Variation in the seasonal patterns of innate and adaptive immunity in the red-eared slider (*Trachemys scripta*)

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SUMMARY

The primary function of the immune system is to protect the organism from invading pathogens. In vertebrates, this has resulted in a multifaceted system comprised of both innate and adaptive components. The immune system of all jawed vertebrates is complex, but unlike the endothermic vertebrates, relatively little is known about the functioning of the ectothermic vertebrate immune system, especially the reptilian system. Because turtles are long-lived ectotherms, factors such as temperature and age may affect their immune response, but comprehensive studies are lacking. We investigated variation in immune responses of adult male and female red-eared sliders (*Trachemys scripta*) across the entire active season. We characterized seasonal variation in innate, cell-mediated and humoral components *via* bactericidal capacity of plasma, delayed-type hypersensitivity and total immunoglobulin levels, respectively. Results indicate that all immune measures varied significantly across the active season, but each measure had a different pattern of variation. Interestingly, temperature alone does not explain the observed seasonal variation. Immune measures did not vary between males and females, but immunoglobulin levels did vary with age. This study demonstrates the highly dynamic nature of the reptilian immune system, and provides information on how biotic and abiotic factors influence the immune system of a long-lived ectotherm.

Key words: immunocompetence, ectotherm, seasonal.

INTRODUCTION

The immune system of vertebrates evolved as a complex and multilayered defense against the near constant threat of parasitism (Kaufmann et al., 2002) that can deprive a host of vital resources and nutrients and negatively impact its survival (Behnke and Barnard, 1990; Gulland, 1995). All jawed vertebrates have both innate and adaptive components of immunity (Sunyer and Lambris, 1998). Innate immunity is the first line of defense; it is nonspecific, rapid, and does not need prior exposure to respond (Medzhitov and Janeway, 2000). By contrast, adaptive immunity is more specific, slower, and requires prior exposure in order to mount a full response (Coico et al., 2003). Adaptive immunity can be further broken down into cell-mediated and humoral branches. Cell-mediated immunity involves T cells, whereas humoral immunity involves the production of antibodies by B cells (Coico et al., 2003). The innate, cell-mediated, and humoral branches of the immune system interact to form a complex network that protects an organism from invading pathogens, but a growing body of evidence suggests that this protection comes at a resource cost (Lochmiller and Deerenberg, 2000).

Despite the fact that one may expect an organism to benefit from a constant, strong defense against invading pathogens, seasonal variation in immune responses is a common occurrence (Nelson, 2004; Martin et al., 2008). Factors that might explain the observed variation include trade-offs with other costly physiological processes, sex differences in investment in immunity, differences in life history strategies, and variation caused by seasonally occurring stressors (Rolff, 2001; Nelson, 2004; Lee, 2006; Martin et al., 2008). However, most of these eco-immune studies have focused on the endothermic birds and mammals (Norris and Evans, 2000; Martin et al., 2008), leaving a deficit in our understanding of the immune responses of ectotherms.

Being long-lived ectotherms, turtles are an interesting taxonomic group in which to investigate how factors such as temperature and age affect the immune system. Reptiles have both innate and adaptive immune systems similar to birds and mammals, but there are functional differences in the immune responses of reptiles relative to their endothermic counterparts. Because reptiles are ectothermic, their immune response can be directly influenced by temperature. The complement system, part of the innate immune response, in crocodilians is able to kill microbes at a range of temperatures between 5°C and 40°C, but there is a significant decrease in complement-based killing at temperatures below 15°C and above 30°C (Merchant et al., 2003; Merchant and Britton, 2006). Phagocytic and cytotoxic ability of splenic macrophages from wall lizards (*Hemidactylus flaviviridis*) was highest at 25°C, with impaired function at lower and higher temperatures, whereas nitric oxide, a compound produced by macrophages that is toxic to bacteria, was only produced at 25°C (Mondal and Rai, 2001). Lymphoid organs of reptiles, including the spleen, thymus and gut-associated lymphoid tissue, undergo seasonal involutions (Hussein et al., 1978; Hussein et al., 1979; El Ridi et al., 1981; Zapata et al., 1992). The thymus of hibernating mammals also seasonally involutes; however, non-hibernating mammals experience an age-related involution (Kruman, 1992; Taub and Longo, 2005). Humoral immune responses of reptiles are much slower in comparison to mammalian responses, often fail to increase in titer upon a secondary immunization, and are of lower affinity than are mammalian responses (Grey, 1963; Marchalonis et al., 1969; Kanakambika and Muthukkarauppan, 1972; Ingram and Molyneux, 1983; Pye et al., 2001; Origgi et al., 2001).

