Humoral immune responses are maintained with age in a long-lived ectotherm, the red-eared slider turtle.

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Abstract

Aging is typically associated with a decrease in immune function. However, aging does not affect each branch of the immune system equally. Because of these varying effects of age on immune responses, aging could affect taxa differently based on how the particular taxon employs its resources towards different components of immune defense. An example of this is found in the humoral immune system. Specific responses tend to decrease with age while non-specific, natural antibody responses increase with age. Compared with mammals, reptiles of all ages have a slower and less robust humoral immune system. Therefore, they may invest more in non-specific responses and thus avoid the negative consequences of age on the immune system. We examined how the humoral immune system of reptiles is affected by aging and investigated the roles of non-specific, natural antibody responses and specific responses by examining several characteristics of antibodies against lipopolysaccharide (LPS) in the red-eared slider turtle. We found very little evidence of immunosenesce in the humoral immune system of the red-eared slider turtle, which supports the idea that non-specific, natural antibody responses are an important line of defense in reptiles. Overall, this demonstrates that a taxon's immune strategy can influence how the immune system is affected by age.

Introduction

Immunosenence, which is a decrease in immune function with increasing age, is a common observation in vertebrates. This process can have important consequences for an organism; for example, an increase in mortality and morbidity in elderly humans is commonly attributed to a decrease in immune function (DiCarlo et al., 2009). However, the aging process does not affect every branch of the immune system in the same manner (Palacios et al., 2007, Ujvari and Madsen 2011). This is highlighted in the humoral immune system, where it has been found in a number of taxa that specific antibody production decreases with age, while non-specific, polyreactive antibodies increase with age (Frasca et al., 2008). Because of these varying effects of age on immune responses, aging could affect taxa differently based on how the particular taxon employs its resources towards different components of immune defense.

An example of how taxa can vary their utilization of immune defenses is found in the differences in timing of the humoral response to antigen exposure in mammals and reptiles. In mammals, the specific humoral response is produced by a subset of B cells called B-2 cells. When a mammal is exposed to an antigen, it will produce antigen-specific antibodies of moderate affinity after a lag period of about one week and peak production at 2 weeks after exposure. After this first exposure to an antigen, memory cells are formed and the lag time to antibody production is reduced upon a second exposure to the antigen, and antibody titers and affinity are increased (Coico et al., 2003). Compared to their mammalian counterparts, the specific humoral system of reptiles is slower and less robust (reviewed in Zimmerman et al., 2010a). Like mammals, reptiles produce antibodies after a lag time of about one week, but production often does not peak until 6 to 8 weeks later. If exposed for a second time to the same antigen, reptiles do not exhibit a memory response typical of mammals. While the lag time is reduced, titers are not increased compared to the primary response. It is generally considered that affinity maturation does not occur in ectotherms, including reptiles, but a small increase in affinity has been found in response to immunization in the rainbow trout, *Oncorhynchus mykiss* (Cain et al., 2002). B cell subsets have not yet been identified in reptiles due to a lack of specific reagents.

Given the less robust antibody response of reptiles, they may rely instead on production of non-specific antibodies in the form of natural antibodies (NAbs). While NAbs have been identified in a variety of taxa, including reptiles, most available information comes from studies of mammals. Natural antibodies are produced by a subset of B cells known as B-1 cells and can be produced constitutively at low levels in the absence of antigen or induced in response to certain antigens including microbial components such as lipopolysaccharide (LPS) and phosphocoline (Baumgarth et al., 2005; Yang et al., 2007), though the antibody produced in response to antigen stimulation is of lower affinity than that produced by B-2 cells. Natural antibodies are polyreactive to evolutionarily conserved components of pathogens, have low binding affinities, and play active roles in triggering both innate and adaptive immunity (Ochsenbein and Zinkernagel 2000). They also bind to a variety of self-antigens, which allows them to play housekeeping roles such as the clearing of apoptotic cells (Binder et al., 2008), and may even act to reduce the risk of autoimmune diseases (Boes et al., 2000). Though they can be of the IgA or IgG isotype, most NAbs are of the IgM isotype and are secreted as pentamers. The pentameric structure

allows them to be a strong activator of complement, and permits more efficient production of antigen-antibody complexes, which helps target antigens to lymphatic tissues and improve adaptive responses (Boes 2000).Both mammals and reptiles show an age-related decrease in specific, B-2 cell produced antibodies and an increase in non-specific, B-1 cell produced NAbs. In humans, this reduction in specific antibody responses has been viewed as a contributor to the rise in mortality and morbidity with increasing age, and while there is also an increase in NAbs with age in humans, they do not appear to be able to compensate for the loss of the specific antibodies (McGlauchlen and Vogel 2003). Interestingly, the less robust specific immune response of reptiles of all ages, combined with the increase in NAbs with age, may constitute an overall positive change in immune defense (Ujvari and Madsen 2011). However, little is known about antibody production in reptiles beyond quantity. Examining other antibody characteristics in addition to quantity would help us to decipher if changes in humoral immune responses with age differentially affect taxa with varying immune strategies. One important characteristic to consider is strength of binding. Affinity is the measure of how well one particular antibody combining site binds to a single antigenic epitope, while avidity is a measure of how well the total antibody population binds to the entire multivalent antigen. Thus avidity can be measured from a plasma sample containing antibodies with different specificities. Knowing the avidity of an immune response can help determine if the antibodies are NAbs (affinity ranges from 10^{-3} to 10^{-7}; Notkins 2004) or specific antibodies produced in response to antigen stimulation (affinity averages 10^{-10} M^{-1}; Foote 1995). Another important characteristic that has been understudied in reptilian immunology is antibody production by individual B cells. Measuring the function of antibody secreting cells (AbSCs) allows us to determine how many cells are producing antibodies and how many antibodies are being produced by each cell. These characteristics are measured frequently in traditional immunology, but thus far have not been examined in any eco-immunology study and can provide a fuller picture of humoral immune functioning.

We examined how the humoral immune system of reptiles is affected by aging and investigated the roles of natural/ B-1-like antibody and specific/B-2-like responses by examining several characteristics of antibodies against LPS. Lipopolysaccharide, which is a component of Gram negative bacteria, is a T-independent antigen meaning that a B cell can respond to the antigen without direct contact with a T cell. In mammals, the humoral immune system responds to LPS with NAbs produced in the absence of antigen stimulation by B-1 cells, antibodies produced as a direct result of antigen stimulation by LPS binding directly to an antigen-specific membrane Ig by B-1 or B-2 cells, or lastly, polyclonal antibody production through a Toll-like receptor pathway by B-1 or B-2 cells (Fig 1). All receptors are constitutively expressed, so a B cell can be activated by any mechanism at any time. The isotypes of both specific and NAbs that bind to LPS include IgA, IgG, and IgM, though NAbs are predominantly IgM. Though germinal centers typically do not form in response to LPS, specific antibodies still have a higher affinity to LPS than NAbs (Bruderer et al., 1992). In mice, an age-associated decrease in antibody responses to T-independent antigens, including LPS, has been reported (Chelvarajan et al., 2005). A similar result was found in a natural population of tree swallows (*Tachycineta bicolor*), where young and mid-age females injected with LPS mounted a specific antibody response, while the oldest individuals did not (Palacios et al., 2011).

