An Investigation Into B Cells In Peripheral Blood And Gut Associated Lymphoid Tissues In The Red Eared Slider Turtle, Trachemys Scripta

Sarah Marie Marrochello
Illinois State University, smarrochello@gmail.com

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As in endotherms, immunity in ectotherms is vital to survival and contributes to the overall fitness of an organism. For reptilians, there are a number of environmental factors, such as temperature, season, and pathogen prevalence, which are known to influence both innate and adaptive immunity. Of particular interest, is the effect of these factors on B cells, a component of adaptive immunity. Reptilian B cells are reported to secrete antibodies, much like their mammalian counterparts, and undergo phagocytosis. The work reported here primarily focused on understanding the effect of environmental temperature, incubation temperature, and nesting season on B cells in the model reptile the red eared slider turtle (Trachemys scripta). First, we investigated the effect of environmental temperature on the functions of peripheral blood B cells isolated from adult red eared sliders. Specifically, we examined B cell phagocytosis and the number of antibody secreting cells at four biologically relevant temperatures, 20°, 25°, 30°, and 35°C. Normal activity range of red eared sliders is reported between 25°-30°C, therefore we hoped to elucidate the effect of temperature on B cell functions above and below this
range. We found that the ability of B cells to phagocytose was not affected by
temperature. Additionally, we report that the number of antibody cells was significantly
affected by temperature with increased and decreased antibody secretion between 20°-
25°C and 30°-35°C, respectively. Our secondary focus was to characterize B cells in the
gut associated lymphoid tissues (GALT) of red eared slider hatchlings. In our study
population, turtles lay two clutches during the summer, an early clutch and a late clutch.
We aimed to examine the effect of incubation temperature and early vs. late season on B
cell distribution in the small intestine of hatchlings derived from the 2014 and 2015
nesting season. To test the effect of incubation temperature, eggs were incubated in
fluctuating temperature treatments with mean temperature of 26.5°C (low), 27.1°C
(medium), or 27.7°C (high). We found that incubation temperature significantly affected
the number of B cells in early season clutches. Specifically, clutches from the low
temperature treatment had more B cells than clutches from the higher incubation
temperature treatments. Additionally, preliminary data suggested that B cell presence in
the small intestine may be significantly affected by nesting season with a large proportion
of late season hatchlings exhibiting no B cells. We also examined maternal antibody
allocation to early and late season eggs. Maternal antibody levels in yolk were not
affected by nesting season; however, they were significantly affected by clutch identity.
Overall, the work presented here explores the importance of B cells in reptilian
immunity.

KEYWORDS: B cell, Immunity, Mucosal immunity, Reptile
AN INVESTIGATION INTO B CELLS IN PERIPHERAL BLOOD AND GUT ASSOCIATED LYMPHOID TISSUES IN THE RED EARED SLIDER TURTLE, TRACHEMYS SCRIPTA

SARAH M. MARROCHELLO

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

MASTER OF SCIENCE

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

2016
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SARAH M. MARROCHELLO

COMMITTEE MEMBERS:
Laura Vogel, Co-Chair
Rachel Bowden, Co-Chair
Benjamin Sadd
ACKNOWLEDGMENTS

First and foremost, I would like to thank my co PIs, Dr. Laura Vogel and Dr. Rachel Bowden for their endless support and advice throughout my project. This work would not have been possible without their guidance and continuous encouragement. I would like to thank all of my past and present lab members for their help both in the lab and out in the field; Hazel Ozuna, Lisa Treidel, Justin Dillard, Amanda Wilson Carter, Ryan Paitz, and Laura Zimmermann. I greatly appreciate all the laughs you held back while watching my fumbles out in the field. I’ve come a long way since my first day out in the field thanks to all of you. I would also like to thank the undergraduates that helped out in various projects along the way; Autumn Joerger, Nicole Wagner, and Piers Hunter. Additionally, many thanks are owed to my committee member, Dr. Ben Sadd, for taking the time to work on my statistics and patiently explain them during our many meetings. I would also like to thank my family and wonderful fiancé, Matt, for supporting me through this endeavor. Finally, I would like to thank my graduate friends for all the fun times and memories we made together.

S. M. M.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>i</td>
</tr>
<tr>
<td>CONTENTS</td>
<td>ii</td>
</tr>
<tr>
<td>TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. AN OVERVIEW OF THE REPTILIAN IMMUNE SYSTEM AND FUTURE DIRECTIONS</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>The reptilian immune system</td>
<td>3</td>
</tr>
<tr>
<td>Innate immunity</td>
<td>4</td>
</tr>
<tr>
<td>Adaptive immunity</td>
<td>6</td>
</tr>
<tr>
<td>Gut associated lymphoid tissues</td>
<td>9</td>
</tr>
<tr>
<td>Temperature and the reptilian immune system</td>
<td>10</td>
</tr>
<tr>
<td>Reptiles as model organisms for immune studies</td>
<td>11</td>
</tr>
<tr>
<td>Conclusion</td>
<td>12</td>
</tr>
<tr>
<td>References</td>
<td>13</td>
</tr>
<tr>
<td>II. THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON REPTILIAN PERIPHERAL BLOOD B CELL FUNCTIONS</td>
<td>23</td>
</tr>
<tr>
<td>Abstract</td>
<td>23</td>
</tr>
<tr>
<td>Background</td>
<td>23</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>27</td>
</tr>
<tr>
<td>Trapping and blood collection</td>
<td>27</td>
</tr>
<tr>
<td>Phagocytic B cell assay</td>
<td>29</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>30</td>
</tr>
</tbody>
</table>
III. THE EFFECT OF NESTING SEASON AND INCUBATION TEMPERATURE ON B CELL DISTRIBUTION IN THE GUT-ASSOCIATED LYMPHOID TISSUE OF RED EARED SLIDER TURTLE (TRACHEMYS SCRIPTA ELEGANS) HATCHLINGS

Abstract 55
Background 56
Materials and Methods 61

Egg Collection and Incubation 61
Immunohistochemistry 63
Yolk Enzyme-linked immunosorbent assay 64

Statistical Analysis 65
Results 68
Discussion 69
References 74
Figures 81

APPENDIX: AN EXPLORATION INTO DUAL ROLES FOR REPTILIAN B CELLS 79
### TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell concentrations pre and post cell separation</td>
<td>94</td>
</tr>
<tr>
<td>2. Cells which had phagocytosed magnetic beads were subsequently</td>
<td>95</td>
</tr>
<tr>
<td>unable to produce ELISpots</td>
<td></td>
</tr>
</tbody>
</table>
FIGURES

1. Schematic representation of predicted temperature effects on innate and adaptive reptilian B cell functions 48
2. Example of flow cytometry phagocytosis analysis 49
3. Temperature did not significantly affect percentages of phagocytic B cells 50
4. Temperature significantly affected antibody secretion in reptilian B cells 51
5. Temperature did not significantly affect the percentage of Ig- phagocytic cells 52
6. Phagocytic B cells are more efficient at phagocytosis than phagocytes 53
7. Ig+ cells are more efficient at phagocytosing fluorescent beads than Ig- phagocytic cells 54
8. Method for enumerating intestinal spots 81
9. B cells are present in hatchling intestinal tissues 82
10. Frequency distribution of B cell clusters in hatchlings indicate majority of late season hatchlings stained negative for B cells in the small intestine 83
11. Lower incubation temperature significantly increases the number of intestinal B cell clusters in early season clutches 84
12. More B cell clusters were found in the small intestine of early season slider hatchlings than late season hatchlings 85
13. Maternal antibody deposition in eggs is not significantly affected by nesting season, but antibody levels are significantly affected by clutch identity

14. Schematic representation of magnetic cell separation
CHAPTER I
AN OVERVIEW OF THE REPTILIAN IMMUNE SYSTEM AND FUTURE DIRECTIONS

Introduction

Currently, many immune studies focus on birds and mammals, leaving aspects of the reptilian immune system understudied. However, it is through work in non-model organisms, that new findings regarding the functions of the vertebrate immune system are discovered. Such novel findings can have important implications for a number of different species. For example, novel B cell functions, such as phagocytosis, were first discovered in non-model immune systems of fish, amphibians, and reptiles (Li et al. 2006, Øverland et al. 2010, Zimmerman et al. 2010b). Phagocytic B cells have now been reported in mice (Parra et al. 2012, Nakashima et al. 2012) and even human B cells from the Raji B cell line can undergo phagocytosis (Zhu et al. 2015). These findings open the door for new research in vaccine development and prophylactics (Parra et al. 2013). Thus, reptilian immune studies are pertinent to understanding the underlying mechanisms of the mammalian immune system and may lead to new and exciting advancements in the field.

Recently, pathogen outbreaks have caused a global decline in amphibians and are anticipated to soon affect reptiles. These widespread amphibian extinctions are driven by global warming and reduced immune defenses to pathogens resulting from the altered environmental conditions (Pounds et al. 2006, Kilburn et al. 2011, Rohr & Raffel 2010).
Temperature can severely affect an organism’s immune response. While temperature sensitivity has been seen in arthropods (Adamo and Lovett 2011), fish (Nikoskelainen et al. 2002, Magnadóttir 2006), reptiles (Mondal and Rai 2001, Merchant et al. 2003, Merchant et al. 2005), and mammals (Carey, Andrews, and Martin 2003), thermal immune performance has been relatively understudied (Angiletta 2009). Immunity is not only vital to survival, it is also associated with life history traits such as growth and reproduction (Graham et al. 2011). During an immune challenge, already limited nutritional and energetic resources are redistributed towards immune defense and away from growth and reproduction (Lochmiller and Deerenburg 2000). Therefore, it is not only important to understand the effect of temperature on the immune system’s ability to combat pathogens, but also how the compromised immune system will affect the organism’s overall fitness.

It has been found by our group that red eared slider turtle (Trachemys scripta) B cells can secrete antibodies and undergo phagocytosis (Zimmerman et al. 2010a). However, it is unknown whether a single B cell can perform both functions, or if separate subsets exist for each role. The work presented here aimed to elucidate the efficiency of reptilian B cell antibody secretion and phagocytosis in regards to temperature in the red-eared slider.

We also explored the role of B cells in gut associated lymphoid tissues (GALT) of hatchlings. The gut is a particularly vulnerable mucosal surface as it is under constant antigenic challenge from food, commensal bacteria, and harmful pathogens (MacDonald 2003). Gut-associated lymphoid tissues, characterized by their high B cell densities, combat an endless amount of pathogenic threats (Nagler-Anderson 2001). Little is known
about GALT composition and function in reptiles. In addition, lower vertebrate GALT studies have mainly focused on tissues in adults; thus, leaving a large knowledge gap on GALT in young individuals.

Specifically, we sought to answer two questions:

1. Are reptilian B cell functions affected by temperature?
2. Can GALT B cells be identified in the small intestine of red-eared slider hatchlings, and if so, are they affected by nesting season or incubation temperature?

We also conducted a preliminary study to examine the ability of a single reptilian B cell to perform dual functions, antibody secretion and phagocytosis (Appendix A).

**The reptilian immune system**

The immune system of all vertebrates is composed of two branches: the innate and adaptive immune systems. The innate immune system acts as the first line of defense, mounting fast, nonspecific responses to pathogens that may or may not have had previous contact with the organism. On the other hand, the adaptive immune system is much slower and can take days to weeks to become fully activated. Unlike the innate system, the adaptive system requires previous exposure to an antigen to mount a full response. It also has “memory,” enabling a much quicker and effective specific response to a previously encountered pathogen (Parham 2014). Despite the presence of innate and adaptive systems in all jawed vertebrates, the components of each branch vary between species.
Innate immunity

With mammals and reptiles alike, the first barrier an intruding pathogen must cross is the integument. Reptiles possess a thick outer keratin layer, which acts as an effective barrier against microbes (Origgi 2007). If the invading pathogen manages to overcome the integument barrier, the innate immune system is activated.