Age can also play a role in the variation of immune responses and generally the two are negatively correlated. In birds, cell-

mediated immunity as measured by the response to phytohaemagglutinin (PHA) is lower in aged birds compared with young adults (Haussmann et al., 2005; Lavoie et al., 2007), and often the humoral response to novel antigens is lowered as well (Lavoie, 2006). However, a complicated relationship between age and the immune branches has also been reported in birds. Palacios et al. (Palacios et al., 2007) reported that cell-mediated responses decreased with age, but acquired and innate humoral immunity did not. In mammals, aging is associated with a decrease in the numbers and function of cells of the innate immune system (Gomez et al., 2008). For example, cell-mediated immunity is impaired because of thymic involution that results in a decrease in the output of T cells (Taub and Longo, 2005). Reduced T-cell activity may also be a contributing factor to the decrease in antigen-specific immune responses that occurs with increasing age (Frasca et al., 2008). Likewise, humoral immunity is impaired with increasing age due largely to the quality and quantity of antibodies produced (McGlauchlen and Vogel, 2003). Limited information is available on the effect of age on innate and adaptive immunity in reptiles. Ujvari and Madsen (Ujvari and Madsen, 2005) used body size as a proxy for age in water pythons (*Liasis fuscus*) and found that humoral responses decreased with increasing body length/age.

The red-eared slider turtle, *Trachemys scripta*, has been the focus of intensive study, but little is known about its immune system. Information on the immune system can be placed in context with information about reproductive behaviors and general physiology in this well-studied species. In the northern part of their range red-eared sliders hibernate in the winter and are not active until water temperatures reach 10°C or higher (Ernst et al., 1994). Mating generally occurs in the spring, and males are more active and move greater distances than females at this time, most likely in search of mating opportunities (Gibbons, 1990). As summer approaches, males are relatively inactive while females finish folliculogenesis and ovulation shortly before they begin nesting (Ernst et al., 1994), which occurs in May and June in our study population. In the fall, females move away from nesting sites while there is some movement of males in search of mating opportunities. Females also initiate folliculogenesis at this time, which results in follicle maturation occurring over a several month period (Ernst et al., 1994). Thus, there is substantial variation in the temperatures experienced by and activity patterns of these turtles across their active season.

The goal of this study was to examine seasonal variation in the immune system during the active season of a natural population of red-eared sliders. We hypothesized that, based upon variation in activity patterns, males and females would differ in their immune responses across the active season. However, if temperature alone drives variation in immunity, then we would expect males and females to have similar patterns of variation in immune responses, but that immune responses would vary seasonally. We also predicted a decrease in immune responsiveness with increasing age based upon our current understanding of age-related effects on immunity in amniotes. To address our hypothesis, we used three common eco-immunology measures: plasma bacterial killing capacity, delayed-type hypersensitivity (DTH) test, and total immunoglobulin (Ig) levels. Bacterial killing capacity is a widely used measure of innate immunity that integrates humoral components of the innate response such as complement, as well as cellular components such as macrophages (Tieleman et al., 2005). The DTH test measures the response to a subcutaneously injected protein such as phytohemagglutinin (PHA), which stimulates local T cells leading to the recruitment of other innate and adaptive immune cells

(Martin et al., 2006; Muñoz et al., 2009). Thus, it is not strictly a cell-mediated response, but provides information on an integrated immune response that is initiated by T cells. To assess humoral immunity, we measured total Ig levels. Previous avian eco-immune studies have quantified gamma globulins (Saino et al., 1997), total Ig levels (Bourgeon and Raclot, 2006), or specific Ig levels in response to a novel antigen (Fairbrother et al., 2004). Each of these methods is thought to provide an assessment of humoral immunity, with the latter being the most specific, but also dependent upon reagent availability in non-model species. Measures of either gamma globulin or total Ig levels, although less specific, also capture variation in natural antibodies, which are thought to integrate information between the innate and adaptive branches (Ochsenbein and Zinkernagel, 2000). In this study we simultaneously assess components of the three major branches of the immune system in an ectothermic vertebrate, and attempt to place the immune response in context by exploring intersexual, age, and seasonal differences in both innate and adaptive immunity of adult red-eared slider turtles.