The present study was conducted on a natural population of red-eared slider turtles (*Trachemys scripta*). The slider is an excellent model for examining humoral responses to LPS with age for several reasons. Sliders grow throughout their lifetime, so plastron length can be used as a proxy for age (Ernst et al., 1994). Due to the challenges of following a turtle population over several decades, it is difficult to give an average life span for the slider turtle, though it is generally estimated to be around 30-40 years (Ernst et al., 1994). Studies in the closely related painted turtle found that they could live upwards of 60 years (Congdon et al., 2003), so it is possible that 40 years is a conservative estimate for sliders. We have previously reported an increase in total Ig levels with age in the slider (Zimmerman et al., 2010b). In addition, sliders are likely to be exposed to LPS in their natural environment, with likelihood of exposure varying seasonally. A previous study in our population has found a high frequency of *Salmonella* prevelance in adult turtles, with prevalence increasing as temperatures increase, and remaining high through the remainder of the active season (Holgersson 2009). It is reasonable to suggest that other bacteria may also increase in prevalence as temperatures increase. Because of this, and the fact that we also have found significant seasonal variation in a number of immune measures (Zimmerman et al., 2010b), we sampled immune responses at three points throughout the active season. Importantly, we have now validated an ELISpot assay to examine the properties of AbSCs along with an avidity assay to measure antibody binding. These new assays, along with the ELISA that we have previously validated and the common practice of using blood smears to examine leukocyte populations, will allow us to gain a fuller understanding of humoral immune responses of sliders.

A wide range of studies in a variety of vertebrate taxa, including mammals, birds, and reptiles have found that specific antibodies decrease with age while natural antibodies increase with age (Candore et al., 1997, Parmentier et al., 2004, Ujvari and Madsen 2005, Lavoie 2006, Benatuil et al., 2008, Frasca et al., 2008,Sparkman and Palacios 2009, Ujvari and Madsen 2011). This pattern, along with the typically slow and less robust specific responses of reptiles lead us to hypothesize that red-eared sliders utilize a predominantly NAb-based response, and thus would not show a deficit in humoral immune defenses with age. Because both NAbs and specific antibodies can be produced in response to antigenic stimulation, we also hypothesized that the humoral response would change across the active season as the animals naturally encounter LPS in the environment, regardless of whether the responses were predominantly NAb-based or specific. If natural antibodies/B-1 cell like immunity are predominant in sliders, we expect immune measures to increase or show no change in function with age. Thus, we would predict that the amount and avidity of antigen-specific antibody as well as total Ig and AbSC number and function should be preserved. Alternatively, if specific responses are predominant in sliders, we would expect immune measures to decrease with age. Thus we would predict that AbSCs would show a reduced response to LPS stimulation and that avidity, LPS-Abs, and percentage of lymphocytes would decrease with age. We also predict that LPS-Abs, total Ig, avidity, the number of AbSCs, the amount of antibody produced by each cell, and the percentage of lymphocytes would increase through the active season as the turtles are more likely to be exposed to LPS. To examine our hypotheses, we determined both the quantity and quality of LPS humoral responses in the slider by measuring the amount of antibodies that can bind to LPS (LPS-Abs), total Ig, antibody avidity to LPS, the properties of AbSCs, and the percentage of leukocytes that are lymphocytes. While the assays used in this study examine variation in both NAbs and specific antibodies, the prediction for the pattern of responses with

age and across the active season differ and thus allows us to distinguish between the two types of responses.

Methods

This study was conducted under IACUC approval (protocol 04-2010) at Banner Marsh State Fish and Wildlife Area, Fulton Co. IL, on a natural population of red-eared sliders from May to August 2011 (IDNR permit NH11.2084). Adult male and female turtles were trapped at three points in the active season: early May, late June, and late August. At time of capture any unmarked individuals were uniquely marked, and plastron length was measured to the nearest 0.1 mm. No individual was used in more than one collection period. Approximately 1 ml of blood was taken from the caudal vein using an EDTA-coated syringe. Plasma was separated from 500 µl of blood by centrifugation at the field site and kept on ice until it was taken to Illinois State University and stored at -20°C. A blood smear was made using 7 µl of whole blood. The remaining blood was diluted approximately 1:2 with a 75% RPMI-25% EDTA mixture for use in the ELISpot.

ELISA

An ELISA was used to measure total Ig and anti-LPS abs (Zimmerman et al., 2010b). Polystyrene 96-well plates (Costar) were coated with either 100 µl of 25 µg/ml dilution of unlabeled anti-turtle light chain antibody (HL673; University of Florida Hybridoma Facility) or 100 µl of a 20 µg/ml solution of LPS (from *Salmonella enterica* serotype typhimurium; Sigma) in PBS and incubated overnight at 4°C. Plates were washed three times for 3 min with 200 µl per well of PBS-1% BSA-0.05% Tween buffer (PBS-BSA-T), which also serves as a blocking step. Plasma samples were diluted 1:1000 for total Ig and 1:50 for LPS-specific Abs in PBS-BSA-T. Goat serum was added as a negative control. Plates were incubated at room temperature for at least 1 hour then washed as before. One hundred microliters of a 1:500 dilution of anti-turtle antibody conjugated to biotin was added to each well, and plates were incubated and washed as before. One hundred microliters of streptavidin-HRP (Southern Biotech; diluted 1:1000 in PBS-BSA-T) was added to each well, and plates were incubated and washed as before. Wells were washed once with 100 µl double distilled water before 100 µl of ABTS (Southern Biotech) substrate powder dissolved in ABTS solution was added to each well. The plate was read at 17 min after adding substrate using a Powerwave 340 plate reader (BioTek Inc) at 405 nm.

Avidity

Avidity was measured using a competitive ELISA. Fifty microliters of diluted test serum (1:50) was added to six 50 _l aliquots of serial diluted LPS (0.08-5 mg/ml) and two tubes of 50 µl of PBS only, and incubated for 18 hours at room temperature in glass tubes. An ELISA was then run as described above with the plates coated with LPS. Avidity values were calculated from the absorbances according to Friguet et al., (1985). Thus, for the individual turtle plasma sample, six values (one from each tube used in the serial dilution) were obtained by subtracting the absorbance in a well from the average absorbance of the wells that came from the tubes that contained no antigen and then dividing that number by the molarity of the antigen present in the glass tube during incubation. Those six values were

then plotted and a linear regression was used and the slope of the line was the dissociation constant. Avidity was then recorded as the reciprocal of the dissociation constant.

ELISpot

Leukocytes were isolated over a Percoll gradient (Harms et al., 2000). Wells of MultiScreen-IP ELISpot plates (Millipore) were coated with 20 _g/ml dilution of unlabeled anti-turtle light chain overnight at 4˚C. The wells were then washed with PBS and blocked for two hours at 37˚C with RPMI (Hyclone) supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin/glutamine, 0.5% 2-mercaptoethanol, and 0.5% sodium pyruvate (cRPMI). A known number of leukocytes in 100 _l cRPMI were added to each well. The number of leukocytes plated ranged from 10^5 to 10^6 cells/well. An additional 100 µl of cRPMI or cRPMI supplemented with 40 µg/ml LPS was added to each well to observe spontaneous antibody production and to stimulate antibody production, respectively. Cells from each turtle were given each treatment in duplicate wells. The cells were then incubated at 31˚C in 5% CO_2 for five days. The cell remains stationary, while any antibody produced remains attached to the capture antibody. After five days, wells were washed with PBS-0.01% Tween to remove the cells. The antibody remains attached and was detected with anti-turtle light chain conjugated to biotin, followed by streptavidin-HRP. AEC substrate (BD Biosciences) was used to develop the wells, which leaves a red spot where antibodies have been detected, with each spot representing one cell. The size of spot indicates how much antibody was produced by each cell. The number of spots was counted to determine the number of AbSCs per 10^5 cells, based on the number of cells added to the well. Size of spots was determined using ImageJ software.

