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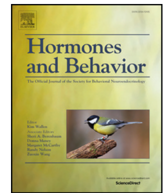

The effect of environmental temperature on reptilian peripheral blood B cell functions

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ABSTRACT

Recent studies have identified phagocytic B cells in a variety of species, yet little is understood about their function and how it is influenced by natural environmental variation, such as temperature. Phagocytic B-cells are present in red-eared slider turtles, *Trachemys scripta*, and the wide range of temperatures experienced by these ectotherms may have an effect on immunity, 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, with maximal antibody secretion occurring at intermediate temperatures (estimated maximum of 28.8 °C). There was no effect of temperature on phagocytosis. We also noted a difference in the efficiency of phagocytosis in this assay between B cells and non-B cells. Interestingly, in our *in vitro* assay, phagocytic B cells engulfed more foreign fluorescent beads per cell than phagocytes lacking surface immunoglobulin. This work sheds light on our understanding of phagocytic B cells and the importance of environmental temperature on the behavior of reptilian immune cells, which may have relevance for organismal fitness.

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1. Introduction

Interactions between the endocrine and immune systems have been well studied in humans and mammalian model systems, and these studies have highlighted the complex and often contradictory nature of how these systems participate in maintaining homeostasis (Demas and Carlton, 2015). Although we are making progress in understanding how these two systems act and interact within an organism, there are critical deficiencies in our ability to examine such interactions in many taxonomic groups (Zimmerman et al., 2010a, 2014; Demas et al., 2011). These endocrine–immune interactions are going to be key in determining the responses of organisms to environmental perturbations. In ectotherms, one critical environmental factor that may affect the interaction between the endocrine and immune systems is temperature. Previous research has shown that the immune response of ectotherms can vary with changing temperature (Wright and Cooper, 1981; Zapata et al., 1992), and this temperature-induced variation could have implications for the survival of the organism. Temperature not only affects host physiology, including the immune system, but also plays an important role in pathogen growth and transmission (Altizer et al., 2013). An organism's fitness may be compromised if there is a mismatch between the temperature at which the host's immune system is most effective and the temperature at which the pathogen is most efficient (Jackson and Tinsley, 2002; Pounds et al., 2006; Rohr and Raffel, 2010; Kilburn et al., 2011). Therefore, it is important to understand how the immune system behaves in response to changing temperatures to understand the outcomes of pathogen 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 for both innate and adaptive immune responses. For example, in American alligators (*Alligator mississippiensis*) complement proteins involved in bacterial lysis function at temperatures between 5 °C and 40 °C, however, activity was lower below 15 °C and above 30 °C relative to intermediate temperatures (Merchant et al., 2003, 2005). Similarly, in the common wall lizard (*Podarcis muralis*), *in vitro* temperature dependent activation of T lymphocytes, in response to phytohemaagglutinin stimulation, was significantly diminished at both 22 °C and 38 °C compared to peak activation at 32 °C (Sacchi et al., 2014). However, not all immunity-temperature interactions display this inverted U-shaped relationship. Butler et al. (2013), found a significant effect of temperature on agglutination and cell lysis in 11 out of 13 species, nine of which were ectotherms, and while some exhibited the standard inverted U-shape, others had a negative linear relationship between innate responses and temperature. In a study of the effects of temperature on

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macrophage activity in four species of turtles, a positive relationship between temperature and the phagocytic ability of macrophages was found (Johnson et al., 1999), and an early study in the painted turtle (*Chrysemys picta*) found that high temperatures may not affect the kinetics of the antibody response (Grey, 1963). It has been hypothesized that temperature dependent changes in immune responses may differ for innate or adaptive functions. Butler et al. (2013) proposed a tradeoff 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, teleost fish exhibit strong complement activity at low temperatures, whereas antibody secretion is strongest at high temperatures (Magnadóttir, 2006).

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 because they display immunoglobulins (Igs) on their surface (Ochsenbein and Zinkernagel, 2000). It is well documented that changes in temperature affect secreted antibody levels across a number of organisms (Grey, 1963; Hrubec et al., 1996; Mikkelsen et al., 2006), however, much like with other immune responses, the patterns are not consistent, thus providing little insight into how temperature may affect B cell performance in reptiles, or ectotherms more generally.