MATERIALS AND METHODS

This study was conducted at Banner Marsh State Fish and Wildlife Area, Fulton Co. IL, USA, on a natural population of red-eared sliders *Trachemys scripta* Schoepff, from March to September 2008. Male and female turtles of reproductive age were trapped throughout the active season: pre-nesting (March to early May), nesting (late May to early July), and post-nesting (late July to October). At the time of capture any unmarked individuals were uniquely marked, and plastron length measured to the nearest 0.1 mm. We used plastron length as a proxy for age because red-eared sliders are indeterminate growers, thus body size should increase over their lifetime (Wilbur, 1975; Gibbons et al., 1981). No individual was used in more than one collection period. Plastron length of individuals used in the study ranged from 88–212 mm for males and 152–241 mm for females. Water temperatures were recorded at each sampling date using a digital thermometer (Fisher Scientific, Pittsburgh, PA, USA). Blood samples were taken from the caudal vein using an EDTA-coated syringe, and plasma was separated by centrifugation at the field site and kept on ice until it was taken to Illinois State University for immediate use in the bactericidal assay. The remaining plasma was stored at –20°C for use in the ELISA at the end of the study. Turtles were transported to Illinois State University and housed in 100 gallon (~450 liter) tanks held at a constant water temperature of 27°C with a light cycle of 12 h:12 h light:dark. All research was conducted with the approval of the Illinois State University IACUC, and under IDNR permit NH.08.2084.

Seasonal variation of innate immunity

A bactericidal assay was used as a measure of innate immunity based on the method of Tieleman et al. (Tieleman et al., 2005). Fresh plasma (5 μl) was mixed with a 10 μl solution containing approximately 400 colony forming units of the bacteria *Escherichia coli* in PBS, and 100 μl of CO_2-independent medium enriched with 4 mmol l^{-1} L-glutamine and 5% fetal bovine serum. Samples were incubated for 30 min at 27°C [red-eared slider activity is greatest between 25°C and 30°C (Ernst et al., 1994)], and 50 μl was plated in duplicate onto agar plates and incubated overnight. The mean number of colonies present on the sample plates was subtracted from the mean number of colonies present on control plates grown only with *E. coli* in medium and divided by the control plate number to determine the proportion of bacteria killed by the plasma.

Seasonal variation of cell-mediated immunity

A DTH test was used as a measure of cell-mediated immunity. A preliminary analysis of the DTH response was first performed on 12 adult turtles to determine peak swelling time. The thickness of the webbing of the back foot was measured before the turtles were injected in that region with 20 μl of a solution of 10 mg PHA in 1 ml PBS. Swelling was measured at 6, 12, 24, 48 and 72 h post injection using a digital thickness gauge (Mitutoyo, Aurora, IL, USA). The turtles were then injected a second time with the same dose of PHA, and measured again at the same time intervals. Peak swelling occurred 12 h after the second injection.

To assess the DTH response of the experimental group, turtles were held for 24 h after capture, then a priming injection was given intraperitoneally. Forty-eight hours later the challenge dose was injected into the webbing of the right foot. The left foot was injected with 20 μl of PBS as a control. The response to DTH was expressed as the swelling in the right foot minus the swelling of the left foot as measured at 12 h post-challenge injection.

Seasonal variation of humoral immunity

Humoral immunity was measured using an ELISA to determine total Ig levels. Polystyrene 96-well plates were coated with 100 μl of 25 μg ml^{-1} dilution of unlabeled anti-turtle light chain antibody (HL 673; University of Florida Hybridoma Facility) and incubated overnight at 4°C. Wells were washed three times for 3 min with 200 μl per well of PBS,1% BSA, 0.05% Tween buffer. The standard curve was determined using 25 μg purified turtle Ig making eight twofold serial dilutions. Plasma samples were diluted 1:1000 and loaded in duplicate. Plates were incubated at room temperature for 1 h and washed as before. 100 μl of a 1:500 dilution of anti-turtle antibody conjugated to biotin was added to each well, incubated for 1 h at room temperature, and washed in the same manner as before. 100 μl of 1:1000 dilution of streptavidin-HRP (Southern Biotech, Birmingham, AL, USA) was added to each well, incubated for one hour at room temperature, and washed again as before. Plates were washed with 100 μl double distilled H$_2$O and 100 μl of ABTS (Southern Biotech) substrate powder dissolved in ABTS solution was added to each well. The plate was read at 405 nm at 10, 15, 17, and 20 min after adding substrate using a Powerwave 340 plate reader (BioTek Inc., Winooski, VT, USA).

