & Pollet, 2015; Kiss et al., 2011; Lee et al., 2012; McDonald, McDonough, & Newberry, 2005).

Virtually nothing is known about reptilian mucosal immunity. While reptiles don't contain PPes or tonsils and most don't produce IgA, they still do contain GALT. There have been studies that have identified a lamina propria region within their digestive tract as well as potential lymphoid cells and aggregations. (Borysenko & Cooper, 1972; Zimmerman, Vogel, & Bowden, 2010). *T. scripta* and many other freshwater reptiles are submerged for the majority of their lives in pathogen-rich environments and constantly ingest these waters, but for the most part, maintain good health. Even those that don't contain life strategies that involve submersion in water are known to carry highly pathogenic bacteria to mammals such as *Salmonella* and don't exhibit signs of health decline (Warwick, Lambiris, Westwood, & Steedman, 2001), which has sparked interest into adaptive immunity in *S. punctatus* for instance (Middleton et al., 2015). ILFs in mammals have been observed to act much like PPs in their ability to mount mucosal immune responses increasing production of IgA in fecal samples when stimulated with an oral inoculation of *Salmonella typhimurium* in mice (Lorenz & Newberry, 2004). In an experiment

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done with transgenic mice, it was shown that absence of PPs decreased antibody production, but antibody production was compensated by other tissues such as the ILF (Yamamoto et al., 2000). So the absence PPs in reptiles shouldn't be thought of as a deficiency in GALT immunity, but rather just a different strategy of gut immunity. I will expand upon the potential presence of these tissues in a study covered in chapter 2.

6 | CONCLUSION

While I mentioned that immunity-based research in reptiles is sparse when compared to other organisms, I should also mention some of the other roadblocks that hinder this progress. With the rise of genomic-based research, there simply are is not enough genomic information of non-model organisms. This problem not only plagues reptile immunity-based research, but research in other areas as well. A common approach to overcome this in our lab has been to utilize avian species as templates, but differences in binding sites, expressed proteins, and other issues have slowed progress. As technology advances and prices decrease for sequencers, however, eventually more data will become available. There also is not a large push to develop transgenic reptiles like those seen in mice and *D. melanogaster*. Many of the breakthroughs that we have seen in immunology have come about precisely because of this ability to manipulate the genome in these organisms. It should also be noted that advancements in research of these model systems have also been accelerated due to their utility in the development of modern medicine. Finally, a lack of reagents available to conduct many experiments due to a lack of knowledge of cell surface markers and reasons listed above.

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The reptile immune system has a unique placement phylogenetically among vertebrates. Reptiles first emerged about 300 million years ago, while other vertebrates such as mammals and dinosaurs did not emerge until about 100 million years later (Buchmann, 2014). With a shared common ancestor with mammals and birds, they give great insight into how immunity may have specialized over time within amniotic vertebrates. While it is known that the reptilian immune system contains both innate and adaptive compartments, an in-depth understanding of these components to the level of other vertebrates such as mammals will require much additional study.

Progress in understanding immunity in reptiles has proceeded notably slower, compared to other model organisms, but important discoveries have come about, nonetheless. For instance, there has been the discovery that not only do reptiles produce AMPs, but also their greater effectiveness against pathogens could potentially yield therapeutic-related outcomes. We now understand that the ectothermic nature of reptiles also has effectiveness on not only metabolism, but also immune function and also that reptiles contain phagocytic B cells, which have also been observed in early jawed vertebrates and mammals. These findings not only defy the traditional humoral role of the B cell, but also give rise to the idea that this cell may have had a less specialized role originally. While these advancements in our knowledge exist for this particular adaptive immune cell, a comprehensive characterization of T cell phenotypes has yet to be conducted. These few studies highlight the need to understand more about the reptile immune system. I have raised more questions than I am able to answer as it seems there are endless topics to research within the reptilian. Our lab and many others have attempted to do

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our part in showcasing this. It is my hope, however, that this brief review on reptile immunity spikes interest in others for potential areas for future research.

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CHAPTER II: *TRACHEMYS SCRIPTA* HATCHLING EXPOSURE TO SALMONELLA ALTERS PRESENCE OF INTESTINAL LYMPHOID AGGREGATIONS

ABSTRACT

Gut-associated lymphoid tissue (GALT) is vital for protection against ingested pathogens and maintenance of normal gut microbiota. In mammals, gut mucosal immunity and the lymphoid tissues associated with it, are well characterized, including isolated lymphoid follicles (ILFs) that consist primarily of B cells, and are found throughout the small intestine. In mammals, the formation of ILFs is not developmentally driven like other lymphoid tissues such as Peyer's Patches (PPs), but is rather dynamic and induced through antigenic stimulation and diet. In reptiles, gut mucosal immunity is much less understood. Reptiles lack lymph nodes and PPs, but whether they have ILF-like structures is unknown. In this study, we first set out to determine if ILF-like structures were present in the red-eared slider turtle, *Trachemys scripta*. We identified that there are B cell aggregates that are contained within the small intestine using paraffin-embedded sections and whole-mount immunochemistry and a primary antibody to turtle antibody light chains; these aggregates appear similar to ILFs in mammals. To determine if the observed ILF-like structures were inducible, similar to those in mammals, we introduced an enteric *Salmonella* species through oral gavage to hatchling turtles. Analysis of intestinal tissues revealed that the presence of the B cell aggregates was much higher in distal sections than in proximal sections, and that turtles exposed to *Salmonella* exhibited significantly more of these aggregates, in general, when compared to those given sham treatments. These studies provide

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novel information about gut immunity in non-mammalian vertebrates and provide the first evidence for ILF-like structures in reptiles.

1 | INTRODUCTION

1.1 | Management of Gut Mucosal Immunity in Mammals

Gastrointestinal (GI) tissues are essential to nutrient absorption in all vertebrates, but also serve as excellent areas for microbial colonization (Kato, Kawamoto, Maruya, & Fagarasan, 2014; Stevens & Hume, 1995). It has been estimated that roughly 90% of the total cells contained within the human body are actually commensal microbiota that colonize the GI tract (Savage, 1977). Because of this, constant monitoring by the immune system must be maintained in these tissues to prevent otherwise benign interactions from becoming pathogenic and to also prevent/limit pathogen establishment. Management of this colonization occurs through lymphoid tissue lining the digestive system such as the spleen, mesenteric lymph nodes, Peyer's patches (PPs), and Isolated Lymphoid Follicles (ILFs). These structures house innate and adaptive immune cells that survey the GI tract and are commonly termed gut-associated lymphoid tissues (GALT) (Brandtzaeg, Kiyono, Pabst, & Russell, 2008; Hamada et al., 2002; Stevens & Hume, 1995). PPs, in particular, are a major inductive site for B cell responses and are the primary site of IgA production in the small intestine in mammals (Jung, Hugot, & Barreau, 2010). Here B and T cells interact in germinal centers to produce high affinity, isotype switched antibodies. Interestingly, in mice, rats, and rabbits that lack functional PPs there was little effect on IgA production, indicating that other gut-associated lymphoid tissues may be able to compensate (Heatley et al., 1981; Keren et al., 1978; Yamamoto et al., 2000). However,

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removal of both mesenteric lymph nodes and PPs in mice results in severely decreased IgA responses and altered lymphocyte distribution, demonstrating that this ability to compensate is limited (Yamamoto et al., 2000).

In addition to PPs and mesenteric lymph nodes, lymphoid aggregates contained within the digestive tract, termed ILFs, have recently gained attention due to their unique inducible nature and protective qualities (Kiss et al., 2011; Lee et al., 2012). ILFs have been found in mammals such as humans (Moghaddami, Cummins, & Mayrhofer, 1998), rabbits (Keren et al., 1978), mice (Hamada et al., 2002), and Guinea pigs (Rosner & Keren, 1984); they have a composition that differs from the "traditional" GALT tissues such as the PP or mesenteric lymph nodes. ILFs are typically visually less distinguishable than PPs or mesenteric lymph nodes in the gut, being much smaller in size, but more numerous (Hamada et al., 2002; Keren et al., 1978). They appear earlier in development than PPs, and are primarily composed of IgA producing B cells (Hamada et al., 2002). ILFs are formed from cryptopatches with RORγt+ lymphoid tissue inducer precursor cells (Bouskra et al., 2008). These precursor cells are necessary to induce the recruitment of B cells and other cells for cryptopatch formation, then through microbial interactions, they are further induced to recruit more cells and form immature ILFs and then mature ILFs (Lochner, 2011). It is important to note that, in mature ILFs have functioning germinal centers, while immature ILFs and cryptopatches instead contain a large population of B cells, dendritic cells, and small numbers of T cells amongst other cell types (Bouskra et al., 2008). Unlike mesenteric lymph nodes and PPs, the distribution of ILFs can be altered in

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response to changes in the surrounding microbial community to maintain homeostasis (Brandtzaeg et al., 2008; Knoop & Newberry, 2012).

While these tissues are compensatory in nature, ILF-deficient mice are shown to have a 10-100 fold increase in commensal bacteria (Bouskra et al., 2008). Furthermore, ILF hyperplasia is observed in Activation-induced cytidine deaminase-/- mice with 100-fold increases in anaerobic bacteria within ILFs in the small intestines (Fagarasan, 2002; Knoop & Newberry, 2012). Upon antibiotic treatment, the presence of ILFs in these animals was observed to be dramatically decreased (Fagarasan, 2002). These results suggest that ILFs play a large role in maintaining populations of enteric bacteria. Beyond their presence in mammals, ILFs have also been described in amphibians (Ardavin, Zapata, Villena, & Solas, 1982; Marshall & Dixon, 1978).

1.2 | Reptilian Gut Mucosal Immunity

While there is extensive knowledge about gut mucosal immunity in mammals, much work needs to be done to understand mucosal immunity in reptilian systems. There are many differences in the physiology of reptiles that are reflected in their management of systemic immunity, but also gut mucosal immunity. Reptiles are vertebrate ectotherms that contain both cellular and humoral immune compartments (Zimmerman, Vogel, & Bowden, 2010). They possess primary lymphoid tissues such as bone marrow and a functioning thymus (Borysenko & Cooper, 1972; Saad & Zapata, 1992; Zimmerman, Vogel, & Bowden, 2010), but they lack certain secondary lymphoid tissues such as lymph nodes and PPs which are vital to mammalian systems (Solas & Zapata, 1980; Zapata & Solas, 1979). It is also thought that they lack germinal

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centers (Pitchappan, 1980), which play a very important role in isotype switching and affinity maturation of antibody responses in mammals. Turtles have been shown to have the ability to produce IgM, IgD, and IgY antibodies (Li et al., 2012; Pettinello & Dooley, 2014). Our lab has also shown that turtles primarily produce natural antibodies (Zimmerman, Bowden, & Vogel, 2013), which are polyreactive, and they possess B cells with phagocytic functionality (Zimmerman, Vogel, Edwards, & Bowden, 2010). Most previous studies on reptile immune function have focused on systemic immunity, leaving important knowledge gaps in other aspects of their immune response.

