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Incubation Environment Affects Immune System Development in a Turtle with Environmental Sex Determination

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ABSTRACT.—**The developmental environment can have lasting effects on posthatching phenotype in oviparous animals. Innate immune response is one important component of fitness in vertebrates because it provides a generalized defense against infection. In addition, because male vertebrates are at a higher risk of infection than females, males may benefit more from increased innate immunity than females. We determined the effects of incubation temperature on the innate immune response of hatchling map turtles (*Graptemys*) by incubating eggs at a range of male and female producing-temperatures and assessing plasma complement activity in the resulting hatchlings. We found a significant effect of incubation environment on circulating complement in hatchling *Graptemys ouachitensis*, with male-producing temperatures yielding the highest innate immune response. Most important, these results demonstrate that immune response is affected by developmental environment in a species with environmental sex determination, potentially resulting in sex differences in the ability to fend off pathogens.**

Incubation temperature can have strong effects on several posthatching traits in reptiles. Research in a wide range of oviparous reptiles has revealed significant effects of incubation temperature on growth, behavior, metabolism, and survival (reviewed in Rhen and Lang, 2004). Because factors such as maternal nest site choice and climatic variation can produce substantial variance in nest temperatures in the wild, it is important to understand what effects this variance in incubation temperature can have on posthatching phenotype.

In reptiles, several antigen receptor systems have been characterized and are known to mediate a wide range of immune responses (Glinski and Buczek, 1999). Immunocompetence may be particularly important in turtles in light of their extremely long generation times. Specifically, long generation times can make hosts more susceptible to parasitic infection, owing to their decreased evolutionary rate relative to their coevolving parasites (Gandon and Michalakis, 2002). The innate immune system is likely to be important for survival in the wild, because it is nonspecific, constitutively expressed and, thus, can have an immediate response to pathogens, as opposed to the slower, antigen-specific response of acquired immunity (Nielsen et al., 1978; Lochmiller and Deerenberg, 2000). Innate immune function may be particularly important in determining the survival of an animal upon its first encounter with a disease, and a successful innate response may help avoid a costly specific response (Lochmiller and Deerenberg, 2000). Complement, a collection of circulating proteins that neutralize foreign (pathogenic) cells via cellular lysis, is a critical component of innate immunity that acts as a first line of defense against pathogens (Mayer, 1948; Song et al., 2000) and also signals the activation of other immune responses (Frank and Fries, 1991; Frank et al., 2000; Ochsenbein and Zinkernagel, 2000; Carroll, 2004).

In vertebrate immune systems, there is often a clear difference in the selective environment of each sex. Numerous studies have demonstrated that males are characterized by higher rates of parasitism than females in a range of taxa (Moore and Wilson, 2002; Amo et al., 2005; Hoby et al., 2006). This disparity is generally attributed to a reduction in immune response in males because of the effects of circulating hormones associated with sexual maturity. Specifically, both testosterone and dihydrotestosterone (DHT) have generally been found to impair immunological responses, whereas estradiol is actually more likely to enhance immunological function (Uller and Olsson, 2003; Klein, 2004). In many systems, males may also have a greater opportunity for exposure to infection as a result of behaviors such as increased aggression and dispersal (Klein, 2004). The trend toward increased prevalence and intensity of parasitic infection in males has been demonstrated across a wide range of parasite and host species (Klein, 2004).

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Incubation temperature may affect posthatching phenotype through two different pathways in reptiles with environmental sex determination (ESD). Because temperature determines sex, any inherent sex-specific differences in the expression of a trait (such as higher circulating testosterone in males) will become associated with incubation temperature. Alternatively, temperature may act directly on the trait, for instance, by activating or deactivating enzymes necessary for the development of the trait. Under this pathway, theory predicts that the sex that is expected to benefit most from an enhanced state of a trait should most often be produced at temperatures that optimize this trait (Charnov and Bull, 1977). Because males may have a greater risk of infection, they may, relative to females, have a greater need for optimized innate immune system development to compensate for their higher inherent susceptibility. Consequently, if incubation environment directly affects immune system development in turtles, selection is predicted to favor the production of males in environments that enhance immunity.