Statistical analysis

Bacterial killing capacity, DTH responses, and total Ig levels were square root transformed prior to statistical analysis to meet the assumption of normality. Owing to technical difficulties with the bactericidal assay, the first sampling date includes only DTH response and Ig levels. One week later, we sampled again but have only bacterial killing capacity and Ig levels for those individuals. The other sampling periods include all three immune measures for each individual. Different individuals were used for each sampling period to avoid any issues with prior exposure. A multivariate analysis of variance (MANOVA) was used for sampling dates that included all three immune measures. Date, sex and plastron length as a continuous variable were included as main effects. All interactions were tested and non-significant effects were removed from the model. In order to include all sampling dates in the analysis of seasonal differences, separate ANOVAs were used to compare each immune measure for effect of sex, date of sampling, and plastron length. All interactions were tested and non-significant effects involving plastron length were removed from the model. Tukey's *post-hoc* comparisons were conducted.

RESULTS

For the MANOVA, a significant effect of date (Pillai's trace: $F_{15,90}$=7.54, P<0.0001) was detected and two significant axes were found. The first significant canonical variate (eigenvalue=2.3846, P<0.0001) was primarily a function of bacterial killing capacity as measured by the bactericidal assay, and explained 86.35% of the overall variation in immune responses among dates (Table 1). The standardized canonical coefficient (SCC) for bacterial killing capacity was of the opposite sign to the coefficients for DTH and Ig levels, but because the DTH and Ig coefficients were close to zero, there appears to be no strong relationship among means of these immune measures across dates (Table 1). The second significant canonical variate (eigenvalue=0.3117, P=0.0014) explained 11.29% of the variation among dates and was primarily a function of DTH response, with the other variables having very small SCCs (Table 1). The strong association of bacterial killing capacity and DTH responses with separate, orthogonal canonical axes of variation suggests little relationship among these immune measures (Table 1).

A significant date by sex interaction was found ($F_{6,197}$=2.17, P=0.048; Fig. 1) for bacterial killing capacity. Females generally had higher bacterial killing capacity than males except in late June when killing capacity was higher in males. However, bacterial killing capacity did not significantly differ between the sexes after correcting for multiple *post-hoc* comparisons. The mean proportion of bacteria killed at the first sampling date in May was 0.15 and then peaked in late May and June with mean proportion killed being 0.60 for both sampling periods. Bacterial killing capacity dropped dramatically in August to a mean proportion killed of 0.01 and remained low throughout the remainder of the active season. Plastron length was not associated with our measure of innate immune function ($F_{1,197}$=2.51, P=0.11).

There was a significant effect of date on the DTH response ($F_{6,117}$=5.00, P=0.0002; Fig. 2). Swelling peaked in late May at 0.18 mm but then dropped to 0.04 mm in June and stayed near this level for the remainder of the active season. There was no significant effect of sex ($F_{1,117}$=0.02, P=0.90) or plastron length ($F_{1,117}$=2.22, P=0.14) on our measure of cell-mediated immunity. The date by sex interaction was also not significant ($F_{6,117}$=1.07, P=0.39) for the DHT response.

Ig levels varied significantly by date ($F_{7,215}$=3.59, P=0.0012), with levels increasing across the active season. Levels of Ig in May were 1.58 μg ml^{-1} and peaked in August at 7.63 μg ml^{-1}. Plastron length

Table 1. MANOVA summary statistics for effect of date on immune response

Source	Variance accounted	d.f.	F-value	P-value	Standardized canonical coefficients		
					Killing capacity	DTH response	Ig
Canonical variate 1	86.35%	15	10.17	<0.0001	0.9844	−0.1159	−0.2924
Canonical variate 2	11.29%	8	3.37	0.0014	−0.1000	1.278	−0.0784

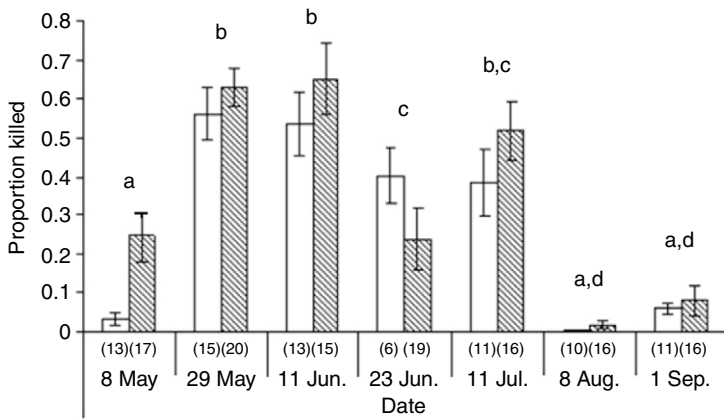d.f., degrees of freedom; DTH, delayed-type hypersensitivity; Ig, immunoglobulin.