Turtles have long lifespans and live in pathogen-rich environments; it is very likely that they have robust mucosal immunity in their gut, but virtually nothing is known about reptilian mucosal immunity. Given that turtles lack IgA, PPs, and germinal centers, although some non-chelonian reptiles have IgA-like genes, it is unclear how their microbiome is managed (Sun, Wei, Li, & Zhao, 2012). GALT has not been extensively characterized in reptiles, with much of the literature consisting of descriptive studies with simple histological stains that are not able to identify specific lymphocyte populations. From these studies, it is reported that there are possible lymphoid structures in the intestines of the Common snapping turtle (*Chelydra serpentina*), with researchers noting that they observed diffuse aggregates that were composed of small lymphocytes in the submucosa (Borysenko & Cooper, 1972). In a later comparative study, similar lymphoid structures were reported in close association with the lamina propria throughout the small and large intestine and in the cloaca in the Caspian turtle (*Mauremys capsica*) (Zapata & Solas, 1979). They also observed similar structures in the Viperine water

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snake (*Natrix maura*), the Algerian sand lizard (*Psammodromus algirus*), and in the Ocellated skink (*Chalcides occelatus*) (Zapata and Solas, 1979). Thus, there is a possibility that reptiles could rely on small immune aggregates like the ILFs of mammals.

We hypothesized that, due to lack of traditional GALT, mesenteric lymph nodes, and PPs, turtles will rely heavily on B cell lymphoid aggregates (e.g., ILFs) in their intestines to regulate gut immunity. We first sought to identify lymphocyte aggregations using histological analysis. We were able to locate a structure reminiscent of a lymphoid aggregation. Like mammals, we predicted that the introduction of a novel microbe would increase the presence of these structures in the small intestine. In order to test this, we used *T. scripta* hatchlings to detect if we could identify populations of B cell aggregates within their small intestines using immunohistochemistry with a turtle light chain-specific mAb. We were able to successfully identify numerous small lymphoid aggregates that are composed of B cells. We then tested whether these aggregations had the ability to respond to changes within the gut microenvironment by introducing *Salmonella* to a subset of hatchlings.

2 | MATERIALS AND METHODS

2.1 | Egg and Hatchling Care

All animals and eggs involved in the studies below were obtained from Banner Marsh State Fish and Wildlife Area in Canton, IL under an approved IACUC protocol. Eggs were collected from freshly laid nests, or from gravid females that were collected from baited traps. Gravid females were transported to the laboratory and induced to oviposit by oxytocin injection

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(Ewert & Legler, 1978). Eggs were incubated in moist vermiculite (approximately -150 kPa) until hatching. Once hatched, hatchlings were moved to individual containers and held for 8-10 weeks before experimental treatment. Hatchlings were then randomly assigned to either the sham (*Salmonella-*) group (N=37) or the exposed (*Salmonella+*) group (N=39) (Figure 1).

2.2 | *Salmonella* Exposure

Salmonella enteritidis (ATCC 13076) was grown to an OD of 0.3 (≈$5x10^5$ cells/ml) in LB broth the night before inoculations. 1ml of these cells were concentrated via centrifugation, then resuspended in 20µ of LB broth on the day of the inoculations. A 1ml feeding tube containing 20µl of *Salmonella* in LB broth or sterilized LB broth for the sham treatment was administered to hatchlings via oral gavage. Water samples were collected from individual containers immediately before inoculation to determine *Salmonella* presence pre-inoculation. Cups were sterilized with a 10% bleach solution, rinsed thoroughly, and the oral inoculations were administered. Hatchlings were held for 48 hours to allow for colonization to take place. Water samples were taken after 48 hours to determine *Salmonella* presence, and hatchlings were euthanized. Following euthanasia, intestinal contents were flushed using a butterfly needle with 1x PBS, pH 7.4 and plated to determine if any *Salmonella* was present in the intestinal content. Testing for *Salmonella* presence was determined using Difco™ XLT4 (Thermofisher) selective agar.

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2.3 |Small Intestine Histology

Sections of hatchling small intestine were collected from two individuals and fixed in a 4% paraformaldehyde solution for 24hrs, then stored in a sucrose solution. Intestinal sections were then embedded in a paraffin wax cassette and sliced into 7µM sections using a microtome. Sections were then stained using Hematoxylin and Eosin. Protocols for slices were adopted from Fletcher and Wibbles (Fletcher & Wibbels, 2014). Sections were photographed using a Leica DMRBE. Histological sections were analyzed using the image processing software suite Fiji ImageJ v2.00 (http://fiji.sc/; Schindelin et al., 2012). Images were modified for correct white balancing using a macro written by Vytas Bindokas; Oct 2006, Univ. of Chicago. Modified by Patrice Mascalchi.

2.4 | Whole-Mount Immunohistochemistry

After being flushed with PBS, intestinal tissue was prepared for immunohistochemistry. Two 1-2 cm sections were collected from each hatchling; one section was excised proximal to the yolk sac and one section distal to the yolk sac. Intestinal sections were prepared using a modified version of whole-mount immunohistochemistry according to a protocol previously used on adult mice (McDonald & Newberry, 2007). Next, sections were split longitudinally and mounted on Sylgard 184 Silicone epoxy (Dow Corning Corporation) in six-well tissue culture dishes (humidity chambers) lumen side up. They were kept hydrated with room temperature 1x PBS, then were washed three times for five minutes each while shaking at 600 rpm in warm (37°C) Hanks balanced salt solution with 0.1M EDTA (HBSS-EDTA) to remove the surface epithelial layer and expose the intestinal lumen.

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The intestinal lumen was then washed with HBSS-EDTA using a 30cc syringe with a 23-gauge needle to ensure complete removal of the epithelial cell layer. Sections were then shaken for 10 minutes in cold 1x PBS and fixed with a 4% paraformaldehyde solution in 1x PBS for one hour. Following fixation, tissues were treated with 1% hydrogen peroxide in 1x PBS for 15 minutes to inactivate any native endogenous peroxidases. Intestines were then blocked overnight in a 50mM Tris buffer (pH 7.2) supplemented with 150mM sodium chloride, 0.06% Triton-X 100, and 0.1% bovine serum albumin (BSA) shaking at 4°C. Following incubation, intestines were once again incubated overnight with a 1:500 dilution of HL673 mAb conjugated to biotin in buffer solution. Next, intestines were washed three times for ten minutes each with 1%BSA-1x PBS and incubated with streptavidin-horseradish peroxidase (SA-HRP) (BD Biosciences) diluted 1:1000 for one hour. Three more subsequent washes occurred, and then intestines were treated with diaminobenzidine peroxidase substrate (Metal Enhanced DAB Substrate Kit, ThermoFisher Scientific) for 15 minutes. Finally, the intestinal sections were washed twice with dH2O to stop any reactions and returned to 1x PBS. Images were taken at (2.5X) magnification using a Leica camera attached to a Leica dissecting scope.

2.5 |Spot Proportion Determination

Intestinal images were analyzed using the image processing software suite Fiji ImageJ v2.00 (Schindelin et al., 2012). A grid pattern was placed on the images 2.5X magnification on the dissection scope. All squares within grids had a surface area of 0.1mm^2. Grids containing small intestine were marked and then area added. Then, grids containing spots (developed through

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immunohistochemistry) were marked and added separately. The proportion of spots marked compared to the entire intestine was then calculated. Images were also edited for white balancing using the aforementioned method in ImageJ.

2.6 | Statistical Analysis

All statistical analyses were performed in R statistical software v3.3.3 (R Development Core Team, 2016) and the *lme4* package (Bates, Mächler, Bolker, & Walker, 2014). A generalized mixed model with a binomial distribution was performed on the presence or absence of *Salmonella* in water samples collected both before and after treatment, and additionally on the proportion of small intestine grid areas with positive B-cell staining. For *Salmonella* presence in water, a model was fitted with *Salmonella* treatment as fixed effect, and clutch as a random effect. The presence or absence of B-cell staining within small intestine grids was analyzed with treatment, location (proximal/distal), and season (early/late) as fixed effects, with clutch origin and hatchling as random effects, to account for multiple measurements being taken from the same individual. Maximal models including possible interactions between fixed effects were simplified by sequentially eliminating non-significant terms through likelihood ratio tests (LRTs), and best-fitting models were chosen based on the Akaike information criterion (AIC). For factor level comparisons, the package *lsmeans* (Lenth, 2016) was used to extract predicted probabilities for treatment levels from the best fitting models.

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3 | RESULTS

3.1 |Intestinal Histology Yielded ILF-like Structures

While early histological studies identified lymphocytes by morphology in these tissues (Borysenko & Cooper, 1972; Solas & Zapata, 1980; Zapata & Solas, 1979), the distribution of specific B cells is unknown. Previous studies have identified B cell aggregations within the small intestine of reptiles (Zapata & Solas, 1979). Intestinal histology slices showed an aggregation of cells with lymphocyte-like cell morphology and placement in the lamina propia (Figure 2-3). There were no apparent afferent or efferent vessels, indicating that aggregate was formed locally, as opposed to a PP with drainage to a local lymph node (Figure 2B and 2D). Slices with these structures were not continuous in all sections of the intestine and were easily distinguishable from a blood vessel (Figure 2A), which was observed to be continuous in all sections. Our initial observation support that these structures are lymphoid aggregates, but this method does not allow us to confirm what the specific cell types are present.

3.2 |ILF-Like Structures are more Abundant in the Distal Portion of the Intestine

With positive identification of lymphoid aggregates, we needed verification through other means to determine cell phenotype. Proximal and distal intestinal sections from untreated hatchling animals were found to contain B cell aggregations within the small intestine (Figure 4). To determine if the presence of these structures corresponded with those observed in mammals, such that more ILFs appear distally as opposed to proximally, we determine spots number on proximal and distal section of intestinal tissue. The proportion of spots contained within the small intestine show a highly significant difference by location, with distal sections

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containing a much higher proportion of spots compared to the proximal sections (Figure 5; $\chi 2=175.0$, d.f. = 1, $p < 0.0001$).