In this study, we sought to determine the effects of incubation temperature on the innate immune response, specifically complement activity, of hatchling turtles. Map turtles (*Graptemys*) offer an ideal system to investigate these effects, because they have considerable variation in nest temperatures in the wild (Bull, 1985), a clearly defined pattern of ESD, are strongly sexually dimorphic, and have been studied extensively in this context (Ernst et al., 1994). We incubated eggs of *Graptemys ouachitensis* and *Graptemys pseudogeographica* at a range of temperatures producing male and female offspring and assessed complement activity in the resulting hatchlings.

Materials and Methods

We collected eggs of *G. ouachitensis* and *G. pseudogeographica* in the summer of 2004. *Graptemys ouachitensis* eggs were collected along the West Fork of the White River, Indiana, whereas *G. pseudogeographica* eggs were collected at Reelfoot Lake, Tennessee. Eggs were obtained through collection from newly laid nests and from gravid females (Ewert and Legler, 1978). Eggs were placed in boxes with moistened vermiculite (vermiculite : water, 1 : 1 by mass; approximately 170 kPa) and were distributed among five temperature-controlled incubators until hatching.

In both *G. ouachitensis* and *G. pseudogeographica*, only females are produced above 30.5°C, only males are produced below 28°C, and both males and females are produced at 29°C (Bull et al., 1982a, b; Bull, 1985; Ewert et al., 1994). We distributed a portion of *G. ouachitensis* eggs from eight clutches among incubators set to 24°C, 29°C, and 33°C. We distributed a portion of *G. pseudogeographica* eggs from 12 clutches among incubators set to 24°C, 26.5°C, 29°C, and 31°C. After hatching, the turtles were placed in cups with a small amount of water and maintained at 30°C. At 10 weeks of age, the turtles were sacrificed for the complement assay.

Hemolytic Complement Assay.—On the day of sampling, animals were brought into the surgery room and were decapitated one at a time using surgical scissors. Pooled trunk blood (~50 µl) was collected via a 100-µl pipette and transferred to a microcentrifuge tubes. All sampling was conducted between 1500 h and 1700 h EST. Following blood sampling, all samples were centrifuged at 8°C for 30 min at 2,500 rpm. Plasma aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at −80°C until assayed for complement.

Complement activity in plasma was measured using methods previously described (Sinclair and Lochmiller, 2000; Greives et al., 2006) with modifications described below. Briefly, 80 µl of both 1 : 5 and 1 : 10 dilutions of plasma in dextrose-gelatin-veronal buffer (VB) (BioWhittaker, Walkersville, MD,) were added to a round-bottomed microtiter plate in duplicate. Next 50 µl of a 0.6% suspension of washed sheep red blood cells (SRBC, MP Biomedicals, Irvine, CA) and 50 µl of a 1 : 40 dilution of rabbit anti-SRBC antibody (Sigma, St. Louis, MO) were added. Lysis wells of 0% and 100% were created by adding 130 µl VB or water, respectively, and 50 µl of 0.6% washed SRBC. Next, the plate was gently shaken for 5 min, incubated at 37°C for 90 min, and subsequently centrifuged for 5 min at 500 rpm at room temperature to separate un-lysed SRBC from the supernatant. Sixty µl of supernatant were then transferred to a new microtiter plate, and absorbance was measured at 405 nm with a microplate reader (BioRad, Hercules, CA).