Until recently, B cells were thought to be involved strictly in antibody production, with the innate mechanism of phagocytosis carried out by traditional phagocytes such as neutrophils/heterophils and macrophages/monocytes. Evidence for a developmental relationship between traditional phagocytes and a subset of B cells, B-1 cells, however, suggests that the mammalian B cell may have evolved from a cell with characteristics of both cell types (Borrello and Phipps, 1996; Montecino-Rodriguez et al., 2001). It is likely this relationship between B cells and phagocytes is not restricted to mammals, in fact, Ig+ phagocytic cells have been found in fish, amphibians, and reptiles (Li et al., 2006; Øverland et al., 2010; Zimmerman et al., 2010b; Muñoz et al., 2014). 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) and present phagocytosed antigen to $CD4^{+}$ T cells (Parra et al., 2012). Both murine studies demonstrated that particle uptake by B cells is not mediated by a B cell receptor (BCR), suggesting that the uptake mechanism may induce the production of nonspecific immunoglobulins by phagocytic B-1 cells (Parra et al., 2012). Alternatively, B-1 cells may strictly act as phagocytic antigen presenting cells, thereby avoiding potentially unnecessary costs associated with antibody secretion (Parra et al., 2012).

At present, it is not possible to determine if there are differing subsets of B cells in most groups of organisms, as such studies are hindered by the lack of available reagents and cell markers to appropriately probe the immune systems of non-model organisms (Zimmerman et al., 2010a). For the past decade, our research group has focused on developing immune reagents, validating assays, and ultimately assessing the immune system of the red-eared slider turtle (*Trachemys scripta*), a long-lived ectothermic vertebrate (Zimmerman et al., 2010b, 2013). In addition to our studies on immunity, *T. scripta* has been the focus of behavioral, endocrinological, and developmental studies (Gibbons, 1990; Crews, 1996; Bowden et al., 2002; Les et al., 2009; Zimmerman et al., 2012; Carter et al., 2016), which allows us to contextualize our findings within the broader biology of the species. In the present study, we utilize immune assays we have previously validated for use in *T. scripta* to examine how the behavior of B cells is modified across a range of biologically relevant temperatures.

It is currently unknown how temperature may affect the phagocytic capacity of B cells in any species of reptile. Here, we examined the effect of temperature on antibody secretion and phagocytosis of Ig+ cells *ex vivo* in order to further understand the behavior of B cells in an ectothermic vertebrate. We have previously demonstrated that *T. scripta* possesses B cells that can secrete antibodies (Zimmerman et al., 2013) and phagocytose (Zimmerman et al., 2010b). *T. scripta* are most active between 25° and 30 °C (Ernst and Lovich, 2009), and we assessed B cell function at temperatures above and below the optimal activity range, but still within the bounds of naturally relevant environmental temperatures. As our samples also contained Ig− phagocytic cells, we compared their phagocytic capacities to that of Ig+ cells to further understand the relative role of phagocytic B cells in the reptilian immune system.

2. Materials and methods

2.1. Trapping and blood collection

Adult female 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. Males were excluded as their trap numbers are much lower than females at this time of the year, and we have previously reported no sex differences between the number of antibody secreting cells and amount of secreted antibody (Zimmerman et al., 2013), providing further rationale for excluding males from our sample. In the field, approximately 1 mL 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. Erythrocytes were removed by a Percoll density gradient, using a modification of Harms et al. (2000). Briefly, a diluted Percoll (MP Biomedicals) density gradient (nine parts Percoll, one part sterile 10× saline solution) was prepared, the diluted blood sample layered on top and centrifuged at 400 ×*g* at 4 °C for 5 min with no braking. Following centrifugation, most erythrocytes pelleted and the interface layer, containing leukocytes, was carefully removed *via* a Pasteur pipet and washed twice with RPMI at 1500 ×*g* 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 viable cell counting. This method produced a robust, viable buffy coat and any remaining erythrocytes would not interfere with subsequent assays.

2.2. Immune assays

In order to examine the effect of temperature on B cell function, two immune assays were used; phagocytosis was assessed with a phagocytic B cell assay and antibody secretion by an ELISpot assay. 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 leukocytes were split evenly among four temperatures: 20°, 25°, 30°, and 35 °C, and each temperature treatment was run in a single incubator. The critical temperature maximum for *T. scripta* is between 40 and 42 °C (Ernst and Lovich, 2009), so higher temperatures are not physiologically relevant and were not used. All four temperatures were run simultaneously for each experiment on separate plates with triplicates at every temperature.