Blood Smears

Previous studies in a number of vertebrates have shown that leukocyte ratios, specifically heterophil:lymphocyte ratio, can change significantly as a result from handling stress (reviewed in Davis et al., 2008). A previous study in our lab demonstrated that heterophil: lymphocyte ratio did not vary from baseline after turtles were restrained for two hours (unpublished data). All blood samples used in this study were taken within 90 minutes of the turtle being removed from the trap. Blood smears were fixed in methanol and stained with Wright-Giesma. The leukocyte profile was determined by counting a total of 100 leukocytes and calculating the percentage of lymphocytes, basophils, eosinophils, monocytes, and heterophils. The number of white blood cells per 10,000 red blood cells was determined as a proxy for white blood cell count (WBC) (Norte et al., 2008).

Statistical Analysis

The effect of date of sampling, sex, and plastron length on number of AbSCs when stimulated and cultured in media only, spot size for both conditions, avidity, LPS-Abs, total Ig, and WBC was examined using ANCOVAs. ANCOVA was chosen rather than MANCOVA because sample size did not permit the inclusion of all immune measures and we had no *a priori* reasons to group certain immune measures. The number of AbSCs for stimulated and media-only conditions and total Ig were square root transformed while avidity was log transformed. All interactions were tested and non-significant interactions involving plastron length were removed from the model. Tukey's *post-hoc* tests were

conducted. For the leukocyte profile, a MANOVA was run including the proportions of each cell type as dependent variables. Date, sex, and plastron length were included as main effects. All interactions were tested and found to be non-significant so they were removed from the models. A paired T-test was used to compare spot size and number of AbSCs between stimulated and media-only wells.

Results

First, LPS binding antibody concentrations were examined (n = 56). All animals tested produced detectable anti-LPS antibodies as measured by ELISA. As animals were not deliberately immunized, either pre-existing polyreactive NAbs were detected or animals had naturally encountered the antigen. Quantity of LPS-Abs significantly increased with plastron length (Fig 2a; $F_{1, 49} = 5.09$, $p = 0.029$) and across the active season (Fig 3a; $F_{2, 49} = 3.34$, $p = 0.044$). There was no correlation with sex on LPS-Abs ($F_{1, 49} = 0.17$, $p = 0.69$) and the date by sex interaction was not significant ($F_{1, 49} = 1.39$, $p = 0.26$). The avidity of these anti-LPS antibodies was also measured by ELISA as described (n = 48). Avidity did not vary with plastron length (Fig 2b; $F_{1, 41} = 1.69$, $p = 0.20$), date (Fig 3b; $F_{2, 41} = 1.40$, $p = 0.26$), or sex ($F_{1, 41} = 3.05$, $p = 0.09$). The date by sex interaction was also not significant ($F_{2, 41} = 1.22$, $p = 0.31$).

Next, total Ig levels were examined (n = 55). Total Ig levels ranged from 0-9.7 mg/ml. Total Ig varied significantly by date (Fig 3c; $F_{2, 48} = 7.68$, $p = 0.0013$) and sex ($F_{1, 48} = 4.26$, $p = 0.045$), with males having significantly higher total Ig levels than females. There was no correlation with plastron length (Fig 2c; $F_{1, 48} = 1.87$, $p = .18$) and the date by sex interaction was not significant ($F_{1, 48} = 0.25$, $p = 0.78$).

The number of spontaneous AbSCs in the blood ranged from 0-13.17 cells per 10^5 leukocytes (n = 40) and the number of LPS-stimulated AbSC ranged from 0-22.73 cells per 10^5 leukocytes (n = 44). For cells cultured in media alone in the ELISpot, the number of AbSCs and average spot size did not vary with plastron length (Fig 2d; $F_{1, 34} = 0.02$, $p = 0.88$; $F_{1, 27} = 0.25$, $p = 0.62$, respectively), date (Fig 3d; $F_{2, 34} = 0.57$, $p = 0.57$; $F_{2, 27} = 0.44$, $p = 0.65$, respectively), sex ($F_{1, 34} = 1.45$, $p = 0.24$; $F_{1, 27} = 0.87$, $p = 0.36$, respectively). The date by sex interaction was also not significant ($F_{1, 34} = 0.83$, $p = 0.37$; $F_{1, 27} = 2.24$, $p = 0.15$). For the cells cultured with LPS in the ELISpot, the average spot size was significantly larger in wells that we stimulated with LPS than in wells that received media only (LPS mean ± SE = 35.43 ± 2.18, media only mean ± SE = 28.68 ± 1.79; $T_{32} = 3.49$, $p = 0.001$), and the total number of Ig secreting cells was increased by LPS (LPS mean ± SE = 3.62 ± 0.66, media only mean ± SE = 2.74 ± 0.46); $T_{38} = 2.345$, $p = 0.024$). However, the number of LPS-stimulated AbSCs and spot size did not vary with plastron length (Fig 2e; $F_{1, 38} = 0.00$, $p = 0.96$; $F_{1, 30} = 0.06$, $p = 0.81$), date (Fig 3e; $F_{2, 38} = 1.12$, $p = 0.34$; $F_{2, 30} = 1.30$, $p = 0.29$, respectively), or sex ($F_{1, 38} = 1.85$, $p = 0.18$; $F_{1, 30} = 0.57$, $p = 0.46$, respectively). The date by sex interaction was also not significant ($F_{1, 38} = 1.14$, $p = 0.29$; $F_{1, 30} = 0.31$, $p = 0.58$, respectively).

Finally, WBC differential counts were examined (n = 54). Total WBC number significantly decreased with increasing plastron length (Fig 2f; $F_{4, 49} = 4.45$, $p = 0.0399$) but did not vary with sex ($F_{4, 49} = 3.42$, $p = 0.07$) or date (Fig 3f; $F_{4, 49} = 0.76$, $p = 0.48$). A significant effect of date on leukocyte profile was detected (Pillai's trace: $F_{10, 92} = 3.23$, $p = 0.001$). A significant axis was found (Table 1; eigenvalue = 0.7858, $p = 0.0006$) and it explained 90.07% of the variation among dates. This was mostly driven by eosinophils and heterophils, and to a lesser extent, monocytes (Table 1 and Table 2). Lymphocytes

decreased across the active season, but did not explain much of the variation. There was no significant effect of sex (Pillai's trace: $F_{5, 45} = 0.1.81$, $p = 0.13$) or plastron length (Pillai's trace: $F_{5, 45} = 0.25$, $p = 0.94$) on the leukocyte profile.