Hemolytic-complement activity was expressed as CH_{50} units/ml plasma, where 1 CH_{50} unit equals the reciprocal of the dilution of plasma required to lyse 50% of the SRBC in culture (Mayer, 1948). Because values violated the assumption of normality, all values were increased by one (so that all CH50 values were above one) and then normalized via a log transformation. The resulting values, $\ln(CH_{50} + 1)$, were then used in statistical analyses. Statistical analyses were performed in Minitab 14 for Windows. The effect of incubation temperature on $\ln(CH_{50} + 1)$ was analyzed separately for each species using a one-way

ANOVA. We examined the effects of maternal identity by including clutch as a covariate in a separate ANOVA. Differences between species in $\ln(CH_{50} + 1)$ at 24°C (male only temp.), 29°C (mixed sex temp.), and the all-female temperatures (31.5°C in *G. pseudogeographica* and 33°C in *G. ouachitensis*) were analyzed separately using a one-way ANOVA. Differences in pairwise means were probed using Tukey's Honestly Significant Differences (HSD) post hoc tests when the overall ANOVA's were significant.

Results

Assays were successfully run on a total of 36 *G. ouachitensis* hatchlings and 39 *G. pseudogeographica* hatchlings. For *G. ouachitensis*, assays were run on 11 turtles from 24°C, 13 turtles from 29°C, and 12 turtles from 33°C. For *G. pseudogeographica*, assays were run on 10 turtles from 24°C, 10 turtles from 26.5°C, nine turtles from 29°C, and 10 turtles from 33°C. Insufficient plasma samples from the remaining turtles precluded their inclusion in the assay.

There was a statistically significant effect on hemolytic complement activity in *G. ouachitensis* (Fig. 1A; $F_{2,35} = 3.86$, $P = 0.031$) but not in *G. pseudogeographica* (Fig. 1B; $F_{3,28} = 0.15$, $P = 0.931$). This effect held when clutch was included as a covariate ($F = 3.74$, $P = 0.035$ in *G. ouachitensis*; $F = 0.21$, $P = 0.889$ in *G. pseudogeographica*). The significant result in *G. ouachitensis* can be attributed to higher CH_{50} in turtles incubated at 24°C relative to the warmer temperatures, indicating greater immunocompetence at this temperature. A Tukey's post hoc analysis in *G. ouachitensis* revealed a significant difference in hemolytic complement activity between 24°C and 29°C ($P = 0.030$). No difference was detected between 29°C and 33°C ($P = 0.814$) or between 24°C and 33°C ($P = 0.117$). There was no difference in hemolytic complement activity between species at 24°C ($F_{1,20} = 0.009$, $P = 0.924$), but there was a significant difference between species at 29°C ($F_{1,21} = 11.21$, $P = 0.002$) and at the all-female temperatures ($F_{1,21} = 6.67$, $P = 0.018$), with *G. ouachitensis* exhibiting lower complement activity. Clutch identity did not have a significant effect on complement activity in either species ($F = 0.01$, $P = 0.914$ in *G. ouachitensis*; $F = 2.07$, $P = 0.159$ in *G. pseudogeographica*).

Discussion

We found a significant effect of incubation environment on innate immune response in hatchling map turtles. Specifically, hemolytic complement activity was higher at lower incubation temperatures in *G. ouachitensis* but not in *G. pseudogeographica*. The effect in *G. ouachitensis* is the first evidence of an effect of developmental environment on immunity in reptiles. Because immune response is a critical component of survival, these results suggest that natural variation in the developmental environment may have pronounced effects on fitness. Furthermore, because developmental temperature also determines offspring sex in *G. ouachitensis*, these differences may be associated with sex differences in immune system development.

The innate immune system enables an immediate response to pathogens and, if successful in neutralizing a pathogen, may decrease energetic cost associated with mounting further immune responses; thus the innate immune system is likely important for survival in the wild (Mayer, 1948; Nielsen et al., 1978; Frank et al., 2000; Ochsenbein and Zinkernagel, 2000). Complement, one component of the innate immune system, is able to, in conjunction with natural antibodies, neutralize pathogens through cellular lyses (Mayer, 1948). Higher levels of complement in individuals reared at 24°C may indicate a greater ability of these individuals to neutralize pathogens at early stages of development, a time before the acquired immune system is fully developed. This would likely provide a benefit in terms of immunity to individuals incubated at temperatures producing all males.