Every multicellular organism has some form of innate immunity that is carried out by different players (Medzhitov and Janeway 1997). The innate immune system is composed of lysozymes, antimicrobial peptides, the complement pathway, and nonspecific leukocytes, all of which make up the front lines of protection. Lysozymes hydrolyze the cell wall of bacteria and have been identified in several species of lizards and turtles (Gayen et al. 1977, Ingram and Molyneux 1983, Araki et al. 1998, Thammasirirak et al. 2006). Much like defensins in mammals, reptiles also exhibit a variety of antimicrobial proteins with similar functions. A small protein found in the egg white of the loggerhead seas turtles (Caretta caretta) resembles mammalian β-defensins. This protein exhibits strong antibacterial activity against *Escherichia coli* and *Salmonella typhimurium*. It also displays strong antiviral activity against the Chandipura virus, a virus responsible for encephalitis in humans (Chattopadhyay et al. 2006).

The complement system of plasma proteins contributes to innate immunity via lysis of bacteria and opsonization. During lysis, complement proteins form membrane attack complexes, poking holes in the membranes of bacterial pathogens (Parham 2014). The American alligator (*Alligator mississippiensis*) has an extremely efficient complement system. Alligator serum has been shown to be more effective than human serum at lysing various strains of Gram-positive bacteria (Merchant et al. 2003). It was
also more effective at lysing three strains of *Naegleria* species and four *Acanthamoeba* species, all of which were unaffected by human complement proteins (Merchant *et al.* 2005).

Opsonization of a pathogen, or coating of its surface with specific proteins, enables the pathogen to be recognized and engulfed by phagocytes, such as monocytes, macrophages, and polymorphonuclear cells. These cells then proceed to engulf and destroy the pathogen through a process known by phagocytosis (Rabinovitch 1995). The actual process of phagocytosis involves actin polymerization and reorganization (Aderem and Underhill 1999). After the foreign particle binds to a specific receptor on the cell’s surface, extensions of the cell membrane surround and engulf the foreign particle. The phagosomal vesicle, containing the particle, then fuses with the lysosome forming the phagolysosome. Lysosomal enzymes then degrade the pathogen and, through a series of mechanistic pathways, portions of the degraded pathogen are displayed on the surface of the cell (Kwiatkowska and Sobota 1999, Stuart and Ezekowitz 2005). The presented antigens act as ‘flags’ to alert the rest of the immune system of an infection.

Without an innate immune response, an organism would become overrun with pathogens and quickly succumb to infection. However, while innate immunity plays a substantial role in decreasing pathogen load in the short term, for long term protection innate immunity serves little purpose due to the lack of specificity. Thus, specific adaptive immunity joins forces with innate immunity to efficiently fight off harmful invaders.
Adaptive immunity

Adaptive immunity adds to an ongoing innate immune response, thereby strengthening the overall efficiency of the immune system. The adaptive branch is divided into cell-mediated and humoral immunity. The adaptive response also has immunological memory enabling the immune system to remember a previously encountered pathogen and clear it much more efficiently than the first infection.

Cell-mediated immunity revolves around T cells, and although multiple subsets of T cells have been identified in mammals, two general subsets are present in all jawed vertebrates: cytotoxic CD8+ T cells and CD4+ T helper cells. Cytotoxic T cells initiate apoptosis in infected cells and T helper cells generally aid in activating B cells. However, T helper cells can further differentiate into TH1 or TH2 cells with different roles (Parham 2014). Although their purpose is relatively unknown, T cells have been observed in various reptiles (Burnham et al. 2005) and an early study of immune cells in the lizard Calotes versicolor, propose the presence of both a cytotoxic-like T cell and a T helper cell in reptiles (Pitchappan and Muthukkaruppan 1977). Unlike mammals, reptiles do not have lymph nodes and lack germinal centers, the site of cognate interactions between B and T lymphocytes and the activation of B cells (Hsu 1998, Parham 2014). Without germinal centers, the purpose of reptilian T helper cells becomes more elusive (Kvell et al. 2007). Where are reptilian B cells activated? Do T helper cells even participate in the activation? More studies are required to further characterize the function of T lymphocytes in reptiles.

While the cell-mediated branch of adaptive immunity focuses on T lymphocytes, the humoral branch revolves around its counterpart, B lymphocytes. Upon activation, B
cells secrete antibodies that act to neutralize pathogens, activate complement, and act as an opsonization agent for phagocytic cells (Parham 2014). Reptiles are known to possess two classes of immunoglobulins, IgM and IgY, although others may exist (Natarajan and Muthukkaruppan 1985). IgM is found in all jawed vertebrates and is the very first antibody secreted upon activation of the humoral response. It circulates in the serum in a pentameric form and is very effective at activating complement (Parham 2014). In reptiles, IgY is the most abundant isotype following infections and can be passed from mother to embryo through yolk (Warr et al. 1995). It is proposed that mammalian immunoglobulins IgG and IgE evolved from IgY (Brown 2002). In recent years, IgA-like and IgD-like immunoglobulins have been identified in lizards, however, these immunoglobulins appear to be species specific (Gambón-Deza et al. 2007, Gambón-Deza et al. 2008, Wei et al. 2009).

As with mammals, reptiles can undergo isotype switching and somatic hypermutation after antigen stimulation (Turchin and Hsu 1996), which enhances the specificity of an immunoglobulin for its respective antigen (Parham 2014). However, the humoral responses of reptiles are much slower in comparison to mammals, and antibodies often do not increase in titer or binding affinity upon a second exposure (Grey 1963, Marchalonis et al. 1969, Kanakambika and Muthukkaruppan 1972, Ingram and Molyneux 1983, Work et al. 2000, Pye et al. 2001, Origgi et al. 2001). This may be attributed to the lack of germinal centers seen in reptiles, as amphibians and fish also lack germinal centers and exhibit the same less robust response (Hsu 1998).

Due to their slow humoral responses, it has been suggested that reptiles may rely on another type of antibody response, natural antibodies. In mammals, natural antibodies
are released by B-1 cells in the absence of antigen stimulation. As a result, natural antibodies exhibit a low binding affinity and are polyreactive to evolutionary conserved components of pathogens (Ochsenbein and Zinkernagel 2000). Natural antibodies have been difficult to characterize because their functions seem to be both of the innate and adaptive nature. From an innate standpoint, natural antibodies are non-specific and activate complement, but from an adaptive standpoint natural antibodies can target antigens to the spleen in order to activate B cells (Boes et al. 1998, Ochsenbein et al. 1999, Ochsenbein and Zinkernagel 2000). Natural antibodies have been identified in alligators, water pythons, garter snakes, and red-eared slider turtles (Longenecker and Mosmann 1980, Madsen et al. 2007, Sparkman and Palacios 2009, Zimmerman et al. 2013).

Until recently, B cells were considered to be involved in antibody production and not phagocytosis (Vidard et al. 1996). However, evidence for a developmental relationship between macrophages and nonspecific, antibody secreting B-1 cells suggests that B cells may have evolved from a phagocytic predecessor (Katsura 2002, Li et al. 2006). In fact, bipotential B-macrophage progenitors have been found in mouse fetal livers and adult human bone marrow (Cumano et al. 1992, Montecino-Rodriguez et al. 2001), and various studies document both induced and non-induced reprogramming of B cells into macrophages. In 1957, Dawe and Potter observed that CD5+ lymphoblastic lymphomas spontaneously undergo morphological transitions into macrophage-like tumors after injection into mice. More recently, cancerous immature B cells in B-cell acute lymphoblastic leukemia have been reprogrammed into ordinary macrophages exhibiting macrophage-like functions, such as phagocytosis (McClellan et al. 2015). This
evolutionary relationship is further supported by the presence of immunoglobulin positive (Ig+) phagocytic cells in teleost fish, amphibians, and reptiles (Li et al. 2006, Zimmerman et al. 2010b, Muñoz et al. 2014). B-1 and B-2 cells from the liver and B-1 cells in the peritoneal cavity of mice have also exhibited phagocytic ability (Parra et al. 2012, Nakashima et al. 2012) and can act as antigen presenting cells to CD4+ T cells (Parra et al. 2012). Even human B cells have been documented to undergo phagocytosis (Zhu et al. 2015). Despite our current state of knowledge, we are only beginning to understand the functions of these novel cells. Can phagocytic B cells also secrete antibodies upon stimulation? Or do separate subsets of B cells exist: one designated for phagocytosis and one for antibody secretion? Further investigative studies may shed light to the functions and purpose of these cells.

**Gut associated lymphoid tissues**

Although the innate and adaptive branches are often described as separate entities, they must work together in order to carry out an effective immune response. The interactions between both branches occur throughout an organism in primary, secondary, and tertiary lymphoid tissues, and are best seen in secondary mucosal tissues. Most antigens enter an organism through mucosal surfaces; therefore, those surfaces must be effectively primed to handle an infection. The initial interaction ultimately determines how the immune response ensues. GALT is one of the most important secondary mucosal tissues containing more lymphocytes, than all the secondary lymphoid tissues combined (Nagler-Anderson 2001). In mammalian and avian GALT there are several regions characterized by high B cell densities. These include, Peyer’s patches (PP), the major inherited antibody producing B cell clusters,
and isolated lymphoid follicles (ILFs), much smaller antibody producing B cell clusters produced in response to inflammation and exposure to microbiota (Coico and Sunshine 2009, Eberl and Lochner 2009). While adult reptilian GALT lack PP, lymphoid aggregates, likely similar to ILFs, are expected to be a source of antibody production instead (Zimmerman et al. 2010a). To date, no study has characterized GALT in young reptiles.

**Temperature and the reptilian immune system**

As ectotherms, many physiological processes of reptiles are dependent upon environmental temperature and the immune response is no exception. While ectotherms can initiate an immune response over a broad range of temperatures, an ideal species-specific temperature exists at which these responses are at their optimum. Often times, this optimum correlates with the average body temperature of the organism (Zimmerman et al. 2010a, Butler et al. 2013). Some reports have found that immune functions exhibit an inverted U shape relationship with temperature, with the optimal immune response peaking in the middle surrounded by decreasing immune responses on either side (Angilletta 2006). Conversely, Butler et al. (2013) noted that not all thermal responses reflect the inverted U shape. Organisms not displaying an-inverted U shape thermal response curve showed a general downward trend of innate immune responses with increasing temperatures. It has been suggested that the general downward trend seen in the study by Butler et al. (2013) indicates a trade-off within the immune system itself, with the innate immune system functioning at lower temperatures and adaptive immunity kicking in at higher temperatures. Fish immune responses are a prime example supporting this
notion; innate immune functions in fish appear to dominate at lower temperatures while adaptive immunity is the primary choice at higher temperatures (Magnadóttir 2006).

Many studies have examined the effect of temperature on both the innate and adaptive immune systems in reptiles. In wild alligators, complement functions at temperatures between 5°C and 40°C, however, below 15°C and above 30°C activity was barely observed. This temperature dependent response follows an inverted U shape curve with the optimum temperatures between 15°C and 30°C (Merchant et al. 2003). Not all reptilian immune responses follow the traditional trend. In four species of turtles (*Chrysemys picta, Emydoidea blandingii, Apalone mutica, and Chelydra serpentina*) macrophages phagocytosed *E. coli* at 2°, 7°, 27°, and 37°C, with the highest phagocytic function at 27° and 37°C. Here, the innate immune response exhibited a positive relationship with temperature (Johnson et al. 1999).

The function of basophils, another white blood cell associated with innate immunity, is also reported to change with temperature (Sypek et al. 1984). Very few studies have examined the effect of temperature on reptilian adaptive responses and to date; no study has characterized the effect of temperature on reptilian B cell functions.