Discussion

Aging negatively affects many aspects of the immune response, including the specific antibody response. However, some immune measures are not negatively affected by age, and may even increase, such as the non-specific NAb response (Frasca et al., 2008). In reptiles of all ages, the specific antibody response is slower and less robust than its mammalian counterpart (Zimmerman et al., 2010a). This may force them to rely more heavily on NAbs to deal with pathogens. Thus, an increase in NAbs with age may be viewed as a positive change in immunity with age in reptiles (Ujvari and Madsen 2011).

The primary goal of the current study was to examine the effect of age on humoral immune responses to LPS in a long-lived reptile, the red-eared slider turtle. We hypothesized that red-eared sliders utilize a predominantly NAb response, and thus would be less affected by changes in humoral immune defenses with age. LPS-Abs significantly increased with age. In addition, the avidity of antibodies to LPS was in the range of NAbs (Ochsenbein and Zinkernagel 2000). Thus we are likely measuring predominantly NAbs that are binding to LPS with a low avidity, rather than measuring highly specific antibodies that would bind to LPS with a high avidity. In addition, the number of AbSCs that responded to LPS stimulation did not vary with age, nor did the amount of antibody produced by each cell. Thus, our turtles seem to have an increase in NAb, B-1-like immunity with age, but the specific, B-2-like response to LPS was not significantly impacted by age. While an increase in NAbs with age has been reported in mammals, birds, and reptiles (Candore et al., 1997, Parmentier et al., 2004, Benatuil et al., 2008, Sparkman and Palacios 2009, Ujvari and Madsen 2011), studies in these same taxa have also reported a decrease in specific antibodies with age (Ujvari and Madsen 2005, Lavoie 2006, Frasca et al., 2008, Ujvari and Madsen 2011). These studies on specific immune responses include a wide range of both T-dependent and T-independent antigens, including LPS. Thus, the red-eared slider immune system appears to be unusual in that there is no evidence of declining humoral responses during aging. However, it should be noted that many of these studies measured antibody production in vivo after immunization while our study utilized in vitro measures. Further work is currently being conducted to examine the effect of age on antibody production in vivo in red-eared sliders.

So what could be allowing the sliders to maintain immune responsiveness to LPS as they age? In mammals, there is an age-related shift from a prominently B-2 response to a B-1 response. In some animals, such as rabbits, all B cells are B-1 cells. In general, B-1 cells are thought to maintain their function with increasing age, while B-2 cells do not. For example, a previous study in mice found that a subset of B-1 cells known as CD5+ B-1 cells maintained their ability to respond to antigen with age while CD5- cells did not (Hu et al., 1993). Thus, it is possible that if all of the turtle B cells were CD5+ B-1 cells, their response to LPS would not change significantly during aging. Currently cell markers do not exist that would allow us to determine what type of B cells are present in red-eared sliders, but several lines of evidence suggest they are B-1-like. First is the increase in total Ig reported previously and LPS-Abs reported here. The avidity for LPS reported in this study (10^{-4} to 10^{-6}) is within range of the avidity for

natural antibodies (10^{-3} to 10^{-7}; Notkins 2004). In addition, we have also found an increase in antibodies to several antigens the turtles should not have been previously exposed to including keyhole limpet hemocyanin, ovalbumin, and hen egg white lysozyme. These levels also increase with age (unpublished data). Recently, it has been reported that B-1 cells of mice are capable of phagocytosing antigen while B-2 cells did not have this ability (Parra 2011). We have previously described phagocytic B cells in red-eared sliders, indicating that these may be of the B-1 type (Zimmerman et al., 2010c). In mice, the phagocytic B cells were located only in the peritoneal cavity, but the phagocytic B cells of the turtles were circulating in the periphery, which suggests that the B cells used in the current study were B-1-like cells as well. Many of the negative effects of age on the humoral immune system are attributed to the shift from predominately B-2 cells to B-1 cells (Weksler and Szabo 2000). Thus, if all B cells in the red-eared slider are B-1-like, then their humoral immune responses would demonstrate fewer negative changes with age. Therefore, we hypothesize the antibody produced in response to LPS is polyreactive, of a low-affinity, and produced by a B-1-like cell (Fig 4). Although we are unable to determine through what pathway the B-cells are activated (see Fig 1), we hypothesize that it is through the Toll-like receptor pathway. It is possible that the B-cell could be activated through an antigen-specific pathway, but we feel that is unlikely because studies in mammals have demonstrated that signaling through surface Ig fails to activate B-1 cells (Berland and Wortis 2002).

We did find a decrease in WBC count with age, and this could potentially negatively affect other types of immune responses by directly limiting the cells available for innate responses and also by having fewer cells available to produce cytokines that direct immune responses. However, in a previous study on the same population of turtles, we found no effect of age on bactericidal capacity or the delayed hypersensitivity response to the mitogen phytohemagglutinin (Zimmerman et al., 2010b), again suggesting limited impacts of age on immune responses of sliders. Importantly, though WBC count decreased with age, the distribution of leukocytes did not change with age.

We have previously reported an increase in total Ig levels with age in red-eared sliders (Zimmerman et al., 2010a), but did not find the same pattern in this study. At present, we cannot provide an explanation for the variation in our two datasets from the same population but are continuing to monitor total Ig levels. There may be year-to-year variation in the levels of Igs produced, which could coincide with local pathogen pressures.

A secondary goal of this study was to examine seasonal variation in the humoral immune response. We predicted all immune measures would increase across the active season. Response to stimulation with LPS and antibody avidity did not vary across dates, while LPS-Abs and total Ig increased. NAbs can be produced as a result of stimulation by antigens, so the increase in total Ig and LPS-Abs could have resulted from an increase in exposure to potential pathogens across the active season. This idea of increased pathogen exposure is supported by the changes in leukocyte profiles across the active season. The proportion of cells that were heterophils, eosinophils, and monocytes increased throughout the active season. These cell types are involved in the innate immune response to bacteria and parasites (Zimmerman et al., 2010a). Alternatively, because of the slow humoral immune response of reptiles, the increase in antibodies could be a result of a delay in response to antigen exposures early in the active season.

The leukocyte profile (Table 2) showed several differences from published hematological values for captive red-eared sliders. A previous study found that basophils and monocytes were the most common leukocytes while lymphocytes accounted for around 14% of the leukocyte population (Taylor and Kaplan 1961). We found heterophils and lymphocytes to be the most common cell types, with lymphocytes accounting for an average of 42% of the leukocyte population. The previous report is similar to other counts of captive turtles of various species (Metin et al., 2006, Metin et al., 2008), while our higher number is similar to what is found in most species of lizards, where heterophils and lymphocytes are the most common cell types (Fisse et al., 2004). It is unclear what could explain the different results, but many factors are known to alter leukocyte counts including nutritional status, parasite load, and season (Fisse et al., 2004).

As mentioned earlier, previous studies in a number of vertebrates have shown that capture stress can affect immune measures, including leukocyte ratios (Davis et al., 2008). A previous study in our lab demonstrated that corticosterone levels did not rise until after 60 minutes of restraint and that leukocyte distribution, including the heterophil: lymphocyte ratio, did not vary from baseline even after two hours of restraint (unpublished data). All blood samples used in this study were taken within 90 minutes of the turtle being removed from the trap and thus we do not expect variation in leukocyte profiles to be affected by capture stress. We believe our other immune measures are unlikely to be affected by capture stress as well because antibody measures examine antibodies that are already circulating in the animal, and are unlikely to change in the short time period we held the turtles before sample collection. Conceivably, short term B cell function, as measured by ELISpot, could be affected by capture stress, but we believe this is unlikely because the cells are washed prior to culturing for five days, and any hormones present in the sample should have been removed.