2.2.1. Phagocytic B cell assay

To examine phagocytic capacity of reptilian B cells, we used a modified version of the method described by Zimmerman et al. (2010b). Following cell counting, cell suspension volume was adjusted with cRPMI to allow 2×10^5 cells/well. The cell suspension was then plated in

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triplicate into each of four 48 well plates, with 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 3.45×10^6 cells to 6.4×10^7 beads in 500 μL of cRPMI. Plates were then incubated at 20°, 25°, 30°, or 35 °C for 3 h in 5% CO_2. After incubating, the cells were resuspended by pipetting and transferred into 1.5 mL microcentrifuge tubes labeled according to temperature and triplicate number. The tubes were centrifuged, supernatant decanted, and cells gently resuspended in 400 μL of 1× 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 1500 ×g for 5 min at 4 °C. Following supernatant removal, cells were washed and centrifuged as previously mentioned, resuspended in 400 μL 1× Hanks-0.5%-BSA, and then prepared for 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 min on ice. Samples also included 10% normal rat serum to prevent non-specific binding. After incubation, cells were washed with 1× Hanks-0.5%-BSA and centrifuged at 1500 ×g for 5 min at 4 °C. 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 1× Hanks-0.5%-BSA was added to each sample and incubated in the dark on ice for 15 min. Cells were washed, centrifuged as before, and resuspended in 400 μL 1× 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). FL1 voltage was set based on unstained cells, (see Fig. 1D). Free beads and dead cells were excluded by forward scatter (FSC)/side scatter (SSC) gating (see Fig. 1C).

2.2.2. ELISpot assay

The ELISpot assay was used to measure the number of antibody secreting cells. MultiScreen-IP ELISpot plates (EMD Millipore) were coated with 100 μL/well of 20 μg/mL unlabeled *anti*-turtle light chain (HL673; University of Florida Hybridoma Facility) diluted in sterile 1× 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 1× PBS. Plates were then blocked with 200 μL of cRPMI for 1 h at 37 °C. Following incubation, the blocking medium was decanted and leukocytes were plated at 5×10^4 cells/well and then serially diluted. Initial experiments determined 5×10^4 cells/well as the optimum number of plated cells in order to prevent well membrane overcrowding. Plates were incubated for three days at 20°, 25°, 30°, or 35 °C in 5% CO_2. Following the three-day incubation, plates were decanted and wells were washed three times for 3 min each with 200 μL 1× 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 1× PBS was added and left to incubate for 1 h at room temperature. Following another series of washes, 3-amino-9-ethylcarbazole (AEC) substrate solution (Sigma) was used to develop the wells. The number of spots was determined using ImageJ software.

2.2.3. Data and analyses

Prior to analyses, repeatability of technical replicates was checked and subsequently the mean of the technical replicates for each assay used. All statistical analyses were performed in R statistical software (version 3.3.1 for Mac). In all cases test assumptions were checked, *e.g.* normality and homogeneity of variance. Given the limited number of discrete values from the temperature treatments and to enable the detection of differences between the means of any of the treatments, independent of their values, ANOVAs were performed for the dependent