**Reptiles as model organisms for immune studies**

The work described here is focused on exploring B cell functions in the model reptile, the red-eared slider turtle. As long-lived organisms subjected to a wide range of environmental temperatures with well adapted immune responses, reptiles are a great model system for this study. Furthermore, the importance of immunity to other life history traits provides clearer perspective into overall reptilian fitness. From an
evolutionary perspective, reptiles are the only living ectothermic amniotes representing a
link between ectothermic anamniotic fishes and amphibians and the endothermic
amniotic birds and mammals. Therefore, a better understanding of their immune system
may uncover important clues into the evolutionary history of the immune responses in
vertebrates. Studying the reptilian immune system could also play a potential role in
conservation efforts. Reptiles play vital roles in the functioning of ecosystems. Not only
do they control their prey populations as predators, but they also serve as prey themselves
for birds and mammals. Hence, reptiles can serve as biological markers displaying the
health of their environment. Finally, study of reptilian immunity could also play a role in
the potential treatment of human diseases (Swei et al. 2011, Merchant et al. 2003,
Merchant et al. 2005).

Conclusion

Despite current efforts, many aspects of the reptilian immune system remain
unclear. Of particular interest for future research is the effect of temperature on reptilian
B cells. B cells are critical for adaptive protection from many pathogens and in reptiles,
exhibit both antibody secretion and phagocytic ability. As with all ectotherms, reptilian B
cell responses are effectively studied under the control of temperature. However, how
temperature affects the functions of reptilian B cells is unknown. It is also important to
study B cells in various immune tissues, such as GALT, where pathogen threat is
unceasing. Overall, reptilian GALT is understudied, especially in young individuals. The
answers to these and other questions will lead to a better understanding of the immune
strategy of reptiles and the evolution of the immune system, improved conservation
efforts, and possible veterinary and human disease treatments.
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CHAPTER II
THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON REPTILIAN
PERIPHERAL BLOOD B CELL FUNCTIONS

Abstract

Recent studies have identified phagocytic B cells in a variety of species, yet little is understood about how they function. Our lab was the first to identify these cells in red-eared slider turtles, *Trachemys scripta*. As ectotherms, turtles experience a wide range of temperatures that may have an effect on immune function, including B cell antibody secretion and phagocytosis. We examined the impact of environmental temperature on B cell function *in vitro* using phagocytic and ELISpot assays conducted at biologically relevant temperatures. We found a significant effect of temperature on antibody secretion, where maximal antibody secretion occurred at the 28.8°C with antibody secretion decreasing above and below this temperature. There was no effect of temperature on phagocytosis. We also compared the efficiency of phagocytosis between B cells and non-B cells. Interestingly, phagocytic B cells exhibited higher median fluorescence intensity than phagocytes, by engulfing more fluorescent beads. These experiments may shed light on the importance of environmental temperature on reptilian immune responses and the overall fitness of an organism.

Background

As ectotherms, reptiles can exhibit variations in immune responses due to changing temperature, and this temperature-induced variation could have serious
implications for the survival of an organism. Temperature not only affects host physiology, such as the immune system, but it also plays an important role on the life cycle of a pathogen. An organism’s fitness is severely compromised at a temperature where the efficiency of the pathogen is greatest compared to host defenses (Jackson and Tinsley 2002, Pounds et al. 2006, Kilburn et al. 2011, Rohr and Raffel 2010). Therefore, it is important to understand how the immune system responds to changing temperatures in order to determine whether an organism can effectively combat an infection.

Typically, ectothermic vertebrates produce immune responses over a wide range of temperatures with a species specific optimal temperature and decreased responses above and below this optimum (Zimmerman et al. 2010b, Butler et al. 2013). This inverted U shaped relationship with temperature has been noted in both innate and adaptive functions. For example, in wild alligators complement functions at temperatures between 5°C and 40°C, however, below 15°C and above 30°C activity was minimal (Merchant et al. 2003, Merchant et al. 2005). Sacchi et al. (2014) reports in vitro temperature dependent activation of T lymphocytes in common wall lizards (Podarcis muralis) in response to phytohaemagglutinin stimulation. T lymphocyte proliferation was significantly diminished at both 22°C and 38°C compared to 32°C, which was the highest level of activation (Sacchi et al. 2014). However, not all immunity-temperature interactions display inverted U shaped relationships. A previous study conducted by Butler et al. (2013), found a significant effect of temperature on the innate immune measures of agglutination and cell lysis in 11 out of 13 species, nine of which were ectotherms. While many of the reported thermal performance curves exhibited the standard inverted U shape, those that did not showed a negative linear relationship
between innate responses and temperature (Butler et al. 2013). Likewise, Johnson et al. (1999) described a positive relationship between temperature and macrophage phagocytosis in four species of turtles (Chrysemys picta, Emydidae blandingii, Apalone mutica, and Chelydra serpentina). Studies in fish suggest that despite delayed antibody secretion in response to cold temperatures, peak antibody titers are not affected upon antigen exposure (Hrubec et al. 1996). Interestingly, Grey (1963) suggested in an early study in the painted turtle (C. picta) that high temperatures may not affect the kinetics of the antibody response. Furthermore, it has been hypothesized that temperature dependent changes in immune responses may be specific, based on their classification as an innate or adaptive functions. Butler et al. (2013) proposed a trade-off between both branches of the immune system in response to increasing temperature, with innate responses having a negative relationship with temperature, and adaptive responses having a positive relationship. In support of this, Magnadóttir (2006) found that teleost fish exhibit strong complement activity at low temperatures, whereas antibody secretion is strongest at high temperatures.

Despite previous studies, thermal immune performance has been relatively understudied in reptiles (Angilletta 2009), especially aspects of the adaptive immune system. Of prime importance to adaptive immunity are B cells. B cells are widely known for their ability to secrete antibodies upon activation and are easily distinguished from other cells in the adaptive branch by the display of immunoglobulins (Ig) on their surface (Ochsenbein & Zinkernagel 2000). Previous literature reports changes in secreted antibody levels due to temperature in a number of organisms (Grey 1963, Hrubec et al. 1996, Mikkelsen, Lindenstrom, and Nielsen 2006), however, results are not consistent
therefore providing little insight on how temperature may affect B cell performance in reptiles.

Until recently, B cells were considered to be involved strictly in antibody production and not the innate mechanism of phagocytosis carried out by phagocytes such as neutrophils and macrophages/monocytes. In reptiles, heterophils are the reptilian counterpart of neutrophils (Zimmerman et al. 2013). Evidence for a developmental relationship between phagocytes and B-1 cells, suggests that the mammalian B cell may have evolved from a cell with characteristics of both cell types (Borello and Phipps 1996, Montecino-Rodriguez et al. 2001). In fact, Ig+ phagocytic cells have been found in fish, amphibians, and reptiles (Li et al. 2006, Øverland et al. 2009, Zimmerman et al. 2010b, Muñoz et al. 2014). Even more recently, B-1 and B-2 cells from the liver and B-1a and B-1b B cells from the peritoneal cavity of mice have been found to possess phagocytic ability (Nakashima et al. 2012, Parra et al. 2012) and present phagocytosed antigen to CD4+ T cells (Parra et al. 2012). Both murine studies demonstrated that particle uptake by B cells occurs in a non-B cell receptor (BCR) manner. Parra et al. (2012) suggests this uptake mechanism may induce the production of non-specific immunoglobulins by phagocytic B-1 cells. Alternatively, they suggest B-1 cells strictly acting as phagocytic antigen presenting cells, thereby avoiding potentially unnecessary costs associated with antibody secretion. It is unknown if there are separate functional B cell subsets in reptiles or if a single cell can carry out both phagocytosis and antibody secretion. Likewise, it is currently unknown how temperature may affect the phagocytic capacity of B cells in any species.
Here, we examined the effect of temperature on antibody secretion and phagocytosis of Ig+ cells in order to further understand ectothermic B cell functions. Our study organism, the red-eared slider turtle, *Trachemys scripta*, possesses B cells that can secrete antibodies and phagocytose, but at present we do not know if one cell is capable of performing both functions. Phagocytic ability and antibody secretion was measured at 20°, 25°, 30°, and 35°C. As slider activity is greatest between 25° and 30°C (Ernst and Lovich 2009), B cell functions were measured temperatures above and below the optimal activity range. We hypothesized a trade-off between B cell phagocytosis and antibody secretion in response to increasing temperatures. We predicted:

1) A negative relationship between temperature and phagocytic ability of B cells

and

2) A positive relationship between temperature and number of antibody secreting cells (Figure 1).

As a secondary focus, we also examined the phagocytic capacities of phagocytes (Ig-) in response to temperature and in comparison to Ig+ cells to further elucidate the role of phagocytic B cells in the reptilian immune system.

**Materials and Methods**

**Trapping and blood collection**

Female adult red-eared sliders (n=140) were trapped at Banner Marsh State Fish and Wildlife Area (Illinois, USA) during the months of May through August 2015. Male red-eared sliders were excluded as their trap numbers are much lower than females at this time of the year. Previous studies conducted in our lab reveal no sex differences between the number of antibody secreting cells and amount of secreted antibody (Zimmerman *et al.*
al. 2013) providing further rationale for excluding males from our sample. In the field, approximately one milliliter of blood was drawn from the caudal vein of each turtle with a coated ethylenediaminetetraacetic acid (EDTA) sterile syringe, to prevent clotting. Immediately following collection, blood samples were diluted 1:1 with cold RPMI 1640 (Life technologies), a supplemented medium that supports growth of cells. Diluted blood samples were then transported back to Illinois State University on ice. Leukocytes were separated from red blood cells via a diluted Percoll (MP Biomedicals) density gradient (nine parts Percoll, one part sterile 10X saline solution) and centrifuged at 400xg at 4°C for five minutes with no break (Harms, Keller, and Kennedy-Stoskopf 2000). Following centrifugation, the interface layer, containing white blood cells, was carefully removed via a Pasteur pipet and washed twice with RPMI at 1500xg for 5 min at 4°C. Cells were resuspended in 1.5 mL of RPMI supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin/glutamine, 0.5% 2-mercaptoethanol, and 0.5% sodium pyruvate (cPRMI) then placed on ice for cell counting.

In order to examine the effect of temperature on reptilian B cell function two immune assays were used, the phagocytic B cell assay for phagocytosis and the ELISpot assay for antibody secretion. For each immune function tested, ten individual experiments were run. Each experiment required lymphocytes from pooled blood samples of seven adult females. Following cell counting in a 1:10 dilution with trypan blue, cell suspension volume was adjusted accordingly for each of the immune assays. Isolated white blood cells were split evenly amongst four temperatures: 20°, 25°, 30°, and 35°C. The critical temperature maximum of the red-eared slider is reported between 40-42°C, so temperatures higher are not physiologically relevant and were not used (Ernst
and Lovich 2009). It is important to note that all four temperatures were run simultaneously for every experiment on separate plates with triplicates at every temperature.

**Phagocytic B cell assay**

To examine phagocytic capacity of reptilian B cells we used a slightly modified version of the method described by Zimmerman *et al.* (2010b). Following cell counting, cell suspension volume was adjusted with cRPMI to allow $6 \times 10^5$ cells/plate or $2 \times 10^5$ cells/well. The cell suspension was then plated in triplicates into four 48 well plates, one plate for each of the temperatures tested. A proportionate amount of cRPMI and 1 µm fluorescein isothiocyanate (FITC) fluorescent beads (Fluoresbrite Plain Yellow Green Microspheres, Polysciences) were added to the wells in order to maintain the same ratio described in Zimmerman *et al.* (2010b), which was $3.45 \times 10^6$ cells to $6.4 \times 10^7$ beads in 500 µl of cRPMI. Plates were then incubated at either 20°, 25°, 30°, or 35°C for three hours in 5% CO$_2$. After incubating, the cell suspensions were removed from wells and pipetted into 1.5 mL microcentrifuge tubes labeled according to temperature and triplicate number. The tubes were centrifuged, supernatant decanted, and cells resuspended in 400 µl of 1X Hanks-0.5% bovine serum albumin (BSA). To remove unphagocytosed beads, cell suspensions were layered over a cushion of 3% BSA-4.5% dextrose-phosphate buffered saline and centrifuged at 1500xg for 5 min at 4°C. Following supernatant removal, cells were washed and centrifuged as previously mentioned, resuspended in 400 µl 1X Hanks-0.5%-BSA, and then prepped for flow cytometry.
Flow Cytometry

Cells were stained with a primary pre-determined dilution of anti-turtle light chain monoclonal antibody (mAb) conjugated to biotin (HL673, University of Florida Hybridoma Facility) and incubated for 15 minutes on ice. Samples also included 10% normal rat serum to prevent non-specific binding. After incubation, cells were washed with 1X Hanks-0.5%-BSA and centrifuged as before. The supernatant was decanted and the remaining cells with bound antibodies were detected with a secondary stain. Streptavidin-spectral red (Southern Biotech) diluted 1:100 in 1X Hanks-0.5%-BSA was added to each sample and incubated in the dark on ice for 15 minutes. Cells were washed, centrifuged as before, and resuspended in 400 µl 1X Hanks-0.5%-BSA. Cells were analyzed immediately on a Becton Dickinson FACSCalibur flow cytometer. A minimum of 20,000 events were collected and data was analyzed using CellQuest Pro software (BD Biosciences). Analyzed samples also included non-phagocytic unstained cells, non-phagocytic stained cells, and free bead samples to ensure accurate gating. An example of flow cytometry gating is presented Figure 2. CellQuest Pro software was also used to analyze MFI data.