O'Steen and Janzen (1999) found that incubation temperature can significantly impact the metabolism and thyroid hormone levels of hatchling turtles. They hypothesized that thyroxine levels may be influenced by a temperature-sensitive regulatory pathway. Our study did not directly address how incubation temperature may affect immune development in turtles, although a similar mechanism seems plausible. Because incubation temperature determines offspring sex, it is also possible that sex-specific differences in physiology may secondarily produce other physiological effects that will be associated with temperature. However, although sex-steroid level appears to be the primary sexually dimorphic trait to characterize turtles at this age (Rhen and Lang, 2004), the higher testosterone level of males is expected to impair immune response and, thus, is unlikely to account for our results. For example, testosterone and complement levels have been found to negatively covary in birds (Greives et al., 2006).

Although we were not able to exclude the possibility that innate immunity is influenced by offspring sex, it is plausible that the differences we observed in innate immunity between 24°C and the warmer temperatures can be attributed to incubation temperature alone.

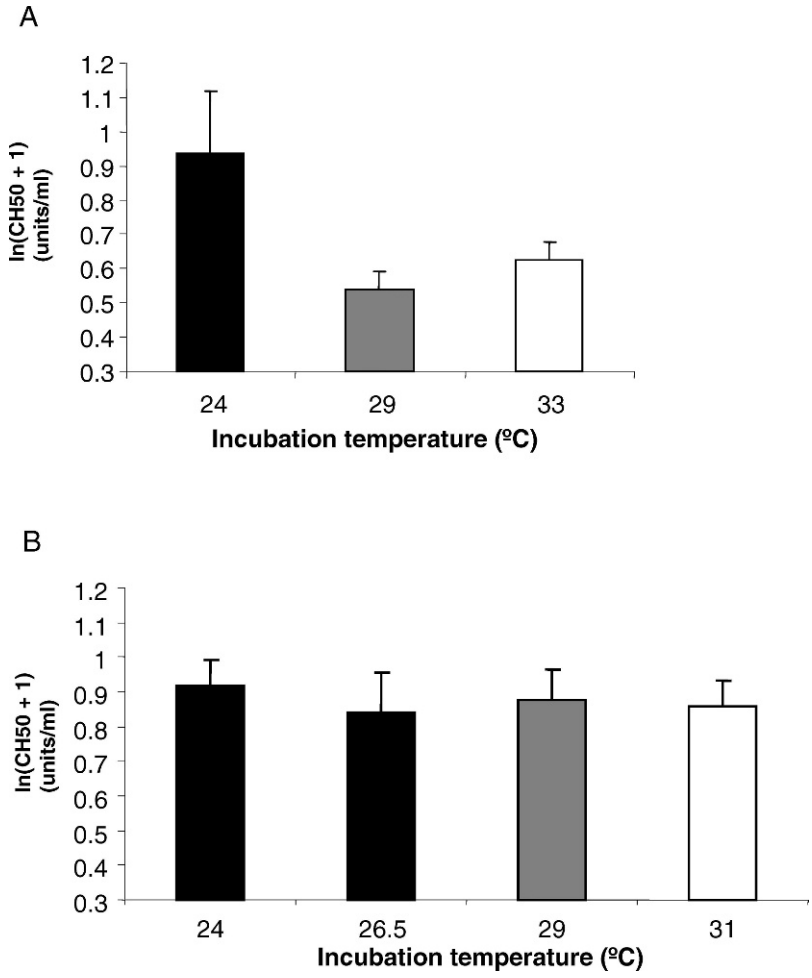

FIG. 1. (A) Incubation temperature versus log transformed hemolytic complement (CH50 + 1) in *Graptemys ouachitensis*. Only males are produced at 24°C (black), only females at 33°C (white); and both sexes are produced at 29°C (gray). Bars denote standard errors. (B) Incubation temperature versus log transformed hemolytic complement (CH50 + 1) in *Graptemys pseudogeographica*. Only males are produced at 24°C and 26.5°C (black), only females are produced at 31.5°C (white); and both sexes are produced at 29°C (gray). Bars denote standard errors.