Interestingly, only total Ig varied between sexes, with males having higher levels. However, we have not found sex differences in any immune response measured in this study or a previous study in this species, which included total Ig (Zimmerman et al., 2010b). No sex differences may be expected for LPS-induced measures as a recent study has suggested that estrogens enhance T-dependent humoral responses but do not affect T-independent responses (Adori et al., 2010). For the other immune measures, including the number of spontaneously secreting AbSCs and total Ig, we would expect greater responses in females (Verthelyi and Ansar Ahmed 1998, Ansar Ahmed et al., 1999).

In conclusion, this study supports the idea that NAbs are an important line of defense in reptiles (Zimmerman, Vogel & Bowden 2010, Ujvari and Madsen 2011). LPS-Abs increased with age while response to stimulation with LPS did not vary with age. Thus, the humoral immune system of sliders may not change as dramatically with age as compared to mammals. An increase in NAbs with age may also be viewed as a positive change in immune function. This contrasts sharply to mammals, where a shift in humoral responses to a predominantly B-1 response is viewed negatively and in humans, is considered a factor in the increase in morbidity and mortality in the elderly (DiCarlo et al., 2009). We also identified seasonal changes in immunity that could be a result of increased pathogen pressure across the active season. Further, we validate the use of both avidity and ELISpot assays for assessing characteristics of antibodies in the red-eared slider turtle.

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Table 1. MANOVA summary statistics for effect of date on leukocyte profile.

				Standardized canonical coefficients				
Variance accounted	**d.f.**	***F*-value**	***P*-value**	**Lymph**	**Het**	**Bas**	**Eos**	**Mon**
90.07	10	3.54	0.0006	-0.432	-0.922	-0.462	-1.215	-0.732

Table 2. Leukocyte profile for each sampling period. Values represent the average percentage ± SE. The range of percentages for each sampling period is in parentheses.

Cell type	May	Late June	Late August
Lymphocyte	44.55 ± 0.73	43.66 ± 1.29	38.31 ± 0.66
	(39 – 50.49)	(40.19 – 56.19)	(34.95 – 42.86)
Heterophil	37.48 ± 0.73	38.4 ± 0.81	38.7 ± 0.56
	(30.1 – 44.55)	(33.03 – 42.16)	(33.64 – 42.57)
Basophil	5.6 ± 0.27	5.25 ± 0.23	6.6 ± 0.22
	(3.7 – 8.4)	(3.81 – 6.93)	(5.61 – 8.91)
Eosinophil	5.49 ± 0.25	6.21 ± 0.33	7.93 ± 0.46
	(2.94 – 7.69)	(3.67 – 7.92)	(5 – 11.54)
Monocyte	6.92 ± 0.39	7.23 ± 0.57	8.46 ± 0.53
	(3.77 – 10.78)	(4.76 – 11.43)	(4.85 – 12.15)

Figure Legends

Figure 1. Modes of B cell activation induced by LPS in mammals. I. LPS is a B cell mitogen that acts through TLR4 and CD14 to induce B cell antibody secretion independent of antigen specificity. This results in the activation of a large number of B cells and polyclonal Ig secretion. In this situation, B-cells A, B, and C would secrete antibody. II. LPS can also activate B cells in the traditional manner by binding directly to antigen-specific surface Ig. In this case, only LPS-specific B cells (B-cell B) are activated to produce Ig. III. Natural antibodies can be secreted in the absence of antigen stimulation. In this situation, B-cell D would spontaneously secrete low affinity antibodies with the ability to bind LPS.

Figure 2. Relationship between plastron length and a) LPS-Abs, b) avidity, c) total Ig, d) number of spots per 10^5 cells cultured in media only, e) number of spots per 10^5 cells cultured in the presence of LPS, and f) WBC.

Figure 3. Mean ± s.e.m. during May (n = 26), late June (n = 22), and August (n = 17) for a) LPS-Abs, b) avidity, c) total Ig, d) number of spots per 10^5 cells cultured in media only, e) number of spots per 10^5 cells cultured in the presence of LPS, and f) WBC. Sampling dates with different letters are significantly different ($p < 0.05$).

Figure 4. Hypothesized response to LPS in red-eared slider turtles. I. In the absence of antigen stimulation, some turtle B-cells secrete antibodies as demonstrated in the media-only wells of the ELISpot. In this case, B-cells D and F are secreting antibodies. All B cells produce a membrane bound antibody, but B cell E is not secreting antibodies. II. When stimulated with antigen, as demonstrated in the wells of the ELISpot that were cultured in the presence of LPS, more cells produce antibodies and each cell secretes more antibodies compared to the unstimulated wells. So in this case, B cells D, E, and F all produce antibodies. We hypothesize that the antibody produced from all B cells is a NAb-like antibody that is polyreactive and of low affinity. Though we cannot determine the mode of activation at this point, we also hypothesize that the B cell is most likely activated via the Toll-like receptor pathway as shown in B cell E.

I)

B-cell A

B-cell B

B-cell C

II)

B-cell A

B-cell B

B-cell C

III)