ELISpot

The ELISpot assay was used to measure the number of antibody secreting cells. MultiScreen-IP ELISpot (EMD Millipore) plates were coated with 100µL/well of 20µg/mL unlabeled anti-turtle light chain (HL673; University of Florida Hybridoma Facility) diluted in sterile 1X PBS and incubated overnight at 4°C. Following incubation, the capture antibody solution was decanted via a multichannel pipette and the plate was washed twice with sterile 1X PBS. Plates were then blocked with 200µl of cRPMI for 1
hour at 37°C. Following incubation, the blocking medium was decanted and leukocytes were plated at $5 \times 10^4$ cells/well and then serially diluted. Initial experiments concluded $5 \times 10^4$ cells/well as the optimum number of plated cells in order to prevent well membrane overcrowding. Previous work in our lab included an additional volume of cRPMI supplemented with 40µg/ml lipopolysaccharide (LPS) in order to stimulate B cell proliferation and spontaneous antibody secretion. This addition was excluded in our protocol because initial experiments showed no difference in the number of antibody secreting cells between plates supplemented with or without LPS. Plates were incubated for three days at the previously mentioned temperatures ($20°, 25°, 30°, \text{ and } 35°C$) in 5% CO2. Following the three day incubation, plated cells were decanted and wells were washed three times for three minutes each with 200µl 1x PBS-1%BSA-0.05% Tween 20 (wash buffer). Next, a 100 µl pre-determined dilution of anti-turtle light chain conjugated to biotin in the wash buffer was added to the wells and left to incubate for two hours at room temperature. After washing as previously described, 100 µl of streptavidin-horse radish peroxidase (SA-HRP) diluted 1:1000 in 1x PBS was added and left to incubate for one hour at room temperature. Following another series of washes, 3-amino-9-ethylcarbazole (AEC) substrate solution was used to develop the wells. AEC substrate detects antibody secreting cells by leaving brownish red spots on the well membrane where adhering antibodies remain, with a spot representing a single cell. Number of spots was determined using ImageJ software, with the number of spots signifying the number of antibody secreting cells per $5 \times 10^4$ cells.
Statistical Analysis

All statistical analyses were performed in R statistical software (version 3.1.2). B cell phagocytosis and ELISpot assay analyses included temperature, B cell function, non-B cell (Ig-) phagocytic ability as fixed effects. Median fluorescence intensity (MFI) data included cell type, MFI values, temperature, and the interaction between the two as fixed effects. For all MFI analyses, the assay number was included as a random effect. All data is presented as mean (± SE). For all analyses, p-values less than 0.05 were considered significant.

To examine the effect of temperature on reptilian B cell functions we performed a fixed effects ANOVA. Characteristics analyzed included temperature and percent of phagocytic B cells/total B cells for phagocytic ability or temperature and mean number of spots per $5 \times 10^4$ cells for the number of antibody secreting cells. Following ANOVA analysis on the ELISpot data, post-hoc pairwise comparisons with false discovery rate corrections were performed where p-values less than 0.05 were considered significant. Models were also were fitted with temperature incorporated as a continuous predictor for the ELISpot data. The best fit model was a quadratic polynomial $f(x) = -86.70 + 20.29x - 0.18x^2$ with 95% confidence intervals fitted to the curve. Additional linear or non-linear models that included temperature as a continuous variable, analogous to the ELISpot models, were performed on significant phagocytic data.

The effect of temperature on Ig- phagocytic cells was analyzed via a fixed effects ANOVA, as used to interpret the B cell data. Ig- phagocytic cells were expressed as a percentage of the total cell population, excluding known B cells. Further linear or non-
linear models that included temperature as a continuous variable, analogous to the ELISpot models, were performed on significant phagocytic data.

Median fluorescence intensity was analyzed via a mixed effects ANOVA, for the effects of cell type (Ig+/− phagocytic cell), temperature, and the interaction between the two. Due the structural nature of the experiment, assay was included as a random effect.

**Results**

*Temperature did not significantly affect B cell phagocytosis but did affect the number of antibody secreting cells.*

When subjected to different temperatures, percentages of phagocytic Ig+ cells were not significantly impacted, n=10 (F_{3,36}=0.67, p=0.577) (Figure 3). Additionally, no significant relationships were found that included temperature as a continuous variable (p>0.3). Temperature did, however, significantly affect the number of antibody secreting cells measured by an ELISpot assay, n=10 (F_{3,36}=5.21, p=0.004) (Figure 4a). The number of antibody secreting cells significantly increased between 20°C-25°C and decreased between 30°C-35°C, respectively (pairwise: p<0.05). The relationship between temperature and the number of antibody secreting cells was further examined by fitting a quadratic polynomial model to the curve (F_{2,37}= 7.44, p= 0.0019). The maximum was at 28.82°C, indicating the optimal temperature for antibody secretion (Figure 4b).

*Temperature did not affect the percentage of other Ig- phagocytic cells.*

Temperature did not significantly affect the percentages of phagocytosing Ig- cells, n=10 (F_{3,36}=1.28, p=0.296) (Figure 5). Furthermore, no significant relationships were found that included temperature as a continuous variable (p>0.155).

*Ig+ phagocytic cells are more efficient at phagocytosing than Ig- phagocytic cells.*
Median fluorescence intensity (MFI) was used to quantify shifts in FITC fluorescence in both Ig+ and Ig- phagocytic cells. The higher the MFI value, the more beads engulfed by either phagocytosing cell population. Cell type had a significant effect on gated lymphocyte MFI, with Ig+ phagocytic cells exhibiting a higher MFI than Ig- phagocytic cells, n=10 (F1,63=1400, p<0.001) (Figure 6). Mean MFI (± SE) values for Ig+ and Ig- phagocytic cells were 6585±700 and 2589±409, respectively. Figure 7 shows MFI histograms for Ig+ and Ig- phagocytic cells. Roughly, 72.5% of Ig+ phagocytic cells exhibit a minimum of 10^4 FITC fluorescence compared to 7.65% of Ig- phagocytic cells. Temperature did not have a significant impact on phagocyte or phagocytic B cell MFI, n=10 (F3,63=0.679, p=0.568) (Figure 6). Finally, there was no significant interaction between temperature and cell type on MFI, n=10 (F3,63=0.528, p=0.665).

**Discussion**

Through our investigation, we successfully examined innate and adaptive reptilian B cell functions in response to biologically relevant temperatures. Moreover, we are the first to measure the efficiency of B cell phagocytosis compared to phagocytes in reptiles.

Contrary to our predictions, temperature did not have a significant effect on the percentage of phagocytic B cells. Phagocytic B cell numbers did not increase or decrease in response to temperature, and cells phagocytosed beads equally well at all temperatures tested. This is rather surprising as there are multiple accounts in previous literature that report a significant effect of temperature on vertebrate innate immune responses (Sypek et al. 1984, Johnson et al. 1999, Merchant et al. 2003, Merchant et al. 2005, Magnadóttir 2006, Butler et al. 2013). On the other hand, an early study observing inflammation processes in higher vertebrates found that the initiation of phagocytosis in fish and
reptiles is relatively temperature insensitive compared to other innate responses (Montali 1988). Previously, we have reported that lymphocytes, both T and B cells, are the most common leukocyte in the red-eared slider, comprising 38-45% of all leukocytes. Here, we report, on average (± SE), the percent of phagocytic B cells in peripheral blood leukocytes ranges from 21-25% of all B cells. The percentage we report for the red eared slider is similar to that reported for Atlantic cod, Gadus morhua (19%) and Xenopus laevis (14%) (Li et al. 2006; Øverland et al. 2010). However, substantially higher and lower percentages have been noted for other species (Li et al. 2006, Øverland et al. 2010, Parra et al. 2012). In mice, phagocytic B cell percentages vary depending on anatomical location. Parra et al. (2012) reports 1.6% of B cells in peripheral blood leukocytes were phagocytic compared to 11-14% of B-1 cells from the peritoneal cavity. Similarly, in Atlantic cod, head-kidney lymphoid organ (HK) contained a higher percentage of phagocytic B cells than the peripheral blood (PB), at 31% and 19% respectively (Øverland et al. 2010). It is currently unknown if anatomic locational differences for phagocytic B cells exist in the red eared slider.

We did find a significant effect of temperature on the number of antibody secreting cells. We observed spot number significantly increase between 20°-25°C and decrease between 30°-35°C, respectively, with a maximum number of antibody secreting cells predicted at 28.8°C. Although our adaptive measure of B cell ability did not exhibit the predicted positive linear relationship with temperature, we did observe an inverted U shape thermal performance curve. Many immune functions demonstrate this relationship with temperature (Angilletta 2006) therefore, our results are not surprising. We observed a rather low percentage of antibody secreting cells given the number of plated leukocytes
(5x10^4 cells) and previously reported percentages of slider lymphocytes (38-45% of leukocytes). Less than 1% of plated cells secreted antibodies at each of the tested temperatures. This was surprising as high temperatures have been reported to increase cell proliferation by shortening the duration of metaphase, thus, speeding up mitosis (DuPraw, 1970). Additionally, previous studies, in both mammalian and reptilian species, report a positive relationship between cell proliferation and temperature (Gelfant 1975, Radmilovich 2003). However, these studies were performed in vivo, while we conducted in vitro experiments. It is important to note plated cells were not stimulated and did not received any B cell mitogen since initial experiments indicated no difference in the number of antibody secreted cells when incubated with or without LPS supplement. Hence, the observed antibody secreting cells are more than likely secreting natural antibodies (Zimmerman et al. 2013). In the absence of antigen, low levels of natural antibodies are produced. In mammals, natural antibodies are produced by B-1 cells and are polyreactive to evolutionary conserved components of pathogens thereby triggering both innate and adaptive immune responses (Ochsenbein and Zinkernagel 2000, Baumgarth et al. 2005, Yang et al. 2007). In fact, natural antibodies have been suggested to be an important immune defense in reptiles (Zimmerman et al. 2010a, Ujarvi and Madsen 2011). Future studies may try incubating cells with levan or dextran, other known B cell mitogens, to increase proliferation and antibody secretion (Ivanyi and Lehner 1974). Future work may also examine slider antibody secretion in response to pathogen prevalence. A previous study in our population has found a high frequency of Salmonella in adult turtles reporting pathogen prevalence increases as temperature
increases (Holgersson 2009). With the addition of a pathogenic stimulus, antibody secretion and temperature may exhibit a different relationship.