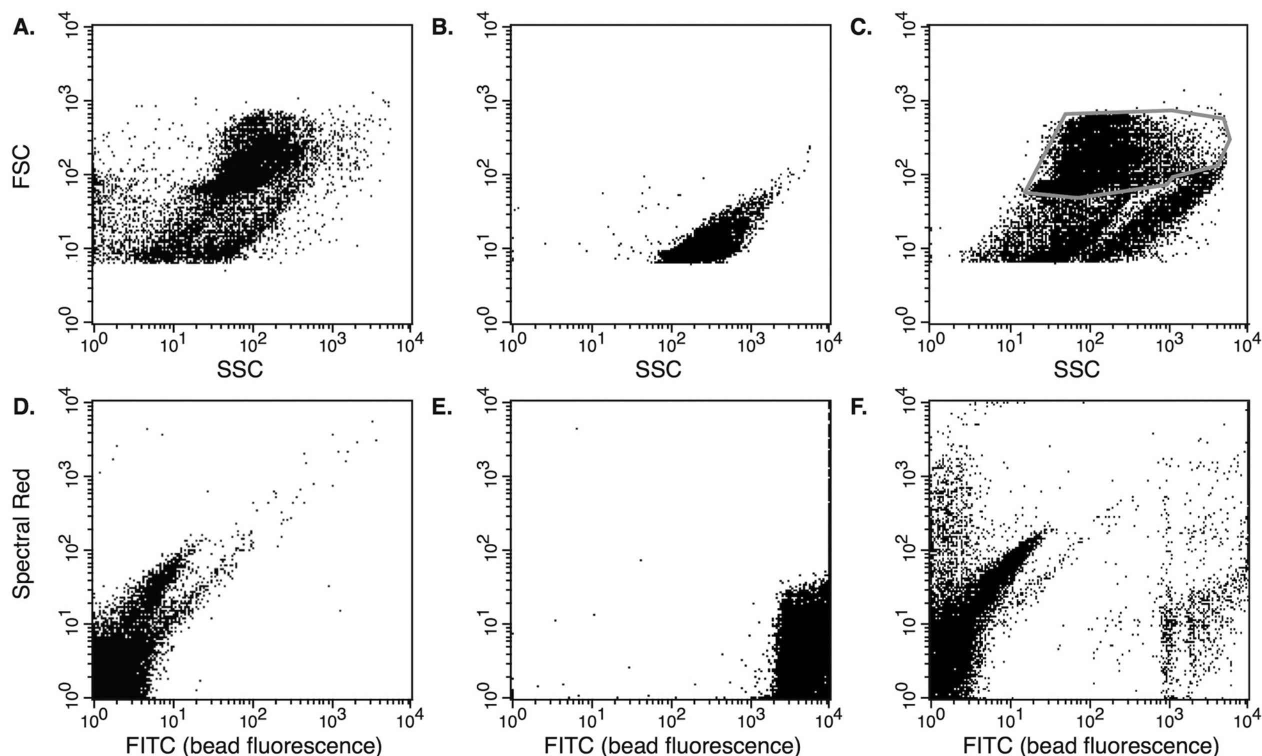

Fig. 1. Sample flow cytometry analysis showing gating of cell populations. Phagocytic cells were detected *via* positive FITC staining. B cells were detected *via* primary staining with HL673-biotin followed by secondary staining with SA-spectral red. Scatter plots (forward scatter (FSC) and side scatter (SSC)) and representative gating are shown on the top (A, C, E) with fluorescence of the same samples on the bottom (B, D, F). Samples are: unstained cells (A, D), free beads (B, E), and stained cells (C, F). In F, the cells are gated as shown in C.

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variables of percentage of phagocytosing cells (Ig+ and Ig−) and ELISpot data with temperature as a categorical fixed factor. Percentage data was normally distributed with no extreme values and therefore assumptions of the linear models were not violated. In the case of significant temperature treatment differences, false discovery rate (FDR) pairwise *t*-test comparisons were performed between temperatures. To further investigate the pattern of any relationships between increasing temperature and the dependent variable of interest, models were also fitted including temperature as a continuous predictor. Linear models and non-linear models with quadratic fits were carried out and their fits compared. Analysis of median fluorescence intensity (MFI) data included cell type (Ig+, Ig−) and temperature, and their interaction as fixed effects. As data from each cell type was collected for each assay run, assay number was included as a random effect in these models.

3. Results

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

Our lab has previously used a fluorescent bead assay to measure phagocytosis (Zimmerman et al., 2010b). Phagocytic B cells are easily distinguished based on their FITC fluorescence (Fig. 1), with cells that internalize a greater number of beads producing a brighter signal (Fig. 1F). A second stain for B cells, *anti*-light chain mAb, distinguishes B cells from non-B cells (Fig. 1F). When subjected to different temperatures, percentages of phagocytic Ig+ cells were not significantly impacted ($F_{3,36} = 0.67$, $P = 0.577$; Fig. 2). Additionally, no significant relationships were found when temperature was included as a continuous variable in linear ($F_{1,38} = 0.488$, $P = 0.489$) or quadratic models ($F_{2,37} = 0.942$, $P = 0.399$). Temperature did, however, significantly affect the number of antibody secreting cells measured by ELISpot assay ($F_{3,36} = 5.21$, $P = 0.004$; Fig. 3). The number of antibody secreting cells significantly increased between 20 °C and 25 °C and decreased between 30 °C and 35 °C, respectively (FDR corrected pairwise t-tests: $P < 0.05$). The relationship between temperature and the number of antibody secreting cells was further examined by fitting a quadratic polynomial model to the curve. The best fit model was a quadratic polynomial ($f(x) = -86.70 + 10.29\boldsymbol{x} - 0.18x^2$, $F_{2,37} = 7.44$, $P = 0.0019$, Fig. 3). The estimated maximum was at 28.82 °C, representing the optimal temperature for antibody secretion.