3.3 |Prevalence of *Salmonella* Pre-inoculation

Before we could determine whether exposure to microbes in the gut would alter the presence of the B cell aggregations, we had to identify if any of our animals were carriers of *Salmonella* prior to our experimental exposure. We predicted natural carriage rates would be low due to being reared under laboratory conditions versus their normal soil habitat. Pre-inoculation water samples for both groups of hatchlings showed a low rate of natural *Salmonella* colonization as expected (Figure 6). There was no significant difference in *Salmonella* prevalence among individuals randomly assigned to the two groups ($\chi 2= 11.5$, d.f. = 1, $p = 0.0007$). Forty-eight hours following inoculation, however, the number of individual water samples testing positive increased significantly in the group that received the oral gavage containing *Salmonella* (Figure 6). Thus, it is likely that these animals were shedding bacteria in water samples. Due to inconsistencies with intestinal flush procedures, that data was not included in our final analysis.

3.4 |*Salmonella* Exposure Increases ILF-like Aggregations

We examined intestinal sections for the presence of spots developed through whole-mount immunohistochemistry. We observed a significant difference in the proportion of spots contained within the distal intestine between our sham and exposed treatment groups. Hatchings inoculated with *Salmonella* had a higher proportion of spots when compared to those who were given the sham treatment (Figure 7; $\chi^2=4.84$, d.f. = 1, $p = 0.028$). These findings

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indicate that exposure of hatchlings to *Salmonella* via oral gavage was successful, and also indicates that the observed B cell aggregations found in the small intestine are responsive to microbe presence.

4 | DISCUSSION

Mucosal immunity and GALT structures in reptiles have received relatively little study compared to those of mammals. In our study, we sought to determine if and how B cells were distributed in the small intestine of the turtle, *T. scripta*. We hypothesized these cells play an important role, similar to mammals, in maintaining commensal microbiota and protection from pathogens. Given the lack of lymph nodes in reptiles, we predicted that the B cells would be found in lymphoid follicle-like structures. We were able to identify lymphoid cell aggregations in the small intestine histologically. Using a monoclonal antibody specific for turtle antibody light chain proteins, we were able to clearly visualize small B cell clusters throughout the intestine. We also wanted to determine if we could induce these structures, with the presence of a newly acquired enteric microbe. It is important to note, that this is also the first study in mucosal immunity in a hatchling turtle, which informs us that hatchlings as young as 8 weeks have potential to develop B cell aggregations in the intestinal tract in response to gut microbes.

We observed that more B cell aggregates were present in hatchlings exposed to *Salmonella* compared to our sham treated hatchings. Spots were also more numerous in distal sections of intestines compared to the proximal sections. This finding is consistent with previous literature using non-specific histological stains examining potential GALT structures and lymphocytes in

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M. caspica, with more migrating lymphocytes and lymphoid tissue being observed as they progressed through the small intestine (Duodenum<Jejunum<Ileum) (Solas & Zapata, 1980; Zapata & Solas, 1979). These findings are also consistent with the increased ILF presence in distal mouse intestines (Hamada et al., 2002; Lorenz, Chaplin, McDonald, McDonough, & Newberry, 2003), suggesting that the distal portion of the small intestine is particularly important for microbe management, as higher loads of bacteria have been recorded further in the digestive tract (Donaldson, Lee, & Mazmanian, 2015). While these differences are congruent with observations from other studies, explanation of the observed differences could also be due to previous microbe exposure via the yolk. It is known that maternal yolk contains antibodies and enzymes to support immunity of offspring in many organisms (Kovacs-Nolan & Mine, 2012), but it is also known that egg yolk can contain pathogens (Parveen, Rahman, Fakhruzzaman, Akter, & Islam, 2017), and it has been shown that microbial incubation in yolk can also promote bacterial colonization in some cases. For instance, in a study examining *Salmonella Enteritidis* infection in mice, scientists discovered that *Salmonella* grown in egg yolk displayed greater illness, disease markers, and colonization than those grown in LB broth or from mice previously infected with *Salmonella* (Moreau et al., 2016). The hatching yolk sac was used as a marker to determine proximal and distal sections of the small intestine, with all proximal sections being located proximally to the sac. Because of this, there was potential differential pathogen exposure from the yolk sac, which may have also aided in the differences observed in these B cell aggregations unintentionally, even though the entire intestinal section should have received *Salmonella* via oral gavage for exposed individuals.

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Pre-inoculation water sample testing for *Salmonella* showed that our hatchings contained a very low prevalence of natural occurrence. Previous work by our laboratory and others did find that hatchling turtles could acquire *Salmonella* from ingesting contaminated eggshells during hatching (Holgersson, Nichols, Paitz, & Bowden, 2016), yet, infection did not persist more than about 8 days (Pasmans, De Herdt, Dewulf, & Haesebrouck, 2002). Post-inoculation water sampling showed a much higher prevalence of *Salmonella* in the exposed group. Ideally, water samples containing shed microbes should serve as a proxy for bacterial colonization in the gut. However, when the hatchlings were given *Salmonella* inoculations, *Salmonella* cells may have coated the mouth or head of the hatchling upon introduction. While this was not ideal, it would still promote ingestion of the pathogen, thus yielding the same result. We attempted to collect intestinal flush samples to confirm colonization, but we experienced difficulty flushing intestinal sections, so methods were changed mid-way through the experiment. Due to this, this data was omitted from analysis, but it should be noted that a there was a low amount of growth observed from flush samples collected. In the future, we hope to measure intestinal colonization with a modified method that will be standardized across treatments. We also hope to examine whether exposure to a more pathogenic enteric bacterium or immune stimulant may cause altered presence of spots, as *Salmonella* is typically asymptomatic in healthy turtles (Chiodini & Sundberg, 1981). It has been shown in mammals that methods of pathogen recognition do not differ between commensal and pathogenic bacteria (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004), but methods for pathogen clearance have been shown to be different (Haller et al., 2000). *Salmonella* is suggested to have potentially have coevolved with reptilians from a more pathogenic organism to a commensal

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organism (Bäumler, Tsolis, Ficht, & Adams, 1998), thus, exposure to a different pathogen may yield different results. Would we see a larger inflammatory reaction from this exposure and larger B cell recruitment as result? This question, among others, remains unknown.

These findings in combination with our post-inoculation water samples strongly suggest that the B cell aggregates are able to respond to microbial presence. Their location is also consistent with those observed in mammals and amphibians. It is of particular interest to know if T cells are also present in the aggregates; however, no turtle-specific T cell reagents are currently available. We also have only examined intestinal tissue in hatchling *T. scripta,* which presumably would have a lower overall microbial load and less B cell development than in adults. Future examination of these tissues in adults could provide even stronger evidence of the ILF-like B cell aggregations.

5 | CONCLUSION

To the best of our knowledge, this is the first study that has specifically identified B cell aggregates within the intestine of a reptile using a primary antibody. We have also shown that these aggregates are present in young hatchling turtles and that their number increases as you progress distally in the small intestine. We have also shown that these aggregates have an inducible nature. Hatchlings exposed to *Salmonella* increased the overall presence of these aggregates when compared to those given a sham treatment containing no bacteria. It is our thought that these aggregates seem to resemble isolated lymphoid follicles, which have similar

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responses to microbes in other organisms. These important questions will be addressed in future studies.

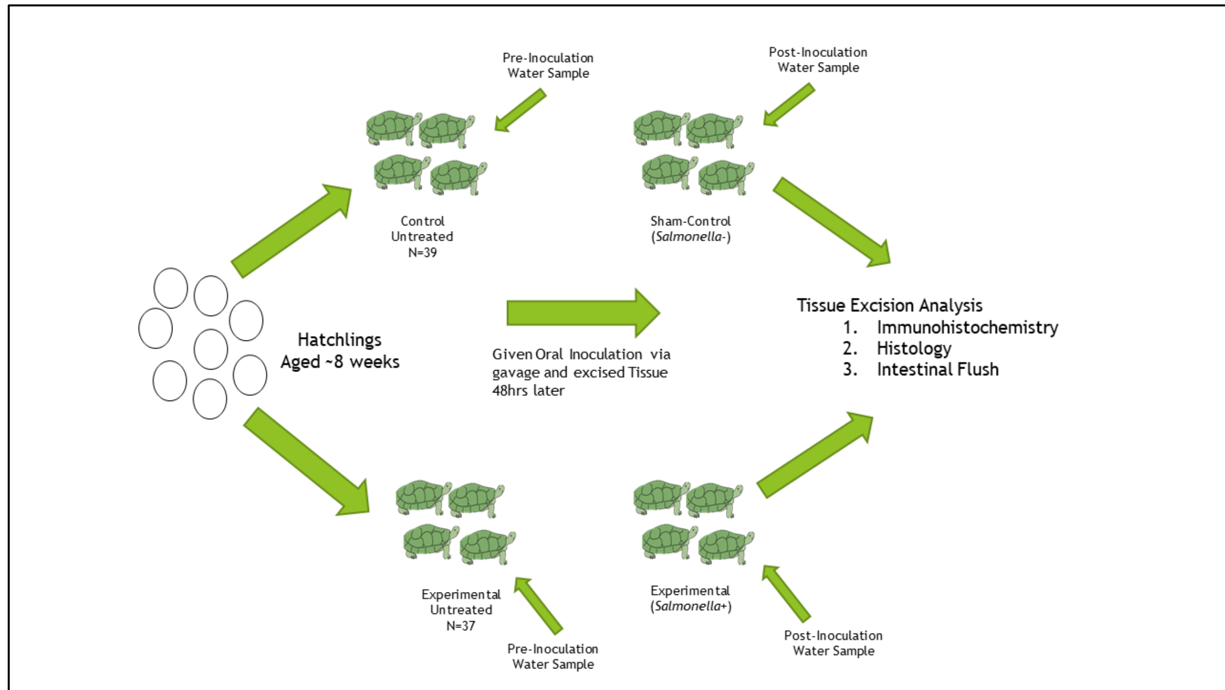

Fig. 1 A graphical representation of the experimental design using hatchling *T. scripta* in our study.