B-cell D

LPS

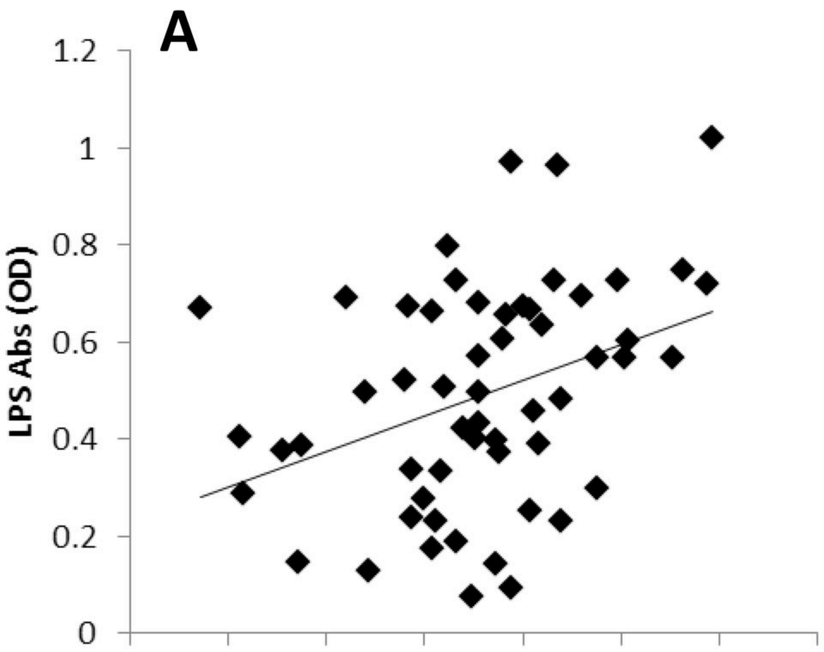
TLR4

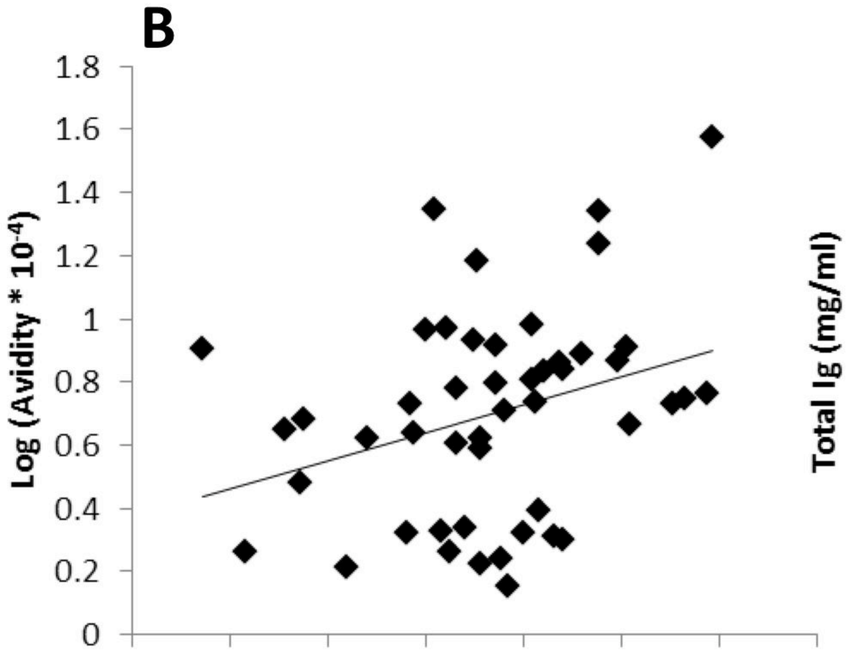
CD14

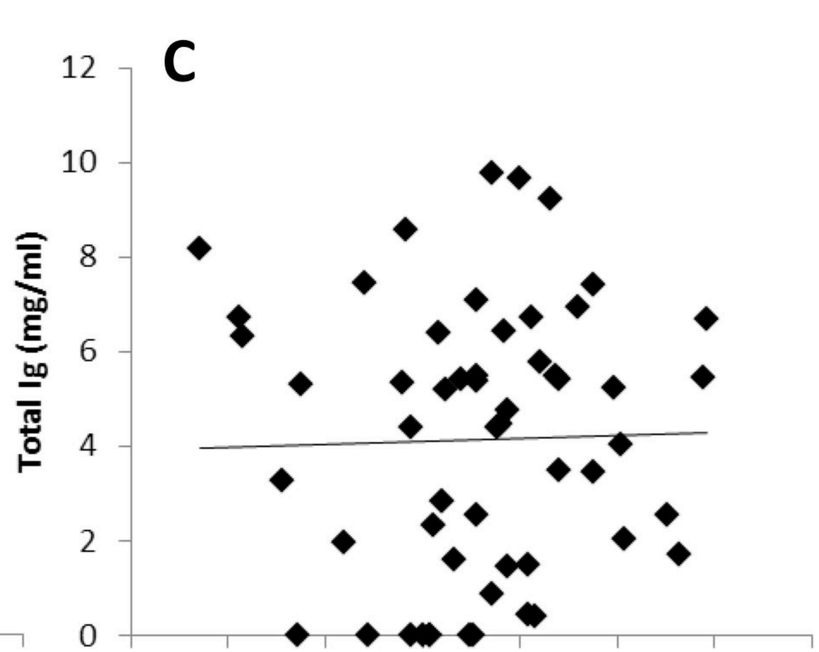
B-1 B cell

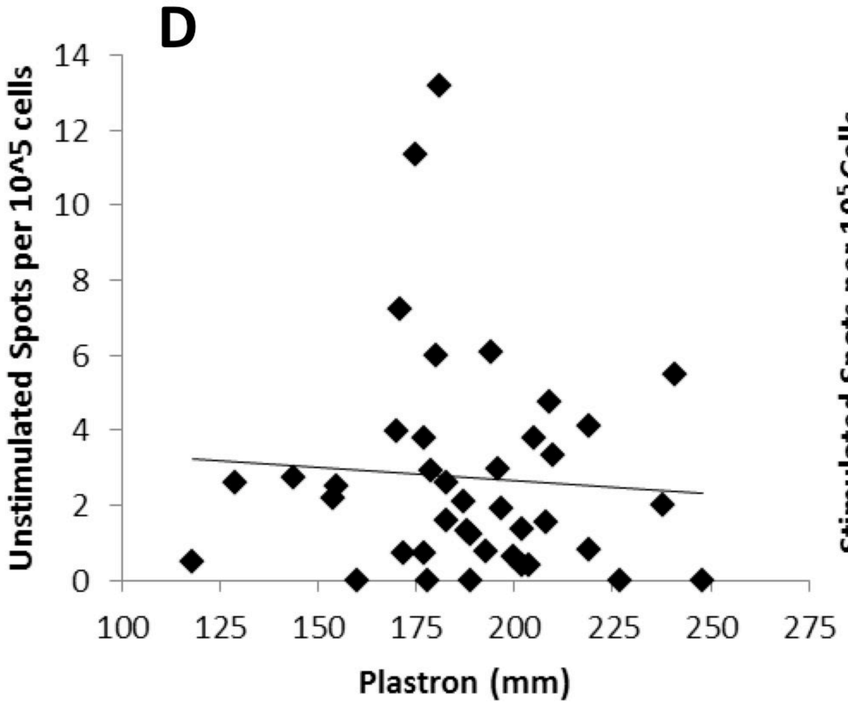
B-2 B cell

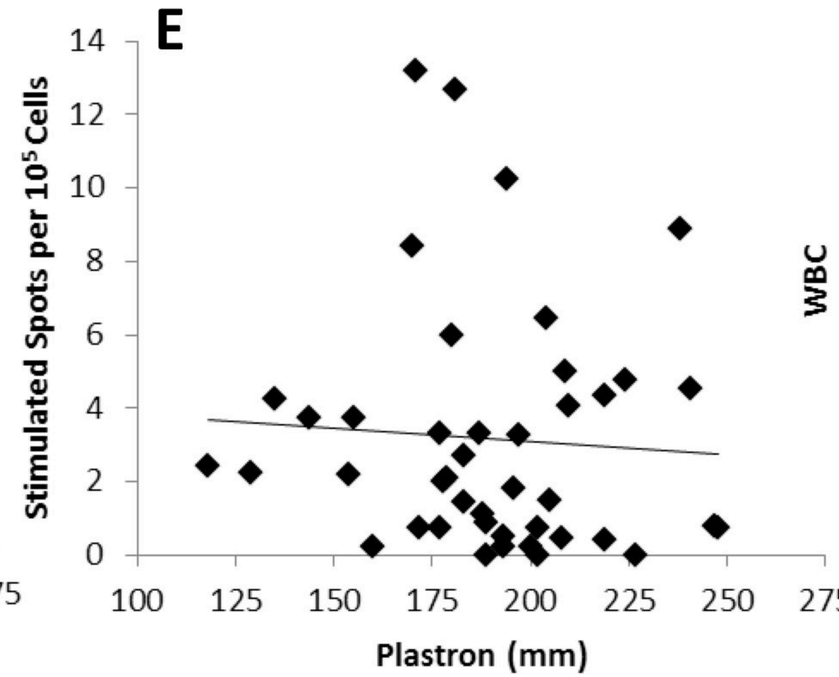
Antibodies with different antigen specificities