The secondary focus of this study was to evaluate the effect of temperature on traditional reptilian phagocytes and compare their phagocytic capacities to B cells. Similar to B cells, we did not observe an effect of temperature on the percentage of Ig-phagocytic cells. Phagocytes engulfed fluorescent beads equally well at all temperatures tested. Previous studies have reported mixed results. Johnson et al. (1999) described a positive relationship between temperature and macrophage phagocytosis, however it has also been reported that phagocytosis is relatively insensitive to temperature changes (Montali 1988). Likewise, in channel catfish (Ictalurus punctatus), a temperature decrease from 18° to 10°C resulted in a decline in the number of lymphocytes in the anterior kidney but not the number of neutrophils, suggesting phagocytes are more resistant to low temperatures than other immune cells (Ainsworth et al. 1991). We have previously reported that heterophils and monocytes compose 44-47% of all peripheral blood leukocytes in sliders (Zimmerman et al. 2013). However, we found that only 1-5% of our Ig- cells were actively engulfing beads. While it appears that only a small percentage of total slider peripheral blood heterophils and monocytes phagocytose, it is important to understand the inflammatory processes that initiate phagocytosis in phagocytes. Macrophages sense an infection through microbial molecules and host derived inflammatory mediators, such immunoglobulin G or complement fragments (Kwiatkowska and Sobota 1999, Torraca et al. 2014). Through scavenger, complement, and Toll-like receptors phagocytes recognize harmful pathogens and then proceed to engulf them (Elomaa et al. 1995). It is likely that our fluorescent beads are not
stimulating a strong enough inflammatory response, due to their lack of microbial particles, to activate phagocytes. Also, studies have reported phagocytosis can be influenced by particles to be phagocytosed, cell to bead/particle ratio, and incubation time (Thuvander et al. 1987, Li et al. 2006; Parra et al. 2012). Perhaps a more accurate percentage might be achieved by using fluorescent bacteria, in place of latex beads, or performing in vivo studies.

Here, we report for the first time a comparison of phagocytic efficiency between B cells and phagocytes in reptiles. We found that Ig+ cells can engulf a significantly larger number of beads than Ig- phagocytes in vitro. MFI histograms show 72.5% of Ig+FITC+ cells exhibiting a minimum of $10^4$ FITC fluorescence compared to 7.65% of Ig-FITC+ cells (Figure 7). These results suggest that reptilian phagocytic B cells may contribute significantly to the clearing of microbes through phagocytosis. Studies in fish have shown similar findings, though phagocytic capacity is dependent upon anatomical location. In cod, although the phagocytic ability of B cells was lower than neutrophils in both HKL and PBL, phagocytic capacity, beads engulfed, was much higher than neutrophils in both locations (Øverland et al. 2010). Despite such findings, studies in mice report that macrophages and neutrophils were able to uptake many more particles than phagocytic B cells (Parra et al. 2012, Nakashima et al. 2012). Moreover, Parra et al. (2012) reports macrophages killed internalized bacteria significantly faster than phagocytic B cells. Therefore, it would be conceivable that the main role of phagocytic B cells may be the clearing of microbes in lower jawed vertebrates and not mammals. In mammals, Parra et al. (2012) proposes the main role of phagocytic B cells is to present of low amounts of particulate antigen to T cells. Temperature did not have a significant
impact on phagocytic capacity of Ig- or Ig+ phagocytic cells. This is not surprising as temperature did not affect phagocytic ability in either population. Finally, we found no significant interaction between temperature and cell type indicating a lack of temperature dependent phagocytic dominance between the two populations.

In conclusion, our slider B cell-temperature results suggest that a trade-off between phagocytosis and antibody secretion in response to temperature does not exist. This suggests that B cell subsets, dedicated to either phagocytosis or antibody secretion, may exist in the red-eared slider that respond to temperature differently from one another. This notion of separate B cell subsets has been suggested about phagocytic peritoneal cavity B-1 cells in mice (Parra et al. 2012). We briefly explore B cell subsets in the Appendix. Our results suggest that phagocytosis, in both Ig+ and Ig- phagocytes, in the red eared slider is unaffected by temperatures experienced just outside and within their normal range. In contrast, temperature had a significant effect on antibody secretion, exhibiting an inverted U shaped relationship. Further work is needed with other immune responses to see if these trends extend to all innate and adaptive immunity. Our findings suggest innate immunity in sliders is not regulated by temperature, unlike adaptive immunity. This may be a result of pathogen prevalence in the slider, as one would assume more specific defenses are required when pathogen load increases. Pathogen studies in the slider may shed light the results reported here. Examining immune responses at extreme low temperatures (10°C) will provide also better picture of how slider immune functions are regulated as sliders hibernate during the winter months under the water. It is important to note, results could change drastically when studying thermal immune performance in vivo. Finally, we found that Ig+ cells have a greater phagocytic
capacity than Ig- cells, which could suggest a primary role of microbe clearance for slider phagocytic B cells.
References


receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell 80: 603-609.


Common wall lizards (Podarcis muralis) in response to PHA stimulation. Acta Herpetologica 9:


Figure 1. Schematic representation of predicted temperature effects on innate and adaptive reptilian B cell functions. We predict a decreased phagocytic ability and increased antibody secretion with increasing temperatures.
Figure 2. Example of flow cytometry phagocytosis analysis. Phagocytic cells were detected via positive FITC staining. B cells were detected via primary staining with HL673-biotin followed by secondary staining with SA-PE. Unstained population (a,d), free beads (b,e), phagocytic sample gated on lymphocytes (c,f).
Figure 3. Temperature did not significantly affect percentages of phagocytic B cells. Temperature did not impact percentages of phagocytic Ig+ cells out of total Ig+ cells. Following the phagocytic assay, samples were analyzed by flow cytometry and all Ig+ cells gated. Percentage of those cells also showing FITC fluorescence was calculated. n=10, ANOVA: F_{3,36}=0.67, p=0.577.
Figure 4. Temperature significantly affected antibody secretion in reptilian B cells.
(a) Temperature significantly impacted the number of antibody secreting cells as measured by ELISpot assay. One spot indicates a single antibody secreting cell with the mean number of spots per $5 \times 10^4$ cells, n=10. Spots increased significantly from 20-25°C and decreased from 30-35°C. ANOVA: $F_{3,36}=5.21$, $p=0.004$, pairwise*: $p<0.05$. (b) Curve fit and confidence intervals from panel a with a maximum number of spots predicted at 28.82°C, the optimal temperature for antibody secretion $f(x) = -86.70 + 20.29x - 0.18x^2$; $F_{2,37}=7.44$, $p=0.0019$. 
Figure 5. Temperature did not significantly affect the percentage of Ig-phagocytic cells. Temperature did not impact percentages of phagocytes (Ig-FITC+) out of total gated lymphocytes excluding known B cells, n=10 ANOVA: $F_{3,36}=1.28$, $p=0.296$. 

![Graph showing temperature vs. percent of phagocytic Ig-cells excluding known B cells](image-url)
Figure 6. Phagocytic B cells are more efficient at phagocytosis than phagocytes. Median fluorescent intensity, or the amount of fluorescent beads engulfed, was significantly affected by cell type. Ig+ cells exhibit a greater MFI than Ig- phagocytic cells, n=10 ANOVA: F_{1,63}=1400, p<0.001. Mean MFI for Ig+ and Ig- phagocytic cells were 6585±700 and 2589±409, respectively. MFI was not significantly impacted by temperature in Ig- or Ig+ cells, n=10 ANOVA: F_{3,36}=0.679, p=0.568. Grey and white bars represent phagocytosing Ig+ and Ig- cells, respectively.
Figure 7. Ig+ cells are more efficient at phagocytosing fluorescent beads than Ig- phagocytic cells. MFI histograms show (a) more than 70% of Ig+ phagocytic cells engulf a considerable number of FITC fluorescent beads compared to less than 10% of Ig- phagocytic cells (b).
CHAPTER III

THE EFFECT OF NESTING SEASON AND INCUBATION TEMPERATURE ON B CELL DISTRIBUTION IN THE GUT-ASSOCIATED LYMPHOID TISSUE OF RED EARED SLIDER TURTLE (TRACHEMYS SCRIPTA ELEGANS) HATCHLINGS

Abstract

As with the mammalian immune system, gut-associated lymphoid tissues (GALT) in reptiles are an important mechanism in controlling pathogens. Little is known about the development and composition of GALT in reptiles. Over the course of the 2014 and 2015 summers, we examined the numbers and distribution of B cells in GALT located in the small intestine of red eared slider turtle (Trachemys scripta) hatchlings. We were also interested in the effects of nesting season variation and incubation temperature on B cells in GALT. We hypothesized that differences in B cell distribution in GALT would be observed for hatchlings from first clutches relative to second clutches, predicting late season hatchlings would have more B cells. Additionally, we hypothesized incubation temperature would affect B cell density, predicting individuals incubated at high temperatures would exhibit more B cells. In the 2014 study, we found that early season hatchlings had significantly more B cell clusters, or large grouping of B cells. Additionally, we report that individuals incubated at low temperatures exhibited more B cell clusters than individuals incubated at higher temperatures. However, the B cell GALT staining could not be replicated for hatchling intestines from the 2015 nesting
season, where no B cells were detected in any tissue stained. While conclusions can be drawn from the initial study results, lack of positive staining in 2015 demands further investigative work in GALT of young reptiles.

**Background**

Mucosa-associated lymphoid tissues (MALT) play a vital role in immune defense throughout ontogeny. From a defensive standpoint, a majority of antigens enter the body via mucosal surfaces and the microenvironment of these mucosal surfaces ultimately determines the subsequent immune response. The gut is a particularly vulnerable mucosal surface as it is under constant antigenic challenge from food, commensal bacterial flora, and harmful pathogens (MacDonald 2003). Consequently, the gastrointestinal track has been equipped with gut-associated lymphoid tissues (GALT) to combat the endless amount of pathogenic threats. In fact, GALT is the largest subdivision of MALT (Brandtzaeg *et al.* 2008) and contains more lymphocytes than all of the secondary lymphoid tissues combined (Nagler-Anderson 2001).

Gut-associated lymphoid tissues are characterized by their high B cell densities and include mesenteric lymph nodes, Peyer’s patches (PP), and isolated lymphoid follicles (ILFs) (Macdonald 2003). Mesenteric lymph nodes and PP have been described as programmed lymphoid tissues meaning their development takes place in the sterile fetal environment (Eberl and Lochner 2009). Despite their early development, these programmed tissues are dependent upon antigenic stimulation, and in germ-free mice which lack normal microbiota in the gut, the further development and function of intestinal lymphoid tissues is impaired (reviewed in Round and Sarkis 2009). Additionally, these tissues lack germinal centers until shortly after birth when exposed to
exogenous antigens (Brandtzaeg et al. 2008). Unlike mesenteric lymph nodes, PP lack afferent lymphatics; thus antigens are sampled directly from mucosal surfaces via dendritic and M cells (Drayton et al. 2006, Nagler-Anderson 2001). Likewise, ILFs also lack afferent lymphatics but unlike the programmed tissues, they are inducible, meaning they form in response to microbiota and inflammation (Fagarasan et al. 2002, Eberl and Lochner 2009). Isolated lymphoid follicles are much smaller than PP and play a major role in intestinal homeostasis by controlling commensal bacteria. In fact, Bouskra et al. (2008) demonstrated in the absence of ILFs commensal flora expanded more than 10-fold in mice. Eberl and Lochner (2009) describe a negative feedback loop between ILFs and bacterial flora where high bacterial numbers in the intestine, induce more ILFs. The more ILFs induced, the lower the bacterial numbers as their presence initiates an immune response within the ILFs.

All jawed vertebrates exhibit variations of GALT. Peyer’s patches (Cornes 1965, Befus et al. 1980, Reynolds and Morris 1983, Bye et al. 1984) and ILFs (Keren et al. 1978, Moghaddami et al. 1998, Hamada et al. 2002) have been described in a number of mammalian and avian species. In contrast, the gut of ectotherms, such as fish and adult reptiles, lack lymph nodes and PP (Hart et al. 1988, Zimmerman et al. 2009). Instead, ILF-like structures, called lymphoid aggregates, are expected to be a source of antibody production and have been documented in fish (Hart et al. 1988, Tacchi et al. 2015), amphibians (Ardavin et al. 1982), and reptiles (Borysenko and Cooper 1972, Zapata and Solas 1979, Solas and Zapata 1980). Despite previous work, little is understood about GALT composition and function in ectotherms. In addition, lower vertebrate GALT
studies have mainly focused on adults, leaving a large knowledge gap on the influence of GALT in young individuals.