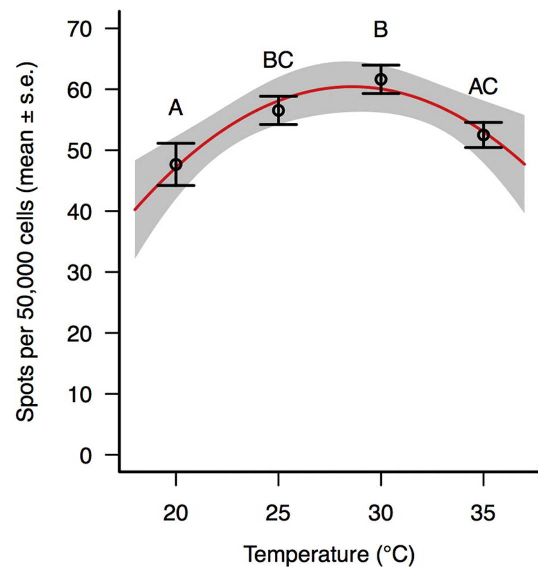

Fig. 3. Temperature significantly impacted the number of antibody secreting cells as measured by ELISpot assay. A spot indicates a single antibody secreting cell with the number of spots per 5×10^4 cells assayed, $n = 10$ per temperature. Temperature treatment had a significant effect on the number of spots (ANOVA: $F_{3,36} = 5.21$, $P = 0.004$), with different letters above bars representing significant differences between treatments (FDR corrected pairwise t-tests $P < 0.05$). A quadratic model fit (red line, $f(x) = -86.70 + 10.29\times - 0.18 \times {}^2$; $F_{2,37} = 7.44$, $P = 0.0019$) and 95% confidence intervals (grey band) predicted a maximum number of spots at 28.82 °C, the optimal temperature for antibody secretion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Temperature did not affect the percentage of Ig− phagocytic cells

Temperature did not significantly affect the percentages of phagocytosing Ig− cells, ($F_{3,36} = 1.28$, $P = 0.296$; Fig. 4). Furthermore, no significant relationships were found when temperature was included as a

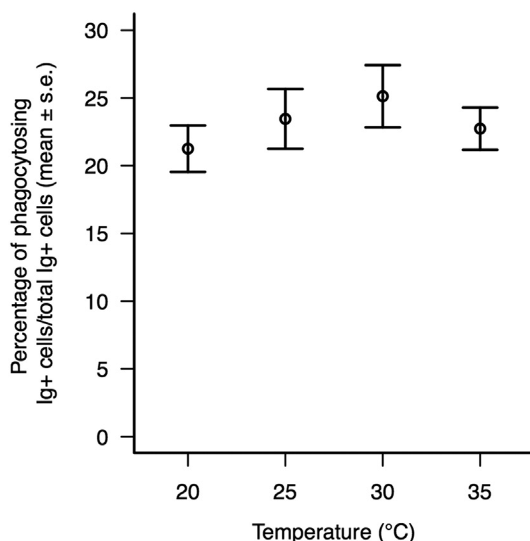

Fig. 2. Temperature did not impact percentages of phagocytic Ig+ cells out of total Ig+ cells. Following the phagocytic assay, samples were analyzed by flow cytometry and all Ig+ cells gated. Percentage of those cells also showing FITC fluorescence was calculated. $n = 10$ per temperature treatment, ANOVA: $F_{3,36} = 0.67$, $P = 0.577$.

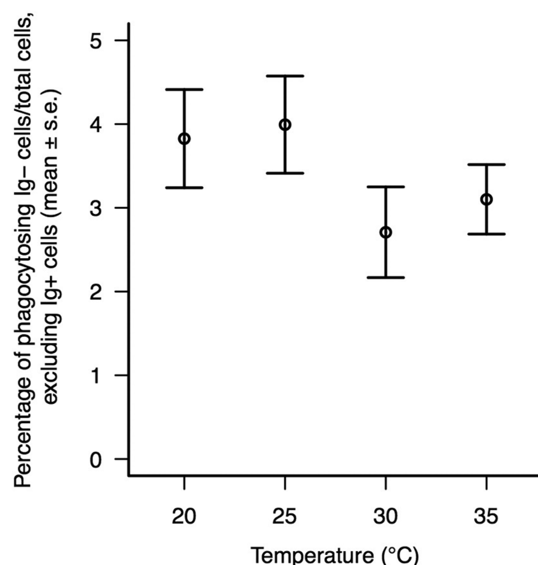

Fig. 4. Temperature did not impact percentages of Ig− phagocytes. Percentages of non-B cells that had engulfed beads ($Ig-FITC^+$) were determined by flow cytometry for each of the various temperatures, $n = 10$ per temperature treatment ANOVA: $F_{3,36} = 1.28$, $P = 0.296$.