Despite the fact that we incorporated an incubation temperature (29°C) that consistently produces both males and females in *G. ouachitensis* (Bull et al., 1982b; M. Ewert, unpubl. data), the immune response at this temperature was no greater than that from the all-female treatment and actually showed a stronger difference from the 24°C treatment ($P = 0.030$) than did the 33°C treatment ($P = 0.117$). Had the effects we observed been sex-specific, we would expect that the immune response at this temperature would be between the responses seen at 24°C and 33°C if, in fact, a mix of males and females were produced at 29°C. This conclusion is consistent with previous research into the effects of incubation temperature on offspring phenotype in turtles, which has consistently found that incubation effects on hatchling phenotypes can be attributed directly to the incubation environment and not offspring sex (Rhen and Lang, 1995; Freedberg et al., 2001; Willingham, 2005).

In the wild, mean nest temperatures of *Graptemys* range from 24°C to 30°C (Bull, 1985), and daily thermal variation causes nest temperatures to fluctuate several degrees beyond this range. Thus, many male *G. ouachitensis* are likely produced in the wild at temperatures that enhance innate immunity relative to females. Because vertebrate males are more susceptible to parasites than females (Klein, 2004), this scenario is consistent with a selective

environment that would favor the pattern of sex determination seen in *Graptemys*. Specifically, because males are at a greater risk of infection (Klein, 2004), ESD would be adaptive if it allowed males to be produced at the temperatures that most enhance innate immunity. Alternatively, the evolution of ESD may have occurred prior to any temperature effects on immune system development, and the latter association may have evolved as a means of compensating male turtles whose immune response are compromised by their elevated testosterone levels.

If other ESD turtles are characterized by greater immune system development in males, it may offset the impaired immune response normally observed in male vertebrates. Despite the consistent observation of male-biased infection in other vertebrate groups, data from turtles with ESD do not consistently reveal a greater rate of infection in males. For instance, aural lesions resulting form bacterial infection occur more frequently in female than male Ornate Box Turtles, *Terrapene ornata* (Christiansen et al., 2005). Although males have higher rates of tick parasitism in *Testudo graeca* (Robbins et al. 1998), the opposite trend was observed in *Rhinoclemmys arreolata,* with females exhibiting higher levels (Robbins et al., 2001). Although dietary differences may contribute to greater Acanthocephalan infection in female versus male Texas Map Turtles, *Graptemys versa* (Lindeman and Barger, 2005), the trend is also consistent with a greater susceptibility to infection in females.

Contrary to previous studies of the effects of incubation temperature on posthatchling phenotype in turtles, we found enhancement of a trait affecting offspring condition in turtles incubated at the low end of the species' natural range. Previous studies have consistently found compromised growth, performance and survival at low incubation temperatures (Freedberg et al., 2004; Rhen and Lang, 2004), making it unclear why female turtles choose to lay in shaded (cool) sites at all. Our findings point to an increase in immune function at cool temperatures in *G. ouachitensis* and, thus, may help to explain patterns of nest site choice observed in some turtle species. In particular, if innate immunity is a vital component of fitness, the benefits of enhanced innate immunity may offset other phenotypic costs associated with incubation at cool temperatures.

Although we found a significant effect of incubation temperature on complement activity in *G. ouachitensis*, we did not find a significant effect in *G. pseudogeographica*. There may be ecological differences between the species that are driving the difference. For instance, Vogt (1980) reported that *G. ouachitensis* are dimorphic in their summer foraging patterns, whereas both male and female *G. pseudogeographica* forage in slow-moving backwaters. If *G. pseudogeographica* females are at as high a risk of infection as males, selection may have favored compensatory mechanisms that prevent a reduction in innate immunity at female-producing temperatures. Consistent with this idea, we found no difference between the species in complement activity at the all-male temperature but found higher activity in *G. pseudogeographica* at the mixed and all-female temperatures. Although little is known about differences in the infectious diseases that characterize these species, additional research in this area may help elucidate the factors shaping the evolution of the immune system in each species.

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