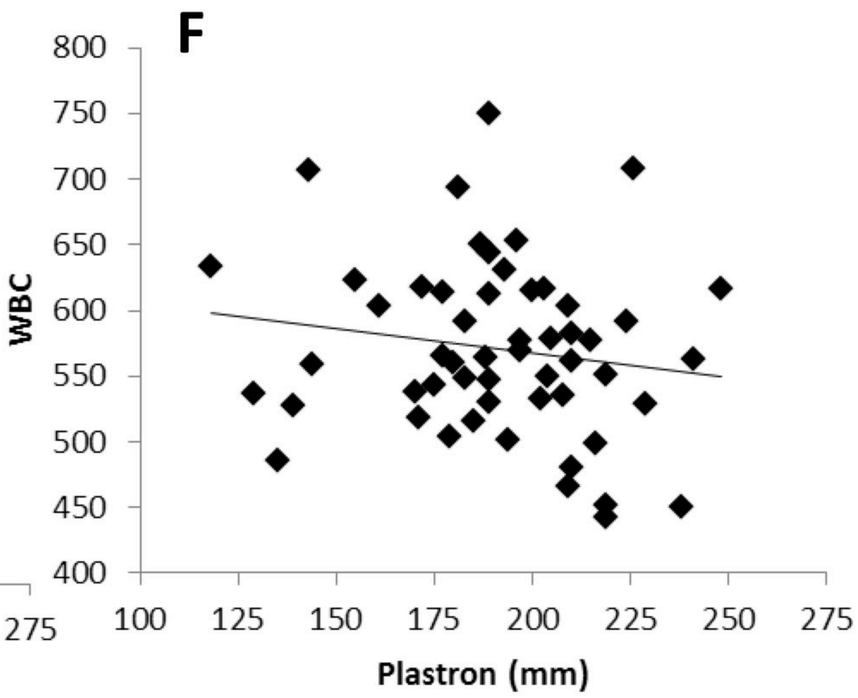

A
LPS Abs (OD)
0
0.2
0.4
0.6
0.8
1
1.2

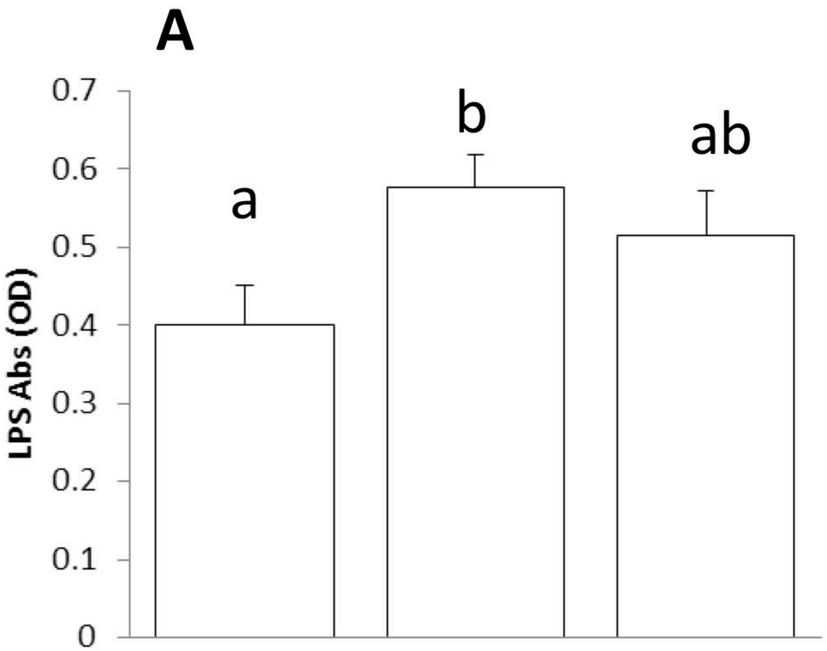

B
Log (Avidity * 10-4)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8

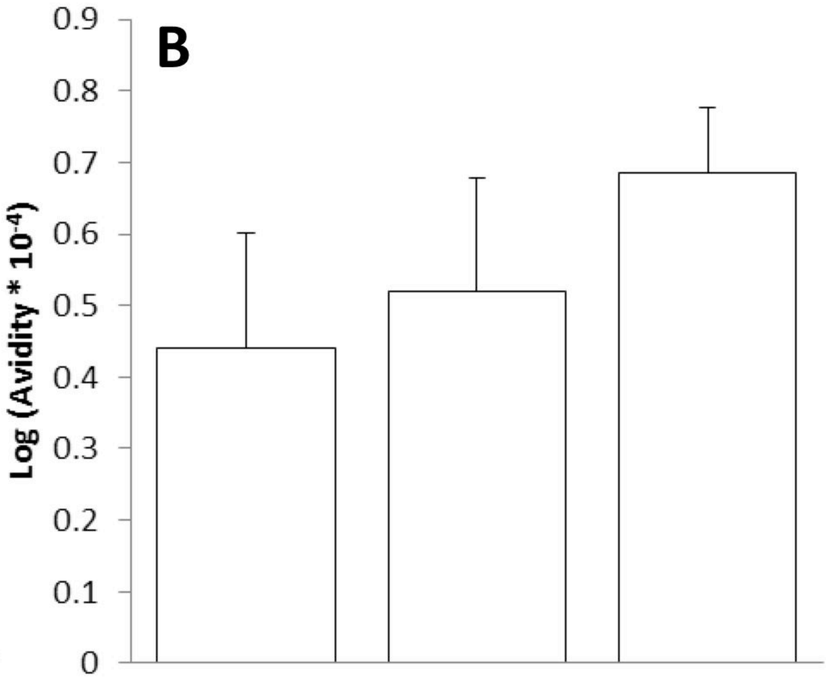

C
Total Ig (mg/ml)
0
2
4
6
8
10
12

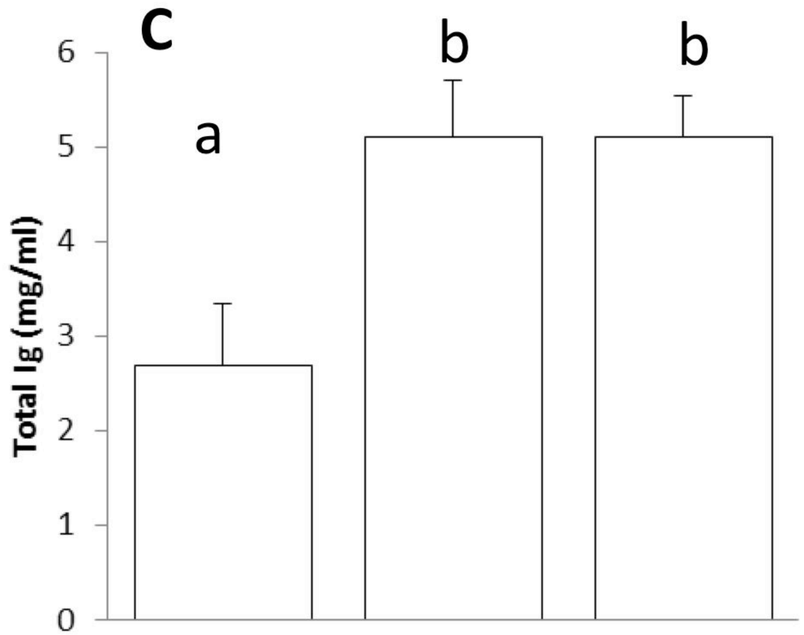

D
Unstimulated Spots per 10^5 cells
0
2
4
6
8
10
12
14
100
125
150
175
200
225
250
275
Plastron (mm)