Fig. 1. Proportion of bacteria killed at each sampling date. Sampling dates with the same letter are not significantly different (*P*<0.05). Males are represented by white bars, females by hatched bars. Values are means ± s.e.m.; sample sizes for males and females are listed below the bars in parentheses.

was significantly and positively related to Ig levels ($F_{1,215}$=5.98, P=0.015; Fig. 3). We found no effect of sex ($F_{1,215}$=0.00, P=0.96), or the date by sex interaction ($F_{7,215}$=1.13, P=0.35) on our measure of the humoral immune response.

DISCUSSION

Immune responses of both ectotherms and endotherms exhibit seasonal variation (Zapata et al., 1992; Martin et al., 2008); however, the factors driving this variation may differ. Previous studies in endotherms suggest these changes may occur independently of temperature (Kruman, 1992; Buehler et al., 2008), whereas the ectothermic immune system is directly affected by temperature (Le Morvan et al., 1998; Mondal and Rai, 2001; Merchant et al., 2003; Raffel et al., 2006). Variation in immune responses may also be affected by intrinsic biological factors including sex and/or age. The goal of this study was to examine seasonal differences in components of the three branches of the immune system of adult red-eared slider turtles in an effort to understand what factors influence variation in the immune system of ectotherms.

We report that the pattern of seasonal variation differed among the three immune measures used to assess innate and adaptive immunity. Plasma bacterial killing capacity peaked in late May and June before dropping off dramatically in August and remained low until our last sampling period in September, near the end of the active season. The DTH response also peaked in late May, but then dropped to a lower but consistent level for the remainder of the active season, whereas Ig levels gradually increased across the active season. To our knowledge, this is the first report of seasonal variation in innate immunity in reptiles, as well as the first report of seasonal variation in DTH responses.

Interestingly, bacterial killing capacity peaked in the spring, well before peak ambient temperatures were reached (Fig. 4). Studies in the leopard frog, *Rana pipiens*, reported that complement activity was not detectable after frogs were held at 5°C for 5 months. However, when the frogs kept at the lower temperature were moved to 22°C for 7 days, complement activity was significantly higher than in frogs held continually at 22°C (Maniero and Carey, 1997). A similar effect may be occurring with the bacterial killing capacity of turtles, where turtles that experience increasing ambient temperatures after prolonged exposure to the cold have elevated responses compared with animals that have been exposed to warmer temperatures for a prolonged period, as would naturally occur seasonally. Maintaining a sufficiently robust immune response while water and ambient air temperatures are rising, but still low, could be important in the spring, as this is a time of high activity for the turtles. Being sick during this time could compromise reproduction as males are actively searching for mating opportunities, and females are finishing folliculogenesis. Bacterial killing capacity remained elevated throughout the spring and early summer in males and females, a period when both water and air temperatures were elevated. However, the rapid decrease in this immune measure in August occurred while water temperatures remained elevated, but air temperatures were declining, suggesting that turtles may use ambient air temperature (and possibly the associated decrease in photoperiod) as a proximal cue for anticipating seasonal changes. A similar pattern of *E. coli* killing capacity has been reported in red knots (*Calidris canutus*), corresponding to the end of molt and the onset of winter (Buehler et al., 2008). Although reptiles do not typically molt just before the onset of winter, they nonetheless must prepare for seasonal hibernation. As part of this preparation, they may undergo
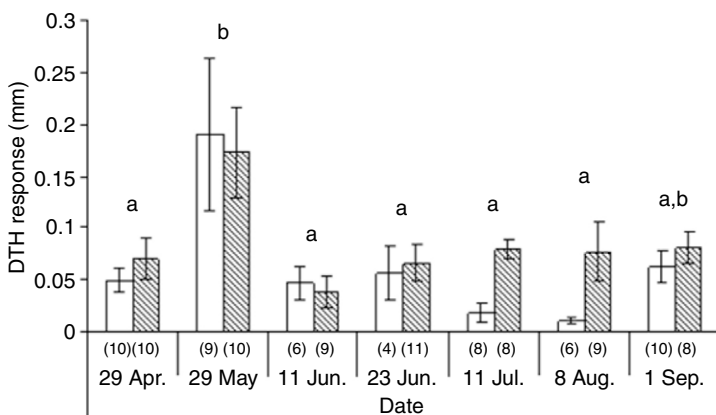