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continuous variable in linear ($F_{1,38} = 2.10$, $P = 0.155$) or quadratic models ($F_{2,37} = 1.05$, $P = 0.361$).

3.3. Ig + cells are more efficient at phagocytosing than Ig − cells

During the analysis of the phagocytic assay, we noted that the majority of Ig + cells that were also FITC positive were highly fluorescent, which reflects the number of beads ingested by a single cell. In fact, most of these events were off the scale when FL1 voltage was set to show unstained cells (see Fig. 1D). While lowering the voltage brought these events on scale, unstained cells went off scale in the opposite direction, thus we opted to keep the voltage the same between all samples. Median fluorescence intensity (MFI) was used to quantify shifts in FITC fluorescence in both Ig + and Ig − phagocytic cells with higher MFI values indicating a greater number of beads engulfed by the cell. Cell type had a significant effect on gated lymphocyte MFI, with Ig + phagocytic cells exhibiting a higher MFI than Ig − phagocytic cells, ($F_{1,63} = 1400$, $P < 0.001$; Fig. 5). Temperature did not have a significant impact on Ig − phagocyte or phagocytic B cell MFI, ($F_{3,63} = 0.679$, $P = 0.568$; Fig. 5). Finally, there was no significant interaction between temperature and cell type on MFI, ($F_{3,63} = 0.528$, $P = 0.665$). Mean MFI (±SE) values for Ig + and Ig − phagocytic cells were 6585 ± 700 and 2589 ± 409, respectively. A representative sample and gating is shown in Fig. 6, where 68% of the Ig + cells had >7000 FITC fluorescence compared to 6% of Ig − phagocytic cells.

4. Discussion

Environmental temperature and immune function will interact, and it is expected that such interactions will be particularly pertinent for ectotherms, subsequently determining their ability to combat pathogenic infections. We examined innate and adaptive reptilian B cell behavior in response to biologically relevant temperatures, and surprisingly noted that Ig + cells phagocytosed more beads per cell than Ig − cells in this assay.

Contrary to our predictions, temperature did not have a significant effect on the percentage of phagocytic B cells. Phagocytic B cell numbers did not change 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, 2005; Magnadóttir, 2006; Butler et al., 2013). On the other hand, an early study observing inflammation processes in amniote 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 (Zimmerman et al., 2013). Here, we report, on average, 21–25% of Ig + cells were phagocytic. The percentage we report for the red eared slider is similar to that reported for other ecotherms, although there is variation among species (Li et al., 2006; Øverland et al., 2010). Interestingly, there is also variation within some species based upon anatomical location. In the Atlantic cod, *Gadus morhua*, a greater concentration of phagocytic B cells was found in the head-kidney lymphoid organ than in the peripheral blood (Øverland et al., 2010), while in mice, there were nearly 10-fold more phagocytic B cells found in the peritoneal cavity relative to peripheral blood leukocytes (Parra et al., 2012). It is currently unknown if anatomic locational differences for phagocytic B cells exist in *T. scripta*, but the observed variation in the percentage of B cells in the blood of *T. scripta* could be the result of migration to other tissues that could be impacted by temperature. Additional studies would be required to confirm this suggestion.

We did find a significant effect of temperature on the number of antibody secreting cells. Spot number significantly increased between 20° and 25 °C and decreased between 30° and 35 °C, respectively, with a maximum number of antibody secreting cells predicted at 28.8 °C. Our adaptive measure of B cell ability exhibited an inverted U-shape thermal performance curve, as has been previously reported for other immune parameters and temperature (Angilletta, 2009). This suggests that, when combating a pathogen, animals could engage in behavioral thermoregulation to maintain a body temperature that will maximize their ability to secrete antibody.