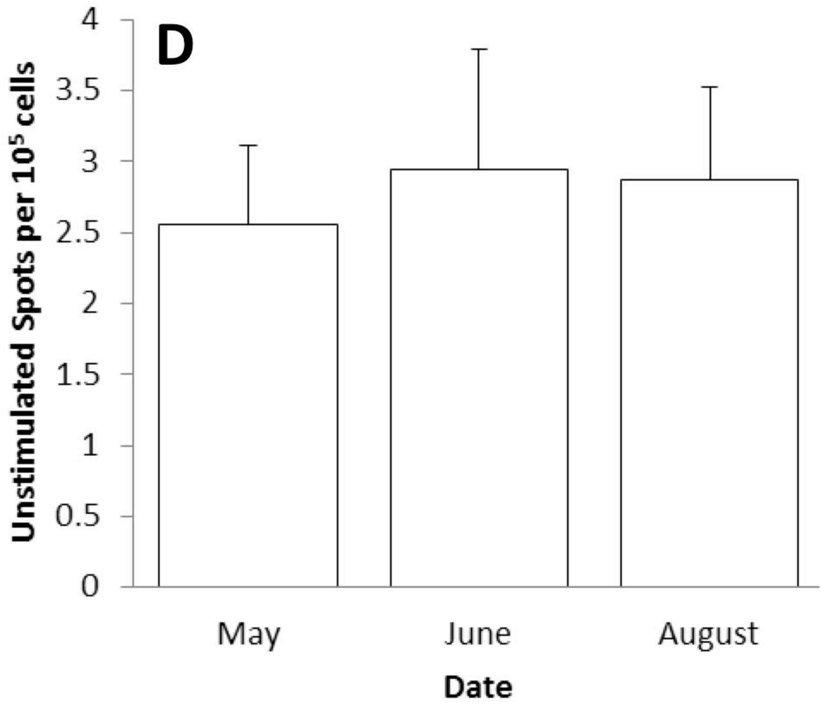

E
Stimulated Spots per 10^5 Cells
0
2
4
6
8
10
12
14
100
125
150
175
200
225
250
275
Plastron (mm)

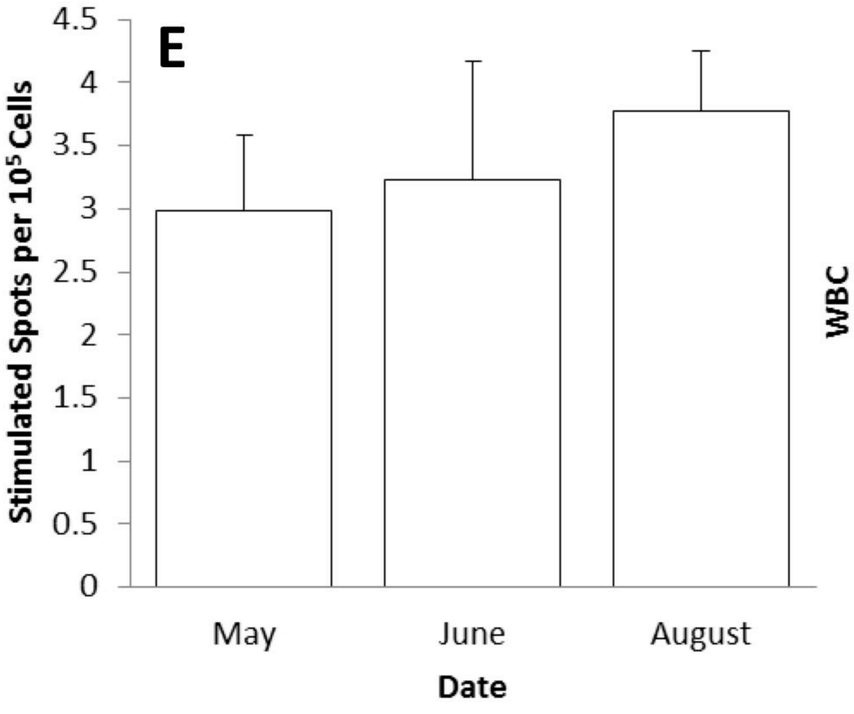

F
WBC
400
450
500
550
600
650
700
750
800
100
125
150
175
200
225
250
275
Plastron (mm)

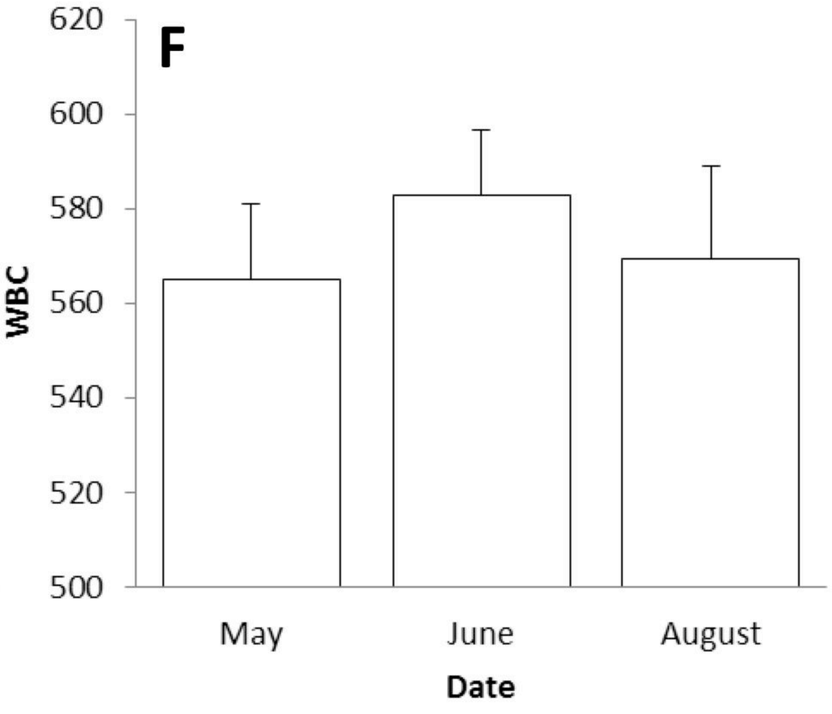

A
a
b
ab
LPS Abs (OD)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7


B
Log (Avidity * 10-4)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9


C
a
b
b
Total Ig (mg/ml)
0
1
2
3
4
5
6


D
Unstimulated Spots per 10^5 cells
0
0.5
1
1.5
2
2.5
3
3.5
4
May
June
August
Date


E
Stimulated Spots per 10^5 Cells
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
May
June
August
Date


F
WBC
500
520
540
560
580
600
620
May
June
August
Date

B-cell D

B-cell E

B-cell F

I. Unstimulated

B-cell D

B-cell E

B-cell F

II. Stimulated with LPS

LPS

TLR4

CD14

B-1 B cell

B-2 B cell

Polyreactive natural antibodies