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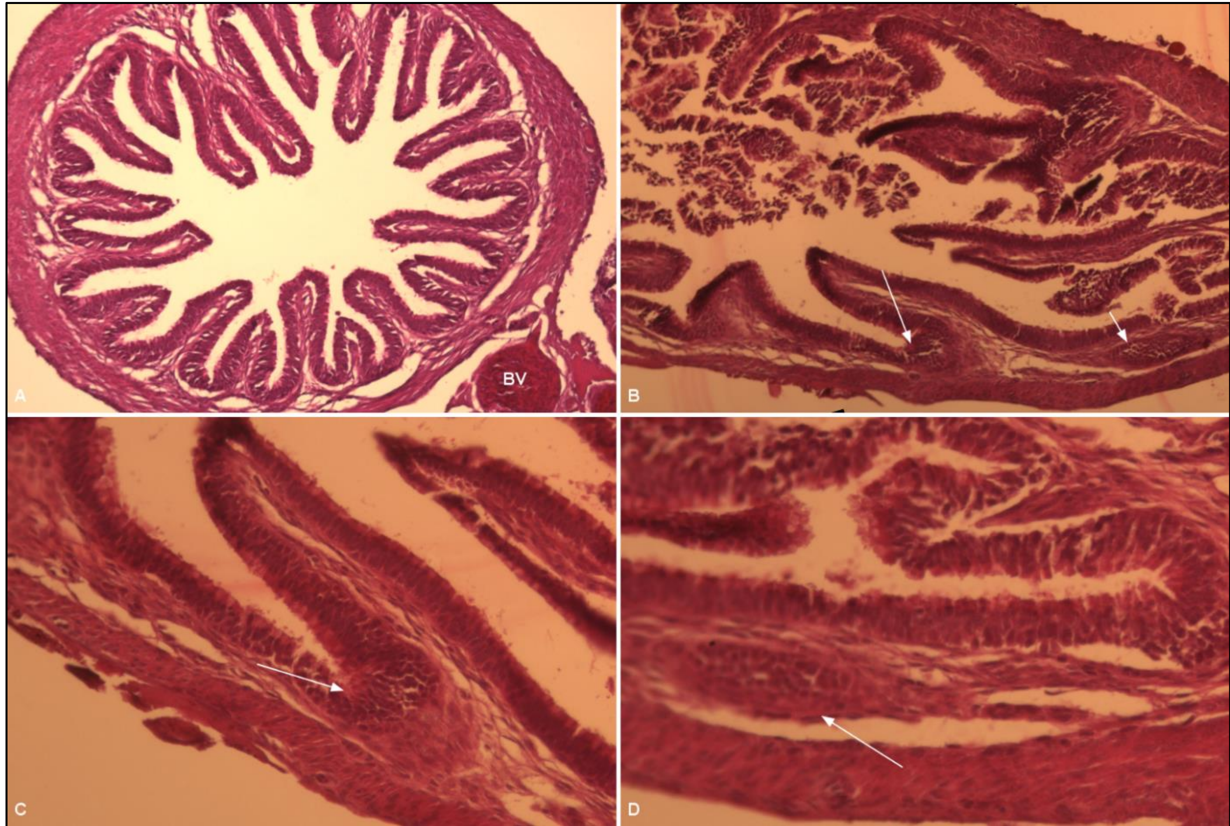

Fig. 2 7μM sliced histological sections of hatchling small intestines stained with H&E. Representative images are shown above all at 10X magnification. A) Intestinal section with no lymphoid aggregation present; BV=Blood Vessel B) Another small intestinal with visible lymphoid aggregations C) Lymphoid aggregation presence at the base of villi D) Lymphoid aggregation presence within the lamina propia

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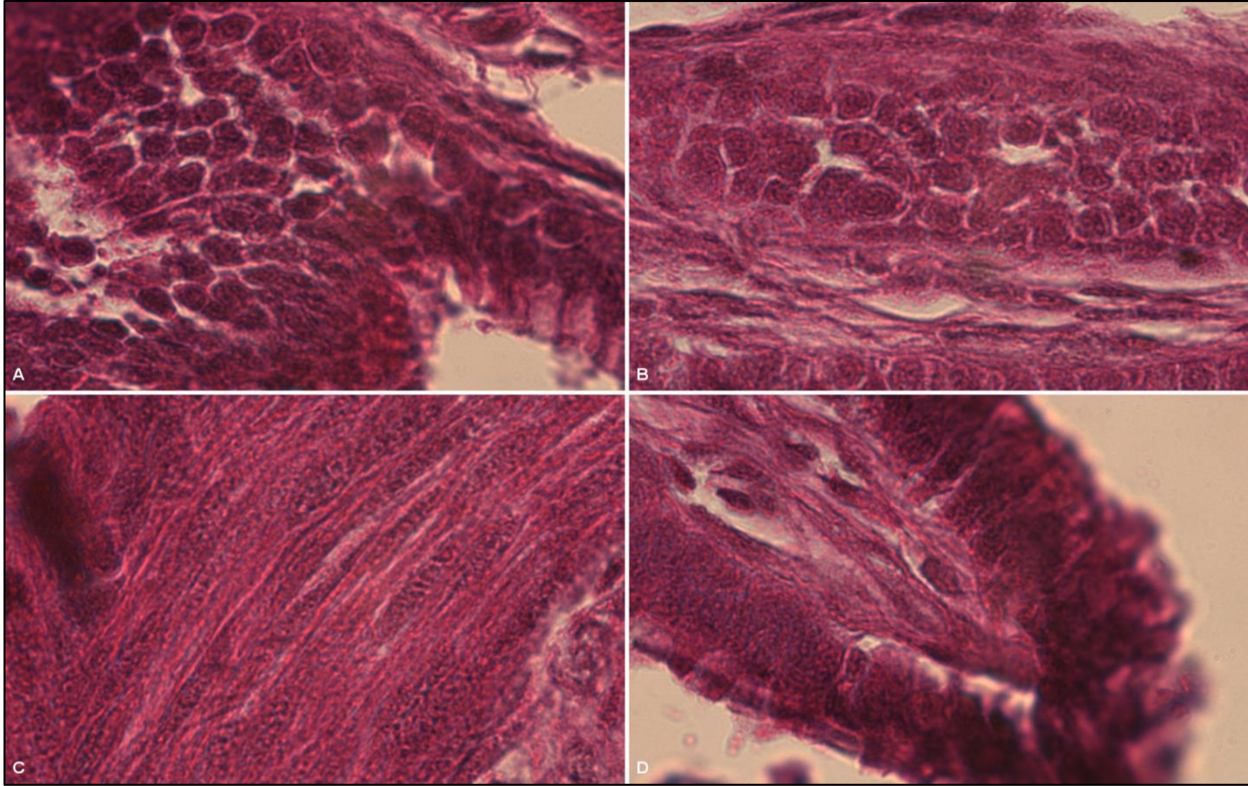

Fig. 3 Oil immersion (100X) of intestinal section containing lymphoid aggregates. A) Small round leukocyte-like cells within the base of the intestinal lumen B) Small round leukocyte-like cells within the lamina propia C) Muscle cells shown within the intestine D) Tip of villi with diffuse leukocytes contained within the interior section surrounded by a single layer of columnar cells

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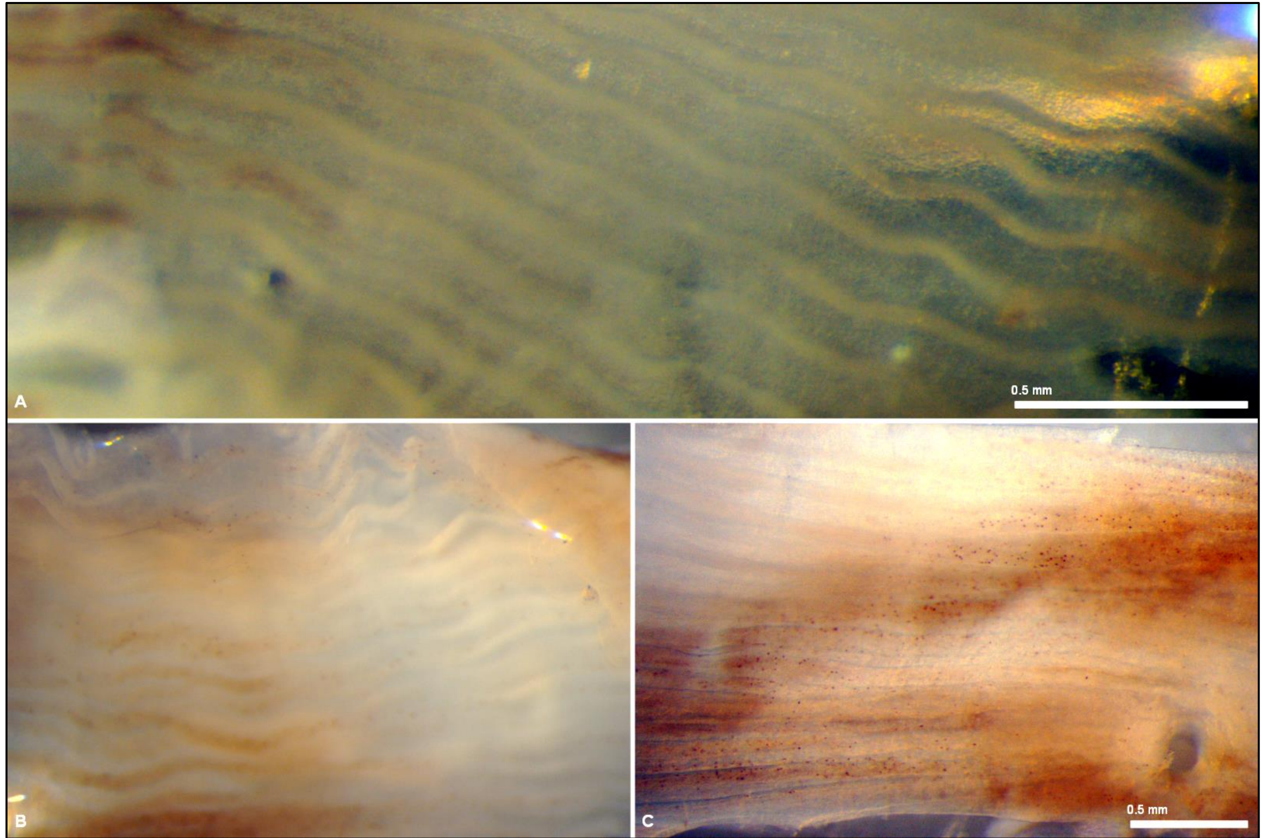

Fig. 4 Clusters of B cells are present throughout hatchling intestines. Representative intestinal images are shown. (A) Negative control intestinal section that received no primary antibody (B) Proximal intestinal sections stained with primary antibody to turtle light chains and (C) distal sections. Scale bars and magnifications are the same for images (B) and (C).