Fig. 2. Delayed-type hypersensitivity (DTH) response at each sampling date. Sampling dates with the same letter are not significantly different (*P*<0.05). Males are represented by white bars, females by hatched bars. Values are means ± s.e.m.; sample sizes for males and females are listed below the bars in parentheses.
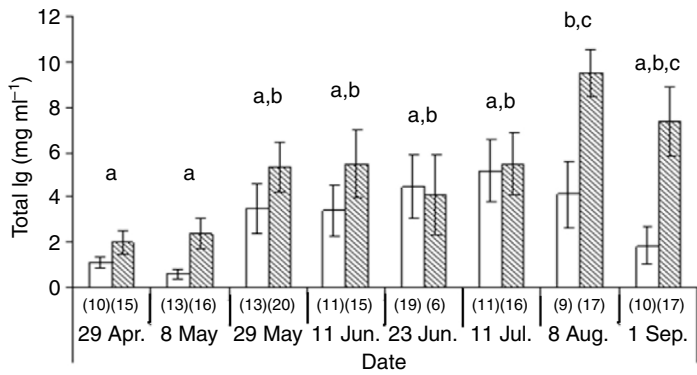
Fig. 3. Total immunoglobulin (Ig) levels at each sampling date. Sampling dates with the same letter are not significantly different ($P<0.05$). Males are represented by white bars, females by hatched bars. Values are means ± s.e.m.; sample sizes for males and females are listed below the bars.

seasonal redistribution of resources away from less essential functions which may include some immune components. Several studies have shown that T-cell proliferation in reptiles varies seasonally, with the strongest responses often occurring in the spring (Farag and El Ridi, 1984; El Ridi et al., 1987; Muñoz and De la Fuente, 2001). In the present study, we found that a T-cell dependent response also peaked in the spring, but was elevated in only one of our sampling periods compared with a much more protracted elevation for bacterial killing capacity. Turtles in the present study were housed under identical conditions at each sampling date for the DTH test, and the elevated responses found in May might reflect the strong proliferation or increased number of cells needed to overcome the decreased efficiency because of lower ambient temperatures during this period (Fig. 4). Although the response to PHA is commonly used in avian eco-immunology studies, DTH responses over the annual cycle are difficult because of the need for naïve individuals at each sampling period. However, our experimental protocol allowed us to use the DTH test across the entire active season. Thus it is difficult to make comparisons with previous studies that use the DTH test, as most focus on comparisons between life-cycle stages such as breeding *versus* non-breeding seasons (Martin et al., 2008).

We report that Ig levels increased across the active season. Our observed increase in Ig levels may be attributed to a build-up of antibodies across the active season resulting from an accumulation of exposures to pathogens. Alternatively, this increase could be due to a direct effect of temperature on Ig production. Studies in fish (*Morone chrysops* × *Morone saxatilis* hybrids and *Oncorhynchus mykiss*) suggest that antibody production is delayed, but peak titer is not affected by decreased environmental temperature when compared with fish kept at higher temperatures (Hrubec et al., 1996; Mikkelsen et al., 2006). The peak in Ig levels at the end of the active season may represent a delay in antibody production resulting from antigen exposures that occurred early in the active season, but at present we are unable to distinguish between natural antibodies and specific antibodies generated in response to a particular pathogen.

A number of other factors may play a role in modulating seasonal variation in immune measures. Sex steroids have been hypothesized to influence seasonal variation of immune function in reptiles (Muñoz et al., 2000). Steroids have also been implicated as a cause of sex differences in immune measures, with estrogens considered immunoenhancing and androgens and glucocorticoids immunosuppressing (McEwen, 1997; Klein, 2000; Bouman et al., 2005). Studies in several species of turtles have shown that males and females have different patterns of seasonal variation in sex steroid levels (Shelby et al., 2000). However, we found no differences in patterns of immune variation between the sexes, and thus it is unlikely that differences in steroids can explain our observed patterns.