Young organisms primarily depend on passive immunity as they are initially immunologically compromised. However, previous studies have shown that maternally derived antibodies do not persist for long periods of time in offspring. In fish and birds, maternally derived antibodies can no longer be detected in offspring plasma after 10 days (van Loon et al. 1981) and two weeks, respectively (Patterson et al. 1962, Liu and Higgins 1990). Additionally, offspring cannot produce antibodies at the same level as adults until 5 - 8 months later for fish (van Loon et al. 1981), and 6 weeks - 6 months later for birds (Brambell 1970, Rose and Orlans 1981). During this transition period from passive immunity to endogenous antibody production, GALT may play a substantial role in immune defense by increasing survival and fitness in young vertebrates. To date, no study has characterized GALT in young reptiles.

Here, for the first time, we investigated B cells in small intestine GALT of red eared slider turtle (Trachemys scripta) hatchlings over the course of the 2014 and 2015 summers. Females in our study population produce two clutches during the course of a single nesting season. Early season clutches are laid toward the end of May - early June, while late season clutches are laid near the end of June. Previous studies in our lab have shown that maternal allocation of resources to freshwater turtle eggs varies intra-seasonally. Specifically, we have shown that late season clutches contain approximately 10-fold higher concentrations of progesterone and estradiol than early season clutches (Paitz and Bowden 2009, Paitz and Bowden 2011). This is important to note because estrogens have generally been attributed to enhanced immunity. In adults, estrogens have
been reported to increase macrophage phagocytosis and natural killer cell activity (Klein 2004). In mammals, estrogens can shift the T helper response from a TH1 to a TH2 response, thereby, increasing humoral immunity (Salem 2004). Likewise, estrogens have been shown to increase antibody production with and without increasing the number plasma cells (Ahmed et al. 1999, Fu et al. 2011). Little is understood regarding the effects of estrogen on immune responses in young organisms. Our lab has also reported that lipid and protein levels differ between early and late clutches in the eggs (Harms et al. 2005). For these reasons, we sought to determine if B cell distribution within GALT varied between early and late season slider hatchlings. We hypothesized B cell distribution in the GALT would differ based on nesting season. We predicted individuals derived from late season eggs that have higher levels of estrogens would have more B cells in their GALT than hatchlings derived from early season eggs.

Among many other fitness related traits, immune responses in oviparous species are known to be influenced by incubation temperature (Booth 2006). A study in young wood ducks (Aix sponsa) demonstrated the effect of incubation temperature on T cell responses, via a phytohemagglutinin (PHA) injection, and antibody secretion. They found that ducklings incubated at 35°C exhibited less swelling and lower antibody responses to sheep red blood cells than ducklings incubated at 35.9°C and 37.0°C (Durant et al. 2011). In reptiles, a recent study reported that hatchling soft shelled turtles (Pelodiscus sinensis) incubated at 24°C had lower cumulative mortalities than individuals incubated at 28°C and 32°C, following exposure to the pathogen Aeromonas hydrophilia. Additionally, hatchlings incubated at 24°C had higher IgM, IgD, and CD3γ expression than individuals from the higher incubation temperatures (Dang, Zhang, and Du 2015). To our knowledge,
the effect of incubation temperature on B cells in the GALT of young reptiles has yet to be described. Thus, we investigated a possible effect of incubation temperature on B cells in GALT in slider hatchlings. *We hypothesized B cell distribution in the GALT would be affected by incubation temperature. Specifically, we predicted that hatchlings incubated at higher temperatures would have more B cells in GALT than hatchlings incubated at higher temperatures.*

As a secondary focus, we measured the effect of nesting season on maternal allocation of antibodies in yolk and explored if maternally derived antibodies influenced B cell distribution within GALT. Following birth, neonatal vertebrates have limited ability to produce endogenous antibodies. Hence, maternally derived antibodies provide the primary form of immune defense until the young are able to establish their own active immunity (Grindstaff *et al.* 2003). While the mechanism is not fully understood, maternal antibody transmission has been reported to affect B cell numbers. Previous literature has described the selection and expansion of B and T cells following neonatal treatment with monoclonal antibodies (Cerny *et al.* 1983, Martinez-*A et al.* 1985). Furthermore, Malanchère *et al.* (1997) reported that progeny of B cell deficient mice that cannot transmit antibodies exhibited two-to-threefold lower numbers of bone marrow pre-B and B cells and splenic B cells than progeny from control mice. In reptiles, maternal antibodies are reported to persist up to a year in the plasma of offspring (Schumacher *et al.* 1998); however, it is unknown when offspring start to produce their own antibodies. Avian active immunity is reported to develop between 6 weeks to 6 months post hatch (Grindstaff *et al.* 2003). Therefore, it is likely that reptile active immunity does not develop until at least six months post-hatch due to a much slower metabolism.
Materials and Methods

Egg Collection and Incubation

Red eared slider turtle clutches were collected during the summer 2014 nesting season at Banner Marsh State Fish and Wildlife Area (Illinois, USA) via excavation of freshly laid nests or from trapped gravid females. All trap caught gravid females were brought back to lab and induced to lay eggs via an oxytocin injection (Ernst & Lovich 2009). Five early and five late season clutches (n=90 eggs) were collected. All eggs were weighed to the nearest 0.01 gram and marked to indicate clutch and individual number. Prior to incubation, one egg from each clutch was removed and immediately frozen at -20°C for yolk antibody (Ab) analysis via enzyme-linked immunosorbent assay (ELISA). Eggs were collected under Illinois Department of Natural Resources permit NH14.2084.

Eggs within each clutch were then randomly assigned to one of three fluctuating temperature incubation regimes: 1) 26.5 ± 2 °C sinusoidal fluctuations every 24 hours (low treatment) (n=30), 2) 27.1 ± 2 °C sinusoidal fluctuations every 24 hours (medium treatment) (n=30), or 3) 27.7 ± 2 °C sinusoidal fluctuations every 24 hours (high treatment) (n=30). These incubation treatments were chosen because they are are suitable for development and within the range of temperatures experienced in natural nests of our study population (Bowden, Carter, and Paitz 2014). Eggs were incubated in vermiculite filled plastic boxes and monitored for moisture conditions. To minimize the effects of temperature inconsistencies within the incubators, box position was rotated every five days.
Following hatching, hatchlings were individually housed and maintained at constant room temperature and dark light conditions. Hatchling mass and carapace lengths were recorded to the nearest 0.01g and 0.01mm, respectively. All hatchlings underwent behavioral testing prior to being sacrificed at 6 weeks post-hatch. Hatchlings were euthanized via an intrapleuroperitoneal injection of pentobarbital sodium solution (60 mg/kg). Within 15 minutes post-mortem hatchling intestinal tissue (n=90) was harvested immediately and placed in cold 1x phosphate buffer saline (PBS). Hatchlings were immediately sexed following removal of intestinal tissue. Due to incubation regimes, all hatchlings from the 2014 nesting season were male.

During the summer 2015 nesting season, 10 early and 10 late nesting season clutches were collected. Eggs were weighed and marked, and prior to incubation, one egg from each clutch frozen at -20°C for yolk Ab analysis via ELISA. Eggs were collected under Illinois Department of Natural Resources permit NH15.2084.

A related study randomly assigned the total number of eggs collected that nesting season to one of three fluctuating temperature incubation regimes: 1) 28 ± 3 °C sinusoidal fluctuations every 24 hours (low treatment), 2) 28.5 ± 3 °C sinusoidal fluctuations every 24 hours (medium treatment), or 3) 29 ± 3 °C sinusoidal fluctuations every 24 hours (high treatment). For the 2015 GALT study, eggs were incubated at the medium treatment regime (n=60) in order to further investigate results from the previous year. Eggs were incubated in vermiculite filled plastic boxes that were rotated within the incubators every five days.
After hatching, hatchling mass and carapace length was recorded. As in the previous year, all hatchlings underwent behavioral testing prior to being sacrificed at 7 weeks post-hatch. Unlike the previous year, intestinal tissue was only harvested from 12 out of the 60 designated hatchlings for the study due to complications with immunohistochemistry.

This work was approved by the Illinois State University Institutional Animal Care and Use Committee (Protocols 08-2014 and 08-2015).

**Immunohistochemistry**

Intestine sections from 2014 and 2015 hatchlings were prepared and stained according an adapted whole mount protocol previously used on adult mice (McDonald and Newberry 2007). After dissecting intestines from turtle hatchlings, tissues were immediately flushed with cold 1x PBS, pH 7.4, to remove fecal matter. Due to the small size of the hatchling intestine, a butterfly needle attached to a syringe was used to purge the tissue. Next, a two centimeter section of small intestinal tissue was isolated near the ileocecal junction and cut open longitudinally under a dissecting microscope. Cut sections were pinned into humidity chambers lumen side up and kept hydrated with room temperature 1x PBS. Sections were washed three times for five minutes each while shaking at 600 rpm in warm (37°C) Hanks balanced salt solution (HBSS) supplemented with 5mM ethylenediaminetetraacetic acid (EDTA) to remove surface epithelial layer. Intestinal lumen surface was then further washed with HBSS-EDTA using a 30cc syringe with a 23 gauge needle to ensure complete removal of epithelial cell layer. Intestinal sections were shaken for 10 minutes in cold 1x PBS and then 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 endogenous peroxidases. Intestines were 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 1:1000 dilution of anti-turtle light chain monoclonal antibody (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 – horse radish peroxidase (SA-HRP) (BD Biosciences) diluted 1:1000 for one hour. After another series of three washes, sections were exposed to a diaminobenzidine peroxidase substrate (Metal Enhanced DAB Substrate Kit, ThermoFisher Scientific) for 15 minutes. Intestinal sections were then rinsed twice with dH2O to stop the enzymatic reaction and returned to 1x PBS. Sections were viewed under the dissecting scope at 60X for brownish/black spots and images were taken. Images were captured using a Leica Camera with associated software. Three images were taken for each section, a promixal, middle, and distal image. Spots were methodically counted using ImageJ software (Figure 8). ImageJ software was used to overlay a grid on top of small intestine images. Grid squares were assigned numbers 1-20 and a random number generator was used to determine which square would represent the overall spot count for a single image. If the random number generate fell on a background square, a new number was generated. Each sample consisted of the average of spots for each of the respectively three images.

**Yolk Enzyme-linked immunosorbent assay**

Egg yolks (n=40, two from each 2014 early and late clutch) were isolated and prepared using a protocol adapted from Jen Grindstaff (2013). Briefly, frozen eggs were
thawed and albumin was separated from yolks. Yolks were then weighed and the mass recorded. Using a small spatula, egg yolks were homogenized and then diluted 1:10 with PBS-0.1% Tween 20. Diluted yolk samples were then vigorously vortexed and centrifuged to remove soluble protein from yolk mass. Supernatant was collected for ELISA analysis.

ELISA 96-well plates were coated overnight at 4°C with 20 µg/ml of HL673 in 1x PBS, 100µl/ well. Following incubation, plates were washed three times for three minutes each with ELISA buffer, 1X PBS-1% BSA-0.05% Tween 20. Yolk supernatant samples, 100 µL/well, were then added and serially two fold diluted down the plate. A positive and negative control of turtle plasma and ELISA buffer, respectively, were included with the yolk supernatant samples. Following a 1 hour incubation at room temperature, wells were washed as previously described and a 1:500 dilution of HL673 mAb conjugated to biotin in buffer was added and allowed to sit at room temperature for an hour. Following another series of three washes, a 1:1000 dilution of SA-HRP in buffer was added and incubated for an hour. Finally, wells were washed twice as before with one extra wash of dH2O. A chromogenic substrate solution with 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (ThermoFisher Scientific) was added and let sit at room temperature for ten minutes. Plates were read on a microplate reader (Biotek) at 405 nm and optical density (OD) values outputted.

