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No evidence that estrogens affect the development of the immune system in the red-eared slider turtle, *Trachemys scripta*

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ABSTRACT

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Exposure to maternally derived substances during development can affect offspring phenotype. *In ovo* exposure to maternally derived steroids has been shown to influence traits such as growth and behavior in the offspring. The development of the immune system also can be altered by exposure to both androgens and glucocorticoids in a variety of species, but much less is known about the potential for estrogens to influence the development of this system. We examined the effect of estradiol on the development of both innate and adaptive immune components in the red-eared slider turtle (*Trachemys scripta*). A bacterial killing assay was used to assess innate immunity, a delayed-type hypersensitivity test for cellular immunity, and total immunoglobulin levels to measure the humoral immune response. We found