Photoperiod has been shown to affect the immune responses of both mammals (Nelson and Demas, 1996) and birds (Bentley, 2001) *via* changes in levels of melatonin. Photoperiod can affect the mean preferred body temperature of ectotherms by altering the production of melatonin (Lutterschmidt et al., 2003), and as discussed earlier, temperature can influence immune responses of reptiles. No studies have addressed photoperiod and the immune system in reptiles, but given the potential for photoperiod and temperature to co-vary, further research aimed at disentangling these two variables seems warranted.

Changes in pathogen pressure might also explain seasonal variation in immune responses. Infectious disease prevalence can vary seasonally as a result of a variety of factors, including changes in weather, fluctuations in vector populations and changes in host behavior (Dowell, 2001; Altizer et al., 2006). In ectotherms, pathogen prevalence can be affected by temperature-induced fluctuations in host physiology as well as direct temperature effects on the pathogen life cycle (Jackson and Tinsley, 2002). In a concurrent study on the same population of turtles used here, Holgersson (Holgersson, 2009) found that *Salmonella* prevalence was low in the spring but increased in the summer months and remained high throughout the rest of the active season. This demonstrates that seasonal variation in pathogen pressure does occur at our study site, but at present we do not know how pathogen pressure may affect the immune response of reptiles.

With respect to immune variation as it relates to either the sex or the age of the animal, we found that while there was a significant date by sex interaction for bacterial killing capacity, there were no differences between males and females once the analyses were corrected for multiple *post-hoc* tests. Other immune measures did
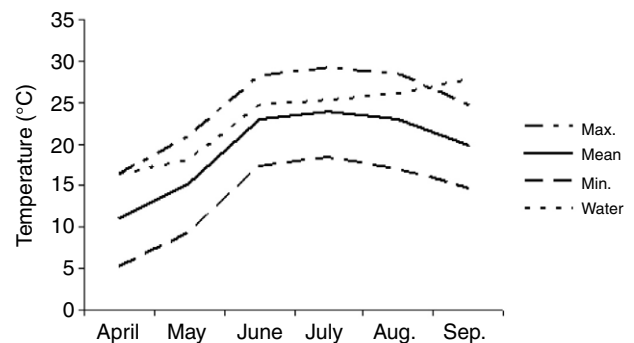

Fig. 4. Monthly air temperatures at Pekin, IL, USA and water temperatures at the collection site (Banner Marsh). Air temperatures were downloaded from www.wunderground.com. During sampling, water temperature was recorded.

not vary between the sexes and there was also no evidence of trade-offs within the immune system based on the results of the MANOVA. We did find a positive correlation between plastron length and antibody levels, indicating that at least the humoral branch of the immune system may vary with age in this long-lived species.

Although antigen specific immune responses typically decrease with age in mammals (Frasca et al., 2008), natural antibody levels tend to increase with age throughout the lifetime of the individual (Candore et al., 1997; Benatuil, 2008). Natural antibodies are produced by a class of long-lived and self-replenishing B cells known as B-1 cells (Baumgarth et al., 2005). They accumulate in the absence of antigen stimulation as well as in response to evolutionarily conserved components of pathogens and can increase survival from bacterial and viral infections by triggering both innate and adaptive immunity (Ochsenbein and Zinkernagel, 2000). Natural antibodies have been previously identified in alligators, pythons and garter snakes, and have been shown to increase with age and/or body size (Longenecker and Mosmann, 1980; Madsen et al., 2007; Sparkman and Palacios et al., 2009). The observed relationship between Ig levels and body size suggests that we are measuring an age-related increase in natural antibody levels in the turtles, but at present we cannot rule out the production of specific antibodies as well.

Reptiles have a complex immune system much like the endothermic birds and mammals, however, there are substantial differences between the groups that make it important to include ectotherms if we are to fully understand vertebrate immunity. We found evidence for seasonal variation in immune responses, with each immune measure having a different response to seasonal variation. This suggests a dynamic relationship between the immune branches that is potentially influenced by a suite of biotic and abiotic factors. This finding, along with other studies that have measured multiple immune responses in the same individual (Blount et al., 2003; Forsman et al., 2008), highlights the complexity of the immune system. Therefore, caution should be taken when interpreting studies that only measure one immune branch, or sample individuals at only one time point. Further study is needed on the reptilian immune system to identify the specific factors driving this seasonal variation, and to understand how these factors shape immune responses.

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