**Statistical Analysis**

All statistical analyses were performed in R statistical software (version 3.1.2); specifically, the lme4 package was used for general and generalized linear and mixed effect models. Models were fitted with fixed effects, including season and incubation
treatment, and the interaction between the two. Clutch identity was included as a random effect. Diurnal temperature fluctuations have been reported to have no effect on immune responses (Paitz et al. 2010), thus mean incubation temperature values were used for analysis.

Due to variation of B cell clusters among early and late season individuals, the 2014 data set was analyzed in two ways:

1. The effect of incubation temperature on the number of B cell clusters was only examined in early season individuals.

2. The effect of nesting season and incubation temperature was examined on the presence or absence of B cell clusters, not the counted numerical value.

To evaluate the effect of incubation temperature on the number of B cell clusters in early season clutches, a generalized linear model with a quasi-Poisson distribution was fitted to the data with number of B cell clusters as a continuous trait. The quasi-Poisson distribution was applied to the model to account for over-dispersion, indicating that greater variability in the data set existed than expected for the generalized linear model.

Due to an unbalanced design and lack of replication in some clutch and treatment combinations, clutch mean values were used for analysis instead of individual hatchling values. Post-hoc pairwise comparisons between differing temperature incubation groups were further analyzed via Tukey’s Honest Significant Difference (HSD) test. P-values less than 0.05 were considered significant.

To evaluate the effect of nesting season, early and late, and incubation temperature on the presence or absence of B cell clusters in slider hatchlings, a
A generalized linear mixed model with binary responses was fitted to the data. With binary responses, the numbers 1 and 0 represented presence and absence of B cell clusters, respectively. Adaptive Gauss-Hermite approximation (nAGQ=15) was used to evaluate log-likelihood in the generalized linear models. Due to lack of replication in temperature incubation groups for late season clutches, this analysis was carried out including only medium and high incubation temperature treatments. Likelihood ratio tests (LRT) simplified models by eliminating non-significant variables. Specifically, the random effect clutch was removed from the model as the variance attributed to this effect was estimated to be zero.

To examine the effect of nesting season on maternal Ig levels in yolk we fit a general linear mixed model to the data. Clutch(season) was included as a fixed effect and clutch as a random effect.

Best fitting models were selected based on the Akaike Information Criterion (AIC) and explanatory power (Somers’ $D_{xy}$). The AIC was used to estimate the best quality model for our data, out of a collection of models. Somers’ $D_{xy}$ was used to analyze if association existed between our independent and dependent variables. Likelihood ratio tests were used to assess significance with chi-square distribution for the binary response data, and F-tests for the remaining data. Significant test values reported below are from are simplified models generated from eliminating non-significant terms through LRTs. Non-significant values correspond to maximal models, pre-removal of non-significant terms. All data is presented as mean ($\pm$ SE).
Results

It is important to note results below are derived from data collected from hatchlings born at the end of the 2014 summer. Positive B cell staining in the small intestines is depicted in figure 9. Based on viewing magnification (60x), it is assumed a single spot represents a cluster of B cells. The following year, positive staining was not repeated in any hatchlings; hence, no data was collected.

*Abundance of B cell clusters varied drastically among individual hatchlings.*

Around 80% of early season individuals stained positive for B cell clusters in small intestinal tissues compared to less than 20% of late season individuals. The majority of late season hatchlings stained negative for B cells (Figure 10).

*Incubation temperature treatment significantly affected the number of B cell clusters in early season clutches*

Clutches from the low incubation temperature treatment group were found to have a greater number of B cells clusters in their intestinal tissue than clutches from other incubation temperature regimes ($F_{2,12}=5.62, p=0.019$) (Figure 11).

*Presence of B cell clusters was significantly affected by nesting season but not by incubation temperature treatment or the interaction between the two.*

Nesting season had a significant effect on the presence of B cell clusters in slider hatchling intestines with individuals from early season clutches having B cell clusters present (LRT: $X^2=19.98, n=54, p<0.0001$) (Figure 12). However, neither incubation temperature treatment (LRT: $X^2=0.388, n=54, p=0.533$) or the interaction between nesting season and incubation treatment (LRT: $X^2=0.466, n=54, p=0.495$) significantly affected the presence of B cell clusters.
Maternal Ig deposition in eggs was not significantly affected by nesting season.

Egg yolk antibody content was assayed via ELISA. Optical density values were used to express relative antibody amounts, with higher OD values indicating a greater amount of antibodies. Total maternal antibody level did not differ between eggs collected in early and late nesting seasons ($F_{1,18}=2.00$, $p=0.174$) (Figure 13a). However, maternal Ig levels in eggs were significantly affected by clutch identity ($F_{19,20}=14.61$, $p<0.0001$) (Figure 13b).

**Discussion**

We are the first to examine B cells in GALT in the small intestine of red eared slider turtles. Despite our findings, much work is still needed to elucidate B cell distribution in the GALT of hatchlings. Nevertheless, our preliminary data could have important implications for the survival of young hatchlings during an immune challenge.

In our 2014 study, due to our experimental design and lack of replication within certain treatment groups we had to analyze our data two different ways. First, the effect of incubation temperature was only examined in clutches laid during the early nesting season. We found a significant effect of mean incubation temperature on the B cell distribution in the GALT of early season clutches. Clutches from the low incubation had a greater number of B cell clusters in their intestine than clutches from the other two treatment groups. This is not the first reported case of an enhanced immune response in individuals incubated at low temperatures. Dang, Zhang, and Du (2015) found that soft shelled turtle hatchlings incubated at 24°C were more immunocompetent when faced with a bacterial challenge and exhibited higher expression levels of IgM, IgD, and CD3γ than hatchlings incubated at higher temperatures. In fact, there is increasing evidence that
The developmental environment may have a significant effect on immune function in the hatchlings of oviparous species. A study in nestling tree swallows found that hatchlings derived from eggs that had been experimentally cooled during incubation had a lower ability to kill bacteria, as assessed by bactericidal killing assays, than hatchlings that had not been cooled during incubation (Ardia, Pérez, and Clotfelter 2010). Another reptilian incubation study showed that when incubated at male producing temperatures (low temperatures), hatchlings had a stronger complement response than individuals from higher incubation temperatures (Freedberg et al. 2008). Alternatively, temperature may affect temporary distribution of lymphocytes within an organism. A study by Engelsma et al. (2003) found that changes in environmental temperature in carp resulted in temporary redistribution of B cells, with B cells leaving the blood stream and migrating into tissues at lower temperatures. Immune function may determine the survival of young and it is also an important determinant of an organism’s fitness. Therefore, our results are pertinent to understanding the developmental role of immunity in offspring.

Second, we examined the effect of nesting season and incubation temperature on the presence or absence of B cell clusters in hatchlings from the 2014 nesting season. We found a significant effect of nesting season on the presence of B cell clusters. Specifically, B cell clusters were more present in early season hatchlings than late season hatchlings. In fact, over 80% of hatchlings derived from late season eggs had no B cell clusters in their small intestine. This finding could be a result of maternal allocation differences between early and late season eggs. Previous studies in our lab have shown that late season eggs have significantly higher amounts of progesterone and estradiol than early season eggs (Paitz and Bowden 2009). While estrogens primarily enhance immune
responses, overly high levels have been shown to inhibit immune responses (Macciò et al. 2008). Therefore, it is possible the high levels of estrogens deposited into late season eggs may be inhibiting B cell activity in the small intestine GALT of hatchlings. Estrogens have also been shown affect B cell migration in genital and intestinal tissues. McDermott, Clark, and Bienenstock (1980), have described the localization of IgA plasma cells from intestinal tissues to vaginal tissues under the influence of the estrous cycle in mice. The high levels of estrogen in late season eggs may trigger the migration of B cells normally present in the GALT to other immune related tissues in late season hatchlings. It is important to note, a previous study in our lab did not find an effect of estrogen on the development of the immune system in hatchlings in regards to innate immunity and total antibody levels in the blood (Zimmerman et al. 2012). However, they did not focus on B cells in resident mucosal tissues. The observed lack of B cells may also be attributed to technical difficulties experienced during immunohistochemical staining. Following staining of early season tissues, the DAB substrate began to develop a precipitate that may have affected staining efficiency. A new solution was ordered and used for the latter half of late season tissues. However, we did not see any positives with the new substrate. We did not observed a significant effect of incubation temperature on the presence of B cells in the small intestine GALT of hatchlings or the interaction between incubation temperature and nesting season. This was surprising as we observed a significant effect of incubation temperature on B cell clusters in early season clutches. This may be attributed to the staining difficulties previously described.

Yolk antibody content in frozen early and season eggs was suspected as a potential driving factor for the effect of nesting season on the presence of B cell clusters
in hatchlings. However, antibody level was not significantly affected by season. We did find a significant effect of clutch identity on maternal antibodies. This suggests that maternal antibody deposition varies across clutches. This could be due to genetic or environmental factors, such as upregulation of genes that determine maternal antibody transmission or local disease environment prior to transmission, respectively. However, it does not appear to be associated with the nesting season from which a clutch is laid.

In our 2015 study, we could not successfully stain B cells in the small intestine GALT of hatchlings using the same protocol from the previous year. First it is important to note, the incubation temperature of the medium group from 2015 was higher than the high temperature group from 2014. This temperature increase could have affected intestinal development resulting in a lack of B cell in the small intestine. It is possible that B cells had migrated to other lymphoid tissues, therefore, they were absent in the small intestine. It is also possible that B cells were present, but they were antibody producing plasma cells and had therefore lost their ability to display immunoglobulins on their surface. The lack of repeatability in our immunohistochemistry questions the validity of B cell staining in the 2014 study. Due to the lack of a good control for the 2014 hatchlings, we may have observed staining artifacts instead of the assumed B cells. Further work must be done to establish a working immunohistochemistry protocol in order to accurately study the B cell distribution in the small intestine GALT in red eared slider hatchlings.

From a morphology standpoint, our work allowed us to examine the intestine structure of 6 week old red eared slider hatchlings. Interestingly, intestine structure varied across hatchlings. While majority of hatchling intestine exhibited ridges (Figure 9), some
lacked this distinct structure (Figure 8). The differences observed may indicate different developmental stages in hatching intestine. A study of gut morphogenesis in chicks describes the formation of different luminal patterns during embryonic development (Shyer et al. 2013). Chicks at embryonic day 6 (E6) and day 15 (E15) exhibit luminal patterns similar to those observed during our GALT study in hatchlings, figure 8 and figure 9, respectively. In chicks, the different luminal patterns are associated with formation of smooth muscles layers. As reptiles develop much slower than birds, it is possible that smooth muscle development might be responsible for the observed intestine structure in slider hatchlings. The structural differences do not appear to be related to nesting season. Further studies should investigate small intestine structure at vary times post-hatch to better understand hatchling gut morphology.