It is important to note that antibody secretion was measured in plated cells that 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; Ujvari and Madsen, 2011). Future studies may try incubating cells with other known B cell mitogens, such as dextran or heat-killed bacteria, to increase proliferation and antibody secretion (Ivanyi and Lehner, 1974). Another avenue for future work may be to examine red-eared slider antibody secretion in response to pathogen prevalence. A previous study in our population has found a high frequency of *Salmonella* in adult turtles, and that pathogen prevalence increases as temperature increases (Holgersson, 2009). With the addition of a pathogenic stimulus, antibody secretion and temperature may exhibit a different relationship as different signaling pathways may be involved.

As our samples also contained Ig − cells, we evaluated the effect of temperature on their phagocytic capacities compared to B cells. Similar to B cells, we did not observe an effect of temperature on the percentage of Ig − phagocytic cells. Ig − cells in our assay engulfed fluorescent beads equally well at all temperatures tested. Previous studies have reported mixed results, with some studies describing a positive

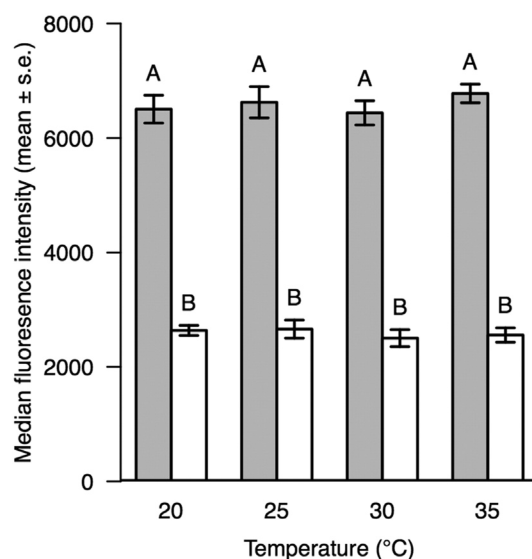

Fig. 5. Median fluorescent intensity (MFI), representing the amount of fluorescent beads engulfed, was significantly affected by cell type. Ig + cells (grey bars, $n = 40$ across temperatures) exhibit a greater MFI than Ig − phagocytic cells (unfilled bars, $n = 40$ across temperatures), ANOVA: $F_{1,63} = 1400$, $P < 0.001$. 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, $n = 10$ per cell per treatment ANOVA: $F_{3,36} = 0.679$, $P = 0.568$. Different letters above bars representing significant differences between means (FDR corrected pairwise t-tests $P < 0.05$).

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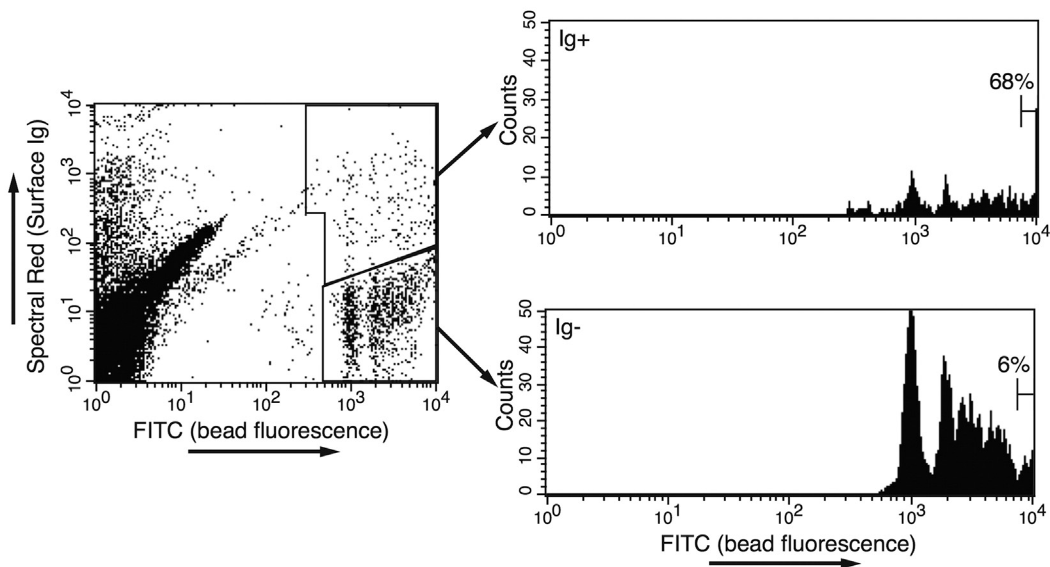