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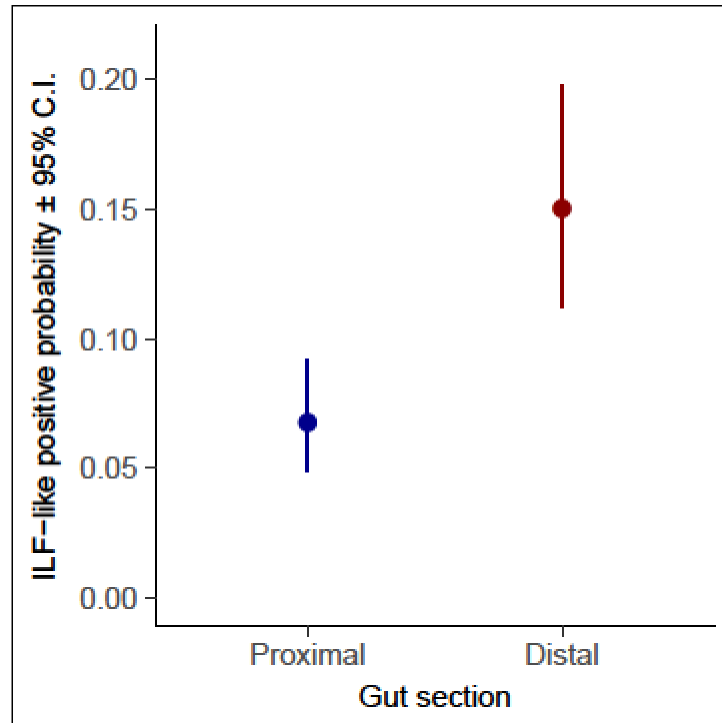

Fig. 5 Frequency of B cell aggregates is increased in distal sections. Spot proportion was determined as described for intestinal sections. Points represent model-estimated probabilities. Distal Sections (n=45), Proximal Sections (n=41).

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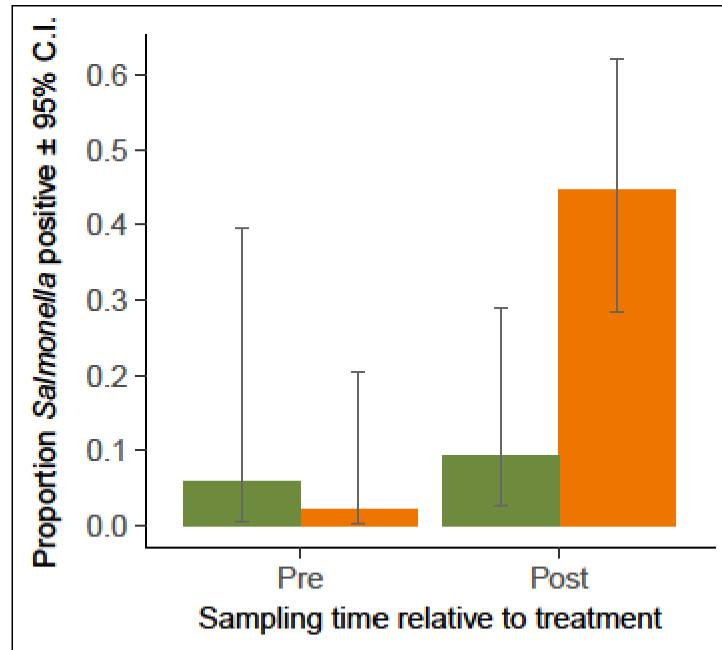

Fig. 6 Prevalence of *Salmonella* in tested water samples before and after inoculation. A low proportion of individual's water tested positive for *Salmonella* before treatment, but a much higher proportion of animals tested positive for Salmonella shed in water following inoculation of *Salmonella* by oral gavage treatment. Hatchlings were randomly placed in sham (green bar) or exposure groups (orange bar) and water samples collected and plated on selective media as described. After 48 hours, a post-treatment water sample was tested. Bars represent model-estimated proportions. Pre-treatment Sham Group (n=72), Pre-treatment Salmonella-exposed Group (n=76), Post-Treatment Sham Group (n=64), Post-treatment Salmonella-exposed Group (n=76).

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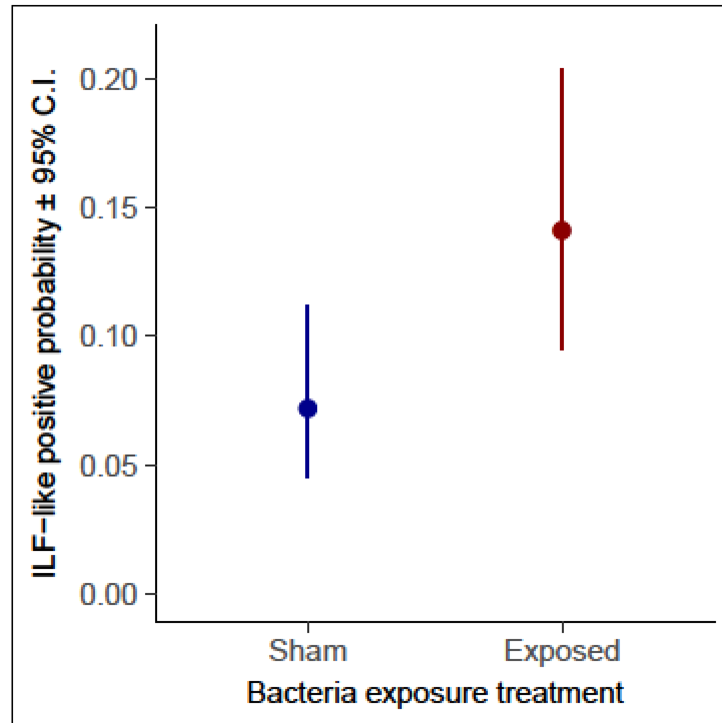

Fig. 7 A higher proportion of spots were observed in hatchlings that were exposed to *Salmonella*. Whole mount immunohistochemistry of the distal portion of hatchling intestines was performed 48 hours after oral gavage. Images were analyzed as described. Points represent model-estimated probabilities. Sham-Treatment Group (n=38), *Salmonella*-exposed Group (n=48)

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CHAPTER III: CHARACTERIZATION OF THE PHAGOCYTIC CAPABILITIES OF THE *T. SCRIPTA* B CELL

ABSTRACT

The B cell is a crucial component of the adaptive immune response. Traditionally, it is thought to function primarily for antibody production, but studies in our lab have identified a population with phagocytic functionality in the red-eared slider (*Trachemys scripta*). Due to many lines of evidence, we believe the phagocytic B cell in *T. scripta* may be very similar to the B-1 cell in mammals. In this study, we aimed to determine whether their B cells had the ability to recognize differing microbes, explore the size limitations of particle consumption, and determine if these cells possess regulatory functionality as well. Unfortunately, the fluorescent bioparticles that were used in our study were not compatible with our machinery and we were not able to accommodate the many issues that arose during throughout our study. It is our hope that in the future we will repeat this study, with new machinery and/or that our particles compatible. We hope that this study will add more knowledge of reptilian immune cell functionality and give more clues on the occurrence of phagocytic B cells in jawed vertebrates.

1 | INTRODUCTION

1.1 | Phagocytic B Cells In A Reptile

Adaptive immunity is essential for mounting specific immune responses by utilizing T and B cells, while innate immunity is fast acting and relies on non-specific responses from innate cells and molecules. Although B cells are typically associated with antibody production, several subsets of B cells have been identified in mammals (Allman & Pillai, 2008). The major subsets of

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mammal B cells are called B-1 cells and B-2 cells. B-2 cells can be thought of as “traditional” B cells that produce many classes of antibodies. They also are located in several lymphoid organs and respond strongly to protein antigens (Allman & Pillai, 2008). B-1 cells, however, have been shown to primarily produce “natural antibodies”, which are also referred to as polyreactive antibodies (Herzenberg et al., 1986; Panda & Ding, 2015). Polyreactive antibodies typically have a low binding affinity to multiple epitopes and are usually of the IgM isotype, but natural forms of IgA has been observed as well (Meyer-Bahlburg, 2015). B-1 cells in mammals are found in niche populations mostly located in the pleural and peritoneal cavities and respond well to carbohydrate antigens (Berland & Wortis, 2002b; Gao et al., 2012; L. Zhu et al., 2016).

While the immune system has been well studied in model species, less is known about immunity in non-model species. Following the discovery that some amphibian species and jawed fish contained B cells capable of phagocytosis (J. Li et al., 2006; Øverland et al., 2010), our lab was able to identify such cells in the red-eared slider turtle (*Trachemys scripta*) (Zimmerman, Vogel, Edwards, et al., 2010). The loggerhead turtle (*Caretta caretta)* and green sea turtle (*Chelonia* mydas) have also been reported to contain phagocytic B cells, giving rise to the idea that these types of cells may be conserved amongst most turtles and potentially all reptiles (Rousselet et al., 2013; Q. Zhu et al., 2016). This innate phagocytic functionality is not exclusive to the turtle however and has been subsequently characterized in many mammals such as mice (Gao et al., 2012), humans (Souwer et al., 2009), and non-human primates (Haas, 2015).

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It is unknown if B cell subsets exist in reptiles, however, several lines of evidence suggest that their B cell function is more equivalent to mammal B-1 cells. First, characteristics of antibody production in reptiles more closely resemble B-1 than B-2 cells. Following immunization, there is a modest increase in antibody titer in the serum which happens slowly over time and titer does not change significantly with a second exposure (T.M Work et al., 2000; Zimmerman, Vogel, & Bowden, 2010). Generally, the antibodies produced are natural antibodies of low affinity (Zimmerman, Bowden, et al., 2013).

Second, reptilian B cells possess phagocytic capability, which is only observed in the B-1 phenotype in mammals (Parra et al., 2012; Popi, 2015). Preliminary data from our lab suggests that reptilian B cells can phagocytose a large number of inert 1um polystyrene beads (Palackdharry et al., 2017), but how they target phagocytosis is unknown. It's been shown that B-1 cells utilize the B cell receptor for specification in mammals (Gao et al., 2012), but toll-like receptor (TLR) recognition is known to play a role in the maturation process and potentially recognition as well (Meyer-Bahlburg & Rawlings, 2012). Reptilian B cells produce a B cell receptor and sequence data from the painted turtle suggests a variety of TLR genes are present as well (Bradley Shaffer et al., 2013).