In conclusion, we examined B cells in small intestine GALT of red eared slider hatchlings. Although much work is still needed to draw solid conclusions, preliminary data shows an interesting relationship between B cells in GALT in response to incubation temperature and nesting season. Also, observed structural differences may result from developmental differences between hatchlings and may affect B cell distribution.
References


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Figure 8. Method for enumerating intestinal spots. ImageJ software was used to overlay a grid on top of small intestine images captured as described following staining. Grid squares were assigned numbers 1-20 and a random number generator was used to determine which square would represent the overall spot count for a sample. In the image above, spots were tallied from randomly selected square #19.
Figure 9. **B cells are present in hatchling intestinal tissues.** B cells in the small intestine of hatchlings were detected with HL673 mAb + biotin, streptavidin-HRP, and DAB peroxidase substrate (a). Control tissue sections were incubated without the primary antibody to ensure B cell cluster authenticity (b). Sections are shown at a magnification of 60X. Due to the low magnification used to view the sections, each individual spot was considered to be an aggregation of B cells, or a B cell cluster. A grouping of many spots, or B cell clusters, was referred to as a B cell patch (a).
Figure 10. Frequency distribution of B cell clusters in hatchlings indicate majority of late season hatchlings stained negative for B cells in the small intestine. Tissues from 35 early and 31 late season individuals were stained and B cell clusters enumerated. Histograms show the frequency distribution of spots.
Figure 11. Lower incubation temperature significantly increases the number of intestinal B cell clusters in early season clutches. Clutches from the medium and high treatments did not have significantly different numbers of B cell clusters. B cell cluster number dropped significantly above 27.1 °C. Different letters above bars represent significantly different groups GENERALIZED LINEAR MODEL (QUASI-POISSON): $F_{2,12}=5.62$, $p=0.019$. 
Figure 12. More B cell clusters were found in the small intestine of early season slider hatchlings than late season hatchlings. Tissues from 35 early and 31 late season individuals were stained and B cell clusters counted. Nesting season significantly affected the presence of B cell clusters. LRT: $X^2 = 19.98$, $n=54$, $p<0.0001$. 
Figure 13. Maternal antibody deposition in eggs is not significantly affected by nesting season, but antibody levels are significantly affected by clutch identity. Yolk ELISA was used to quantify maternal antibody levels in 10 early and 10 late season eggs. Optical density values represent relative amounts of antibody; higher OD values indicate a greater amount of bound antibodies in the ELISA. (a) GENERALIZED LINEAR MIXED MODEL: $F_{1,18}=2.00$, $p=0.174$. Clutch identity significantly affected maternal Ab levels in eggs, suggesting maternal allocation of antibodies differs across clutches (b). Clutches are ordered by mean antibody quantity. GENERALIZED LINEAR MIXED MODEL: $F_{19,20}=14.61$, $p<0.0001$. 

![Graph A: Optical density determined quantity of maternal Ig antibodies (mean + s.e.) vs. Season](image)

- Early Season: Optical density value
- Late Season: Optical density value

![Graph B: Optical density determined quantity of maternal Ig antibodies (mean + s.e.) vs. Clutch](image)

- Optical density values for Clutch 1 to Clutch 20

86
APPENDIX

AN EXPLORATION INTO DUAL ROLES FOR REPTILIAN B CELLS

Phagocytic B cells have been discovered in a variety of species (Li et al. 2006, Øverland et al. 2009, Zimmerman et al. 2010, Muñoz et al. 2014, Nakashima et al. 2012, Parra et al. 2012, Gao et al. 2012) and express both phagocytic and intracellular bactericidal capabilities. More recently, human B cells from the Raji B cell line have been shown to phagocytose both live and dead *Mycobacterium tuberculosis* (Zhu et al. 2015). Moreover, it has been shown that phagocytic B cells in teleost fish and B-1 B cells in mice are able to present particulate antigen to CD4+ T cells (Parra et al. 2012, Zhu et al. 2014). In mice and teleost fish, B cell phagocytosis has been described as occurring in a non-B cell receptor manner (Parra et al. 2012, Nakashima et al. 2012, Zhu et al. 2014). This process has been suggested to trigger the production of natural immunoglobulins (Parra et al. 2012); however, to date no study has examined antibody secretion in phagocytic B cells. Here, we are the first to examine the capacity of reptilian B cells to secrete antibodies following phagocytosis. Initial studies proved cell sorting via the flow cytometer to be too harsh on reptilian cells. Hence, we designed a new method, the dual function assay, to further elucidate the functions of reptilian phagocytic B cells.

Methods

Dual Function Assay

The dual function assay combined the phagocytic B cell assay followed by ELISpot assay in order to determine if phagocytic B cells can subsequently secrete...
antibody. Both assays were completely carried out under sterile conditions. Blood samples were taken from the caudal vein of adult female red eared sliders (n=30) in the summer of 2015. For each experiment (n=6) blood samples from five sliders were pooled and leukocytes prepared for the phagocytic B cell assay as described in Chapter II. Non-toxic magnetic 1 µm beads (ProMag™ 1 Series COOH, Bangs Laboratories) were substituted for fluorescent beads (same cell:bead:media ratio reported in Chapter II) and cells were incubated for three hours at 29°C in 5% CO₂. Following incubation and washes to remove free beads, cells were counted via trypan blue staining. Phagocytic cells were isolated via manual magnetic separation (MACS, Miltenyi Biotec). Briefly, the cell suspension was run through a column attached to a MACS separator magnet. The column, which contains a ferromagnetic matrix, retained cells that had previously engulfed magnetic beads. The first flow-through fraction collected was the negative fraction, or the non-phagocytic cells. Following the removal of the column from the magnet separator, the second eluted fraction contained the positively selected phagocytic cells (Figure 1). Both fractions were then centrifuged at 1500xg for 5 min at 4°C, supernatant decanted, and re-suspended in 150µL of RPMI supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin/ glutamine, 0.5% 2-mercaptoethanol, and 0.5% sodium pyruvate (cPRMI). Then, cells were counted to examine viability following magnetic separation. After counting, adherent and non-adherent cell suspensions were plated for the ELISpot assay along with a control well containing 150 µL of cRPMI. The entire volume of the adherent and non-adherent cell suspensions were plated regardless of total cell number. We did this because we observed a substantial decrease in cell viability following magnetic separation. Each experiment consisted of a single plate with three
wells: control well, non-adherent well, and adherent well. Plates were incubated at 29°C in 5% CO₂ for three days. ELISpot wells were coated with anti-turtle light chain HL673 monoclonal antibody (mAb) and detected with HL673 mAb-biotin, streptavidin-horse radish peroxidase, and 3-amino-9-ethylcarbazole substrate solution as described in Chapter II. Three out of the six experiments included wells supplemented with 40µg/ml lipopolysaccharide (LPS) in cRPMI to stimulate B cell proliferation and antibody secretion. Spots were manually counted after well membrane development.

Results and Discussion

To address the question whether phagocytic B cells were able to subsequently secrete antibody, turtle lymphocytes were subjected to a phagocytic assay using magnetic beads. It is unknown whether a threshold exists for the number of engulfed beads required to retain a phagocytic cell in the column during the first eluted fraction. While it is possible that some phagocytic cells were eluted in the first non-adherent fraction, we assume that those cells had engulfed a small number of magnetic beads (i.e., too few beads to allow attraction to the magnetic column). We have shown that reptilian B cells phagocytose a rather large number of beads compared to phagocytes (Chapter II); therefore we expect most of the phagocytic B cells to have been eluted in the second adherent fraction. Future experiments could elucidate this issue. It is also important to note cell viability substantially decreased following magnetic cell separation (Table 1). In all experiments, a similar level of leukocyte death occurred and only about 20% of the cells were recovered. Trypan blue analysis revealed a large number of dead cells following magnetic cell separation. It appears turtle B cells do not respond well to magnetic sorting, despite the gentleness of this technique. Previous research in our lab
also found difficulty in getting turtle B cells to survive sorting in the flow cytometer. It is possible that phagocytic turtle B cells are weakened from engulfing a large number of beads and, thus, are more prone to cell lysis. Future work may involve cell membrane integrity studies subsequent to B cell phagocytosis.

Following a three-day incubation, reptilian B cells that had phagocytosed beads were not able to subsequently produce antibodies even when stimulated with LPS (Table 2). As expected, our negative control well, lacking cells, did not exhibit spots upon AEC development. Spots were observed in the non-phagocytic cell sample. The number of observed spots was similar to previous experiments in Chapter II despite the lower of number cells plated here. Much like the percentages of antibody secreting cells in Chapter II, we report less than 1% of plated cells secreted antibodies. This could be attributed to lack of antigenic challenge or necessary activation signals received in vivo. More likely, lack of antibody response could be attributed to the low survival of cells following magnetic cell separation. As this is a new method, much troubleshooting is still needed for the dual function assay to be considered a reliable technique. In future studies, it would be of interest to confirm the sorting purity as well as the percentages of B cells in each fraction.

As it stands, our results suggest that either reptilian B cells are terminally differentiated or functionally distinct subsets exist. Hence, the primary roles of reptilian phagocytic B cells could be limited to those exhibited by other phagocytes and antigen presenting cells (Sunyer 2012). Further work may examine microbial killing and antigen presentation in reptilian phagocytic B cells to support this notion. Alternatively,
phagocytic B cells may be stimulated to secret antibodies \textit{in vivo}. Hence, \textit{in vivo} studies will also provide insight to the roles of phagocytic B cells in the reptilian immune system.
References


**Table 1. Cell concentrations pre and post cell separation.** Cell viability substantially decreased following magnetic cell separation. It is important to note, the actual number of cells pre-magnetic separation is 2 times the cells/mL va value as total sample volume was 2 mL. Plated cell numbers in non-adherent and adherent samples are 0.150 times the cells/mL value because total sample volume was 150µL.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cells/mL pre-magnetic separation</th>
<th>Total cells pre-magnetic separation</th>
<th>Cells/mL non-adherent sample</th>
<th>Total plated non-adherent sample</th>
<th>Cells/mL adherent sample</th>
<th>Total plated adherent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay 1</td>
<td>$1\times10^6$</td>
<td>$2\times10^6$</td>
<td>$1.8\times10^5$</td>
<td>$2.7\times10^4$</td>
<td>$4\times10^4$</td>
<td>$6\times10^3$</td>
</tr>
<tr>
<td>Assay 2</td>
<td>$1.1\times10^6$</td>
<td>$2.2\times10^6$</td>
<td>$2.2\times10^5$</td>
<td>$3.3\times10^4$</td>
<td>$6\times10^4$</td>
<td>$9\times10^3$</td>
</tr>
<tr>
<td>Assay 3</td>
<td>$1.5\times10^6$</td>
<td>$3\times10^6$</td>
<td>$2.6\times10^5$</td>
<td>$3.9\times10^4$</td>
<td>$2\times10^4$</td>
<td>$3\times10^3$</td>
</tr>
<tr>
<td>Assay 4</td>
<td>$9\times10^5$</td>
<td>$1.8\times10^6$</td>
<td>$2.2\times10^5$</td>
<td>$3.3\times10^4$</td>
<td>$4\times10^4$</td>
<td>$6\times10^3$</td>
</tr>
<tr>
<td>Assay 5</td>
<td>$1.2\times10^6$</td>
<td>$2.4\times10^6$</td>
<td>$1.6\times10^5$</td>
<td>$2.4\times10^4$</td>
<td>$2\times10^4$</td>
<td>$3\times10^3$</td>
</tr>
<tr>
<td>Assay 6</td>
<td>$1.1\times10^6$</td>
<td>$2.2\times10^6$</td>
<td>$1\times10^5$</td>
<td>$1.5\times10^4$</td>
<td>$2\times10^4$</td>
<td>$3\times10^3$</td>
</tr>
</tbody>
</table>
Table 2. Cells which had phagocytosed magnetic beads were subsequently unable to produce ELISpots. Following magnetic bead separation, phagocytic and non-phagocytic cells were cultured in the ELISpot assay as described. Assay spot numbers were counted and averaged for well contents with or without LPS stimulation. Assay spot numbers for non-phagocytic cells are reported as the average number of observed spots for the six assays per the average of number of non-adherent plated cells for the six assays. Assay spot numbers for phagocytic cells are reported as the average number of observed spots for the six assays per the average of number of adherent plated cell numbers for the six assays.

<table>
<thead>
<tr>
<th></th>
<th>No cells</th>
<th>Non-phagocytic cells spots/ (2.8 \times 10^4) cells</th>
<th>Phagocytic cells spots/ (5 \times 10^3) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No LPS</td>
<td>0</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>LPS Stimulation</td>
<td>0</td>
<td>63</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURES

Figure 14. Schematic representation of magnetic cell separation. Isolated leukocytes were incubated with magnetic beads in the phagocytic B cell assay. B cells and phagocytes are indicated by black and blue circles respectively. Magnetic beads are represented by smaller brown circles. (a). Phagocytic cells are separated from non-phagocytic cells using magnetic column separation. The column contains a ferromagnetic matrix, thus creating a magnetic field. The flow-through fraction contains the non-adherent, non-phagocytic cells (b,c). The adherent cells are eluted as the phagocytic cell fraction when the column is removed from the magnet (d). Separated cell populations are then plated for ELISpot.