Fig. 6. Ig+ B cells engulfed more beads per cell than Ig− cells. Following the phagocytic assay, live cells were gated as in Fig. 1C. Ig+ and Ig− FITC+ cells were then gated (left panel) and gated histograms show (top) 68% of Ig+ phagocytic cells with >7000 FITC fluorescence compared to less than 6% of Ig− phagocytic cells (bottom).

relationship between temperature and macrophage phagocytosis, and others reporting that phagocytosis is relatively insensitive to temperature changes (Montali, 1988; Johnson et al., 1999). 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). Further studies are necessary to characterize these Ig− cell types and their optimal phagocytic capacity. It is also important to understand the inflammatory processes that initiate phagocytosis. 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 generating a strong inflammatory response, due to their lack of microbial particles, to activate phagocytes broadly. 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). It may be possible to get a more accurate estimate of phagocytic capacity by using fluorescent bacteria in place of the latex beads, or performing *in vivo* studies.

Surprisingly, we found that that in our *in vitro* assay, Ig+ cells engulfed a significantly larger number of beads than the Ig− cells in our samples, with Ig+ FITC+ cells exhibiting a nearly 10-fold increase in fluorescence relative to Ig− FITC^{+} cells. The *in vivo* relevance of this finding is not clear and could simply be an artifact of the assay. Further characterization of the Ig− cell population is also necessary as this was an incidental finding and not the original goal of the study. Nonetheless, 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 the head-kidney lymphoid organ and peripheral blood leukocytes, phagocytic capacity 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) report that macrophages killed internalized bacteria significantly faster than phagocytic B cells. Therefore, it is possible that the main role of phagocytic B cells may be the clearing of microbes in lower jawed vertebrates, while in mammals, the main role of phagocytic B cells may be to present of low amounts of particulate antigen to T cells (Parra et al., 2012).

In conclusion, our study demonstrates that biologically relevant temperatures can affect behavior of immune cells in *T. scripta*. Since our studies were conducted *in vitro* at the various temperatures, we were not able to discern other effects of temperature such as number of cells produced from bone marrow and migration to cells to different tissues. Future *in vivo* studies will help address these other potential effects. Although phagocytosis was not impacted by temperature, antibody secretion was, and this may provide important clues as to how these aspects of immunity are utilized by animals in the field. For example, the fact that phagocytosis was unaffected by temperature in either cell population may suggest that phagocytosis is an “essential” immune process that must be maintained under a wider variety of environmental conditions. Recent work in the sea bass (*Dicentrarchus labrax*) indicates that phagocytic activity is decreased by early exposure to 17β-estradiol (Seemann et al., 2016), whether phagocytic activity in adult *T. scripta* is also affected by estrogens is unknown, but endocrine state is known to affect a wide variety of immune responses in vertebrates (Klein, 2000; Casto et al., 2001; Lang, 2004). In mammals, estrogens can affect cells from across all branches of the immune system, including phagocytic activity of macrophages and B cell function (Khan and Ahmed, 2016), and recent research suggests that antibody expression may be directly affected by estrogens (Jones et al., 2016). Given the potential for estrogens to affect immunity, it is clear that studies in non-model systems are needed to better understand the nature of endocrine-immune interactions across taxa. Temperature did affect antibody secretion, and this may allow an individual to modulate this aspect of immunity behaviorally in the field. For example, aquatic turtles, such as *T. scripta* routinely bask as part of their thermoregulatory repertoire and this behavior is thought to be associated with the initiation behavioral fever in reptiles (Monagas and Gatten, 1983; do Amaral et al., 2002). Our data suggests that turtles can enhance their antibody secretion by modulating their body temperature, but that there is an optimal range of temperatures for this response. Future work should explore how the temperatures reported herein compare to temperatures of animals in the field, to the optimal temperatures of growth for their pathogens, and the potential for these processes to be modulated by the endocrine state of the individual. Further, studies should

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incorporate aspects of endocrine function to address questions such as does endocrine status interact with thermal biology to affect immune function? In many species of freshwater turtles, females produce multiple clutches of eggs within a nesting season, with concomitant changes in steroid hormone levels and basking (or thermal) preferences, but we know relatively little about how such factors interact with immune responses in the female or her offspring. Only by taking a multifaceted approach, can we begin to understand the complex interactions between the endocrine and immune systems.

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