Finally, there is potential evidence, that the reptile B cell has regulatory functionality. B-1 cells in mammals have been observed to play a role in inflammation and a subset of B-1 cells have been aptly named, B regulatory cells due to this (Aziz, Holodick, Rothstein, & Wang, 2015). It was observed in preliminary findings, that our B cells seemed to be more aggressive at

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consuming polystyrene beads than the co-incubated adherent cells (i.e. macrophages/monocytes)(Marrochello, 2016). It is known that B regulatory cells possess the ability to secrete IL-10 which decreases the phagocytic ability of macrophages *in-vitro* (Popi, Lopes, & Mariano, 2004).

In order to learn more about these unique cells, we sought out to answer some of the questions mentioned above using *T. scripta*. First, we wanted to determine if the reptile B cell has the ability to recognize different kinds of microbes. Thus, we modified our phagocytic assay to use fluorescent microbes instead of beads as targets. Unfortunately, due to the extreme brightness of the beads, we were unable to distinguish B and non-B cells in the assays. Future studies might resolve the issue by using a fluorochrome to detect B cell staining that is much longer in wavelength than FITC to prevent fluorescent bleed over.

2 | MATERIALS AND METHODS

2.1 | Lymphocyte Collection and Preparation

During the nesting season, female adult *T. scripta* were collected from Banner Marsh (Canton, IL) as approved by the IACUC. A 1:1 ratio of 3-4ml of pooled whole blood was collected and added to RPMI 1640 (Life Technologies) and 1:30 of 0.1M ethylenediaminetetraacetic acid (EDTA; Fisher Scientific) in a 15ml tube. Mixtures were then be carefully layered on a 50% Percoll (MP Biomedicals, LLC) and saline solution with the final result being a 1:1 ratio of blood/RPMI to Percoll/saline. Tubes were then centrifuged at 400xg at 4°C for 5mins without a brake. The resulting buffy coat layer containing leukocytes was extracted and washed with RPMI. A 15ml tube filled RPMI was then centrifuged for 5mins at 1500xg at 4°C with a brake

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applied. RPMI was then decanted and resulting pellet was resuspended in 500µl of RPMI and counted. Ideal cell concentration should be 6-7 x 10^6 cells/ml. One ml of resuspended solution was divided into different tubes corresponding to the specific experiment (see below).

2.2 | Phagocytic B cell Assay

In vitro phagocytic assays were carried out as described in L. M. Zimmerman et al., (2013b). Isolated leukocytes were adjusted to at least 5x10^5 cell/well and incubated with fluorescent bioparticles (20:1), based on another experiment using similar bioparticles (Nuutila & Lilius, 2005) and previous experiments with FITC beads (Fluoresbrite Plain Yellow Green Microspheres, Polysciences) (Zimmerman, Vogel, Edwards, et al., 2010) for control samples. Cells and particles were incubated in 6 well plates at 29-30°C and 5% CO_2 for 3 hours. The cells and bioparticles were collected from the wells by vigorous pipetting and transferred to a collection tube, filled with RPMI and washed for 1500 ×g for 5 min at 4 °C. The cells were resuspended in 2ml of 1x Hanks' Balanced Salt Solution (HBSS, Life Technologies)-1% BSA. In order to wash away non-consumed beads, the beads/bioparticles and cells were layered 1:1 on a cushion of 3%-4.5% Dextrose-Phosphate buffed saline. Following supernatant removal (which contained the free beads), pelleted cells were washed and centrifuged as previously mentioned and resuspended in 400µL 1× Hanks-0.5%-BSA. They were washed again and decanted until only 50µl remain in the tube. A 1:10 ratio of normal rat serum was added to block non-specific binding and 1µl of biotin-labeled anti-turtle light chain monoclonal antibody (HL673 mAb, University of Florida Hybridoma Facility) was incubated for 15mins and the cells were stored on ice. Cells were washed and then stained with 20µl of Streptavidin-spectral red (Southern

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Biotech, 0.5mg/ml) diluted 1:1000 in 1× Hanks-0.5%-BSA and incubated in the dark on ice for 15min. They were washed again and 300µl of 1x HBSS-1% BSA was added to prepare the sample for Flow cytometry. In order to avoid high background from the bioparticles, cells were quenched with 200 µl of 1.2 mg/ml of Trypan Blue (MP Biomedicals) prior to running (Nuutila & Lilius, 2005). Cells were analyzed immediately on a Becton Dickinson FACSCalibur flow cytometer. A minimum of 10,000 events were collected and data was analyzed using CellQuest Pro software (BD Biosciences).

2.3 | Bioparticles and Beads

There were three experimental groups: (Bioparticles from *E. coli* only, *S. aureus* only, and *S. cerevisiae* only (Zymosan) that were all FITC-labeled. As a control FITC-labeled (0.5µm) polystyrene beads were used since turtle B cells have previously been shown to phagocytose these particles (Zimmerman, Vogel, Edwards, et al., 2010). Fluorescent bacteria and yeast bioparticles were purchased from ThermoFisher Scientific. Five replicates were attempted per group for statistical comparisons. Experiments were completed in groups. For example, experiment 1 was completed using one blood pool sample with every experimental group at once. A total of five different blood pools were used on five separate days.

2.4 | Microscopy

We utilized a Leica TCS SP2 for confocal microscopy to locate B cells that consumed particles/beads by spotting them on a microscope slide following the phagocytic assay. We also used a Leica DMRBE equipped with darkfield, DIC, and phase contrast as well as fluorescence filter sets for UV, blue, green and GFP excitation to attempt to locate these cells as well. We

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replaced the Streptavidin-spectral red stain for Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 and 568 (Thermofisher), as our original filter/laser setup on these microscopes were unable to locate the cells.

3 | RESULTS

3.1 | Bioparticles were too Bright for the BD FACSCalibur

Previous research in our lab developed an assay to examine B cell phagocytosis using polystyrene beads (Zimmerman, Vogel, Edwards, et al., 2010). Because different receptors, such as TLR, may be involved in recognition of actual pathogens, we predicted phagocytosis of pathogens may be more efficient than inert beads. Thus, we modified our phagocytic assay to include Bioparticles obtained from Thermofisher. We selected bioparticles from the major different types of microbes: *E. coli* for Gram-negative bacteria, *S. aureus* for Gram-positive bacteria, and *S. cerevisiae* for yeast. In Figure 8, a typical scatter plot with lymphocyte gating is shown (upper left panel). Staining with HL673 reveals specific B cell staining versus control (no fluorochrome; top middle and right panels). Bottom panels show polystyrene beads alone. Scatter plot on left, normal beads in middle and beads quenched by addition of trypan blue on right.

A phagocytic assay was run with beads and bioparticles as described in methods. In Figure 9, scatter, control staining, and B cell staining are shown for cells that did not receive beads (top panels). Middle and bottom panels show cell populations following the phagocytic assay with the indicated targets. In the bead sample, the upper left quadrant is showing an unusually high

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level of B cell staining. Although in the upper right panel we can clearly distinguish phagocytic FITC+ cells, it is not clear they are B cells in this experiment. Likely, too much SA-SR dye was added causing a high background. For the bioparticles, for *E. coli* and *S. aureus*, there is also evidence of a phagocytic population but for the yeast particles, the fluorescent was too intense to distinguish from background.

Based on other reports (Nuutila & Lilius, 2005), we attempted to use Trypan Blue to quench the bright signal. However, the resulted in all cells appearing false positive for the SA-SR dye (Figure 10). Again, we were able to detect phagocytosis in 3 of the 4 samples (not in the yeast sample) but were unable to determine if the cells were truly B cells. Figure 11 illustrates the bioparticles themselves in trypan blue and even with maximal compensation settings the FITC signal was being detected in the FL3 photomultiplier tube. Thus, we were unable to prevent bleed over of the fluorescence into the photomultiplier tube where we collected the B cell signal, and were unable to discriminate phagocytic B and non-B cells.

3.2 | Determination of Phagocytosis by Fluorescent and Confocal Microscopy

Due to our difficulty in obtaining data through flow cytometry, we tried to visualize if our bioparticles were ingested by both fluorescent (Figure 12 and 13) and confocal microscopy. We encountered difficulty locating a single B cell that underwent phagocytosis. Usually, ~2-5 percent of B cells undergo phagocytosis in *T. scripta* (Figure 9, Zimmerman et al., 2010b), so visualizing unpurified populations of cells provided a very low probability of occurrence. We also experienced high cell death, which could be due to slide preparation, as we did not fix our

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cells prior to staining. Fluorescent microscopy showed that FITC-coated particles were so bright, they were visible in both GFP and RFP filters (Figure 13), as was the case for the flow cytometry. We should note that quenching minimally altered the fluorescence for the scope as well. Confocal microscopic analysis showed us a single potential phagocytic B cell. It was very misshaped, however, and we couldn't be sure if it was an actual B cell. Staining with secondary Alexa 568 and 633 antibodies also didn't show many positive-stained B cells (Figure 12). Thus we were unable to confirm the B cells were ingesting the bioparticles.

4 | DISCUSSION

Innate immunity is thought to have evolved prior to adaptive immunity (Buchmann, 2014; Hirano, Das, Guo, & Cooper, 2011). Many vertebrates have populations of innate immune cells with specialized innate functionality, such as macrophages and neutrophils. Thus, it is of interest why innate immune function might have been retained in some cells of the adaptive immune system, such as the phagocytic B cell identified in *T. scripta.* Understanding how and why these cells contain this innate functionality provides novel information into the evolution of the immune system. At first glance, it would seem that in a system where professional phagocytes currently exist, this redundant function is unnecessary. Because of this, there's been much debate of the origin of the B-1 cell in the mammal, with some scientist conceding to this idea that the B-1 cell could be of the myeloid lineage (Popi, 2015). As studies have progressed, however, the literature has now uncovered the importance of these cells for managing mucosal immunity by the production of IgA (Meyer-Bahlburg, 2015) among other functions such as antigen presentation (Popi, Longo-Maugéri, & Mariano, 2016). Examination of

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these cells begs the evolutionary question, are B-1 cells artifacts from innate immunity that specialized to become B-2 cells as the adaptive immune system evolved better microbe recognition strategies or are these cells simply another cell subset? Evidence that these cells are contained within reptiles, amphibians and early jawed fish (J. Li et al., 2006) provide important clues to aid in resolving this argument.

It is unknown whether or not the B cells within *T. scripta* have the ability to recognize and consume different types of microbes or are specialized in their phagocytic targets. Prior phagocytic studies within turtles have only examined the uptake of phagocytic beads, but we suspect pattern recognition receptors for microbial components may also play a role in this process. Particle uptake in professional phagocytes has also been shown to decrease as the size of the target increases in many different studies (Cannon & Swanson, 1992). If the size of the target becomes too large, the B cell may attempt phagocytosis but fail ("frustrated phagocytosis"), or it may switch to produce antibodies instead. As of now, no study has sought out to see the size limitation of phagocytosis in phagocytic B cells or if the cells switch to antibody production in *T. scripta*. Thus, we'd propose to investigate this in future studies using beads and particles of different sizes.

It is currently not known if the B cell has antigen presentation capability following phagocytosis. It has been reported in the literature in mice that both B-1 and B-2 cells have the capability to also aid in antigen presentation (Lee-Chang et al., 2016; Popi et al., 2016). A future goal in our lab is to determine whether or not this ability is retained within the reptilian B cell. A recent

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study was done in the turbot (*Scophthalmus maximus*) that determined that teleosts B cells rely heavily on micropinocytosis for particle uptake (Y. Li, Sun, & Li, 2018). It remains an open question if the reptile B cell utilizes these same mechanisms and future studies aim to explore this as well.

5 | CONCLUSIONS

We originally aimed to learn more about the phagocytic functionality within the B cell in *T. scripta* in the 2017 nesting season. Unfortunately, due to many technical issues, this study was unable to be completed. The various bioparticles we chose to use proved to be much more fluorescent than we expected. Despite many efforts to work around this issue by quenching, altering compensation/voltages in flow cytometry, and utilizing fluorescent and confocal microscopy, we were still unsuccessful in relevant data collection. It is our hope in the future to reattempt the proposed experiments and learn more about the phagocytic B cell in the reptile.

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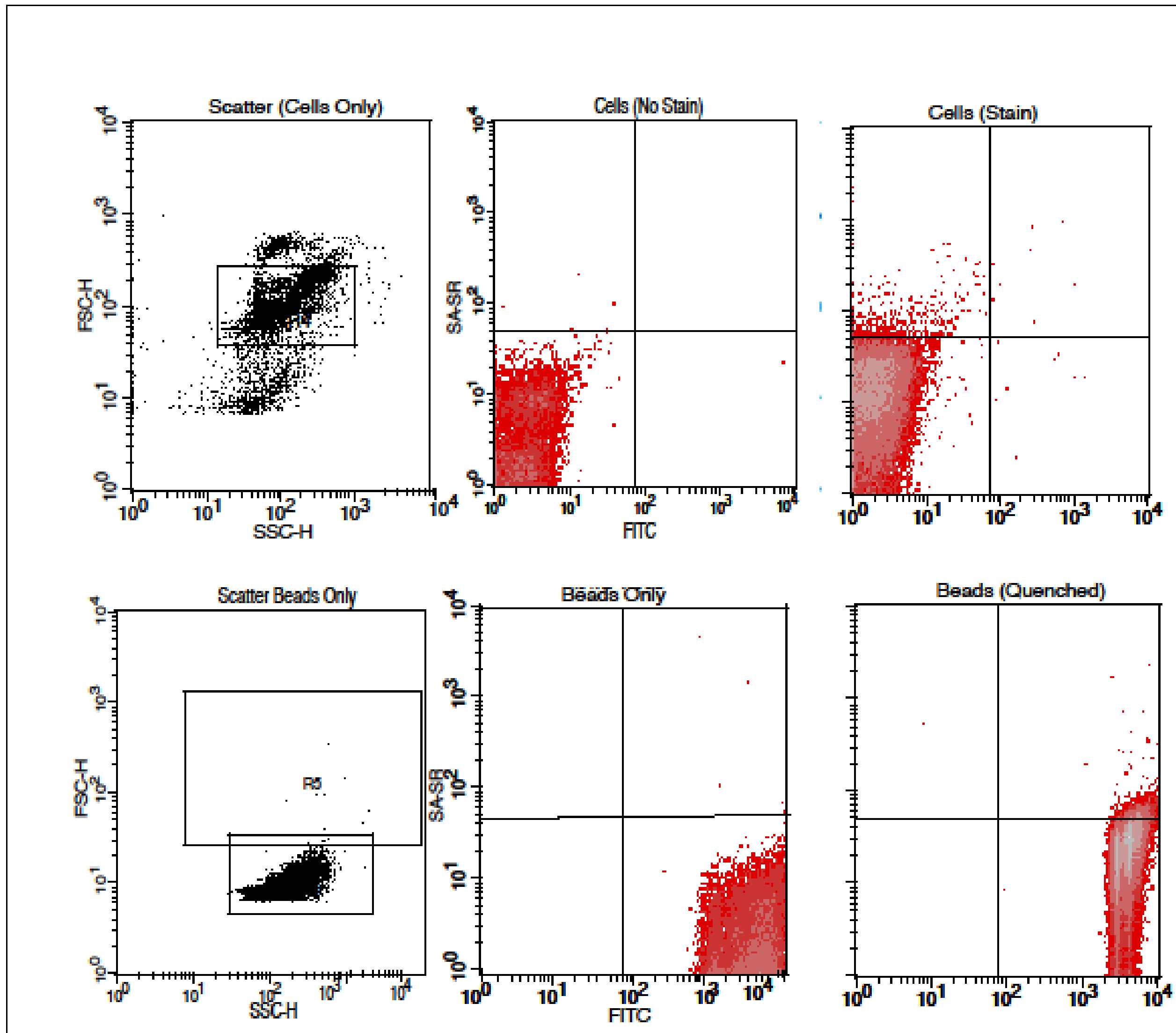

Fig. 8 Sample flow cytometry analysis showing gating of cell/bead populations. Top left: Scatter plots with forward scatter (FSC) and side scatter (SSC) showing gating of lymphocyte populations. Top middle: Cells that did not receive SA-SR. Top right: cells stained with HL673. Bottom left: scatter plot showing beads alone. Bottom middle: fluorescence of beads only and bottom right: beads quenched with trypan blue. Representative histograms from an individual blood pool are shown.

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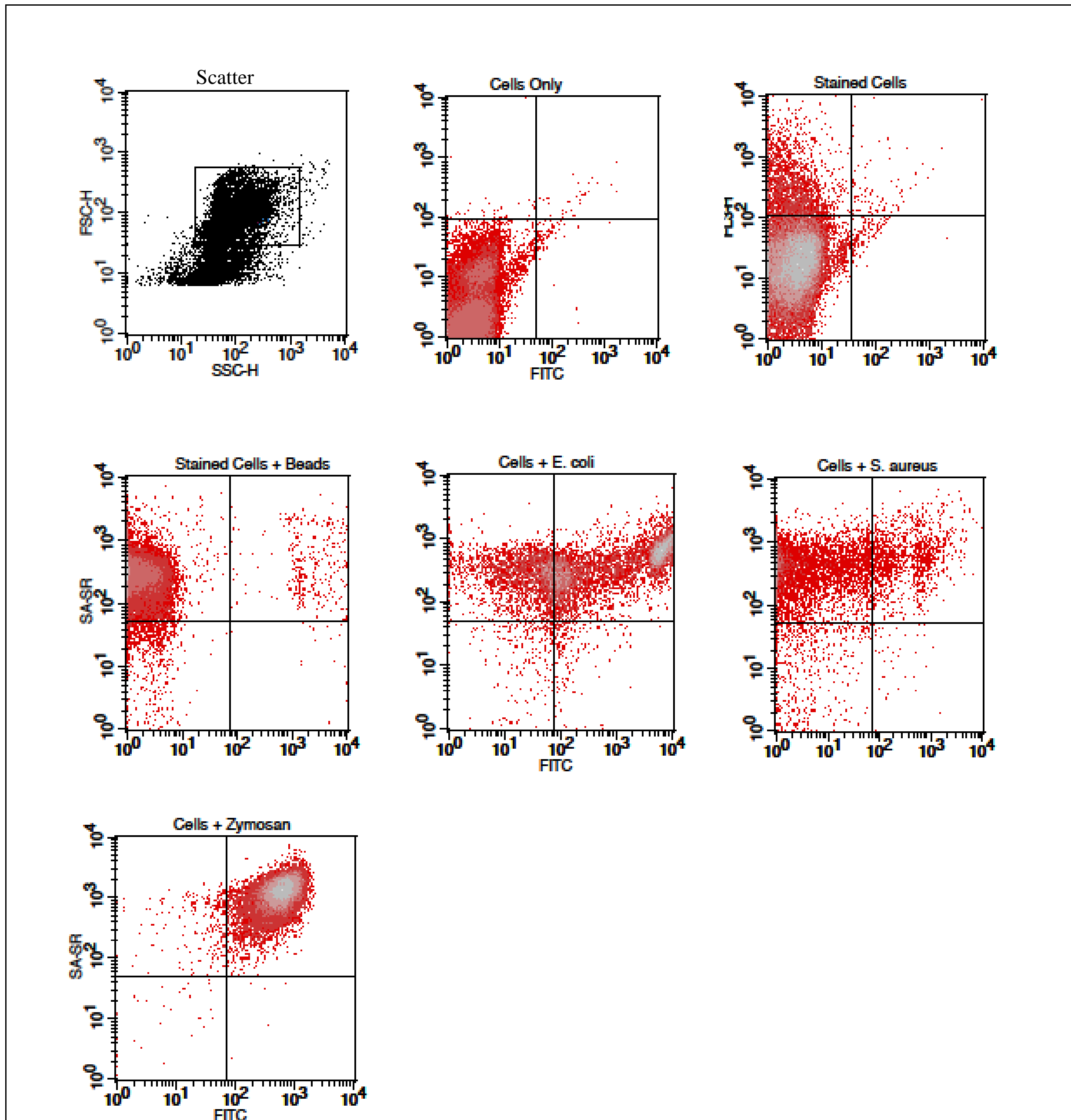

Fig. 9 Turtle blood cells were able to phagocytose bioparticles, but fluorescence bleed over prohibited identification of B cells. Top left: scatter plot of the blood sample. Top middle; Control staining without SA-SR. Top right; Stained cells without beads stained for B cells. Middle and bottom panels: Cells incubated with various indicated target particles and stained with HL673-SA-SR. Representative histograms from an individual blood pool are shown.

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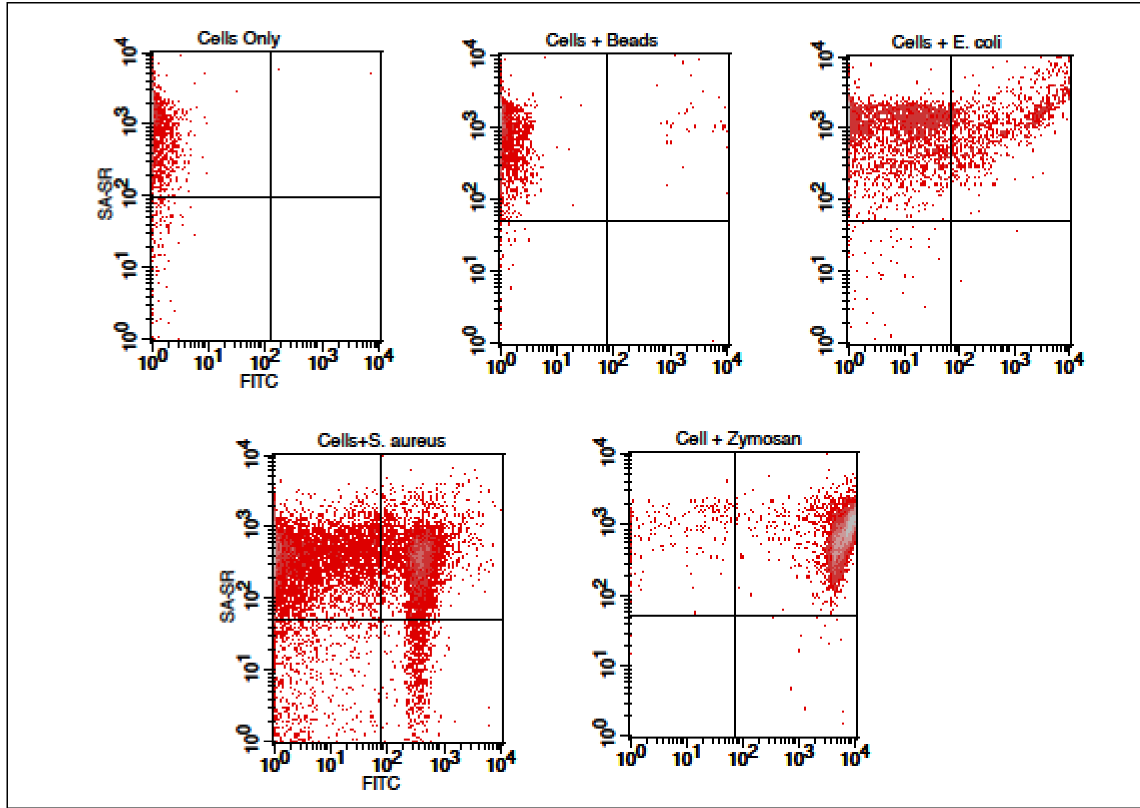

Fig. 10 Trypan blue failed to quench fluorescence bleed over. Samples were prepared as in Figure 9 and trypan blue was added immediately prior to analysis.

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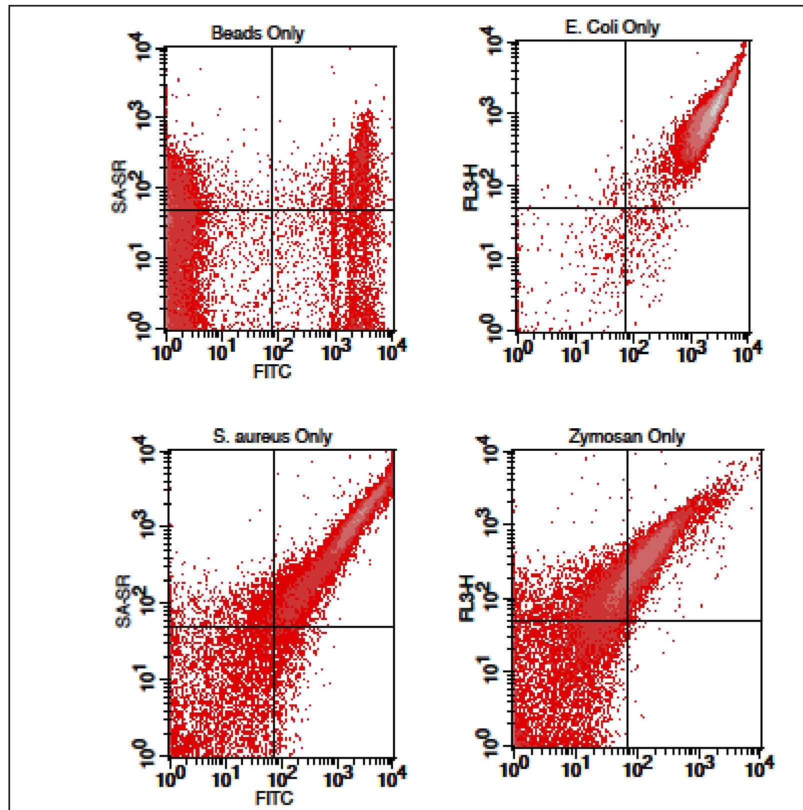

Fig. 11 Bioparticles quenched without cells still prove to be too bright. Bioparticles were incubated directly in trypan blue and analyzed as in Figures 9 and 10

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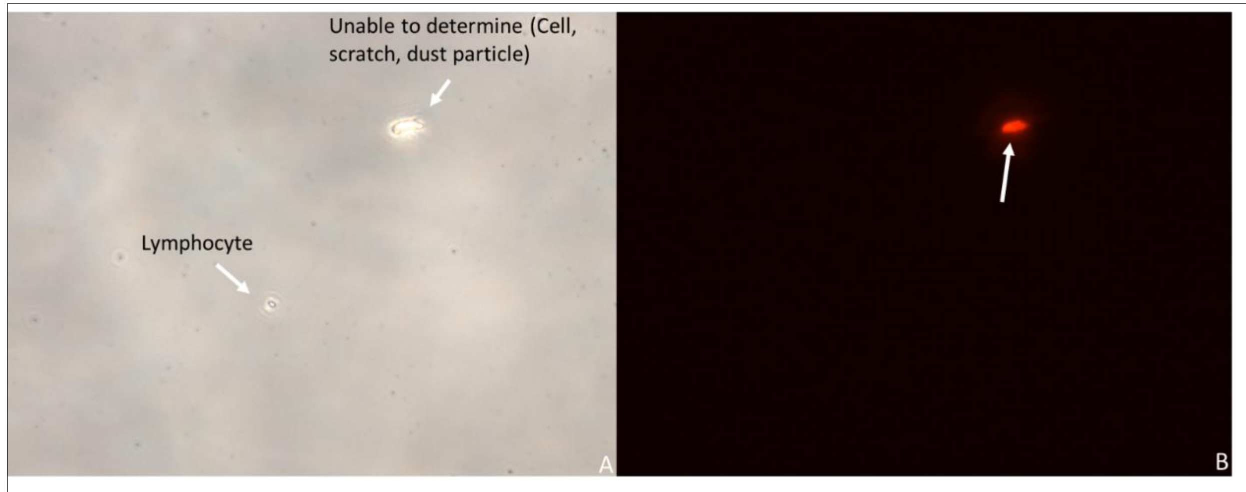

Fig. 12 Fluorescent microscopy failed to detect stained lymphocytes. Following the phagocytic assay with various bioparticles, samples were spotted onto a microscope slide and viewed under the confocal microscope A) Bright field image of a potential lymphocyte and a structure of unknown origin B) Same field of view as A but using the RFP filter.

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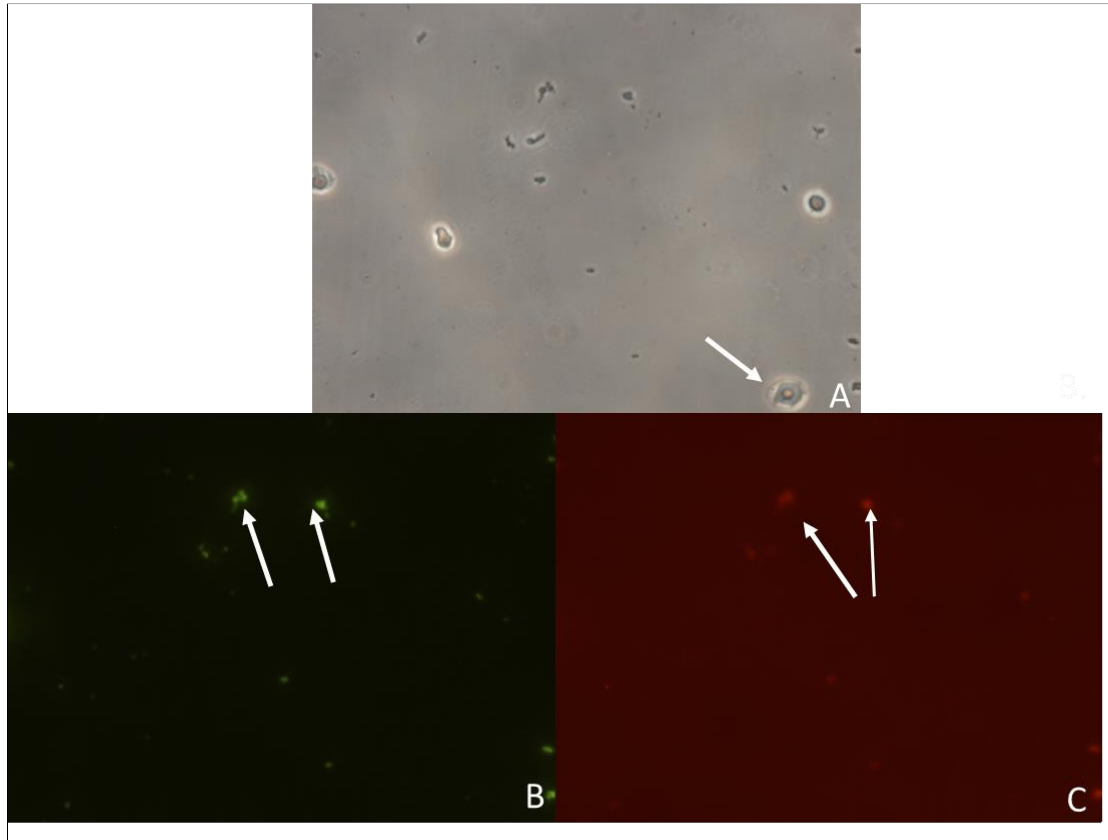

Fig. 13 Bioparticles show false positive in GFP and RFP filters. A) Brightfield image with an arrow indicating a blood cell and bioparticles in the background. B) A GFP-Filter indicating E. coli particles clumped together with no interaction with blood cell. C) An RFP-Filter with arrows indicating that bioparticles show false positive in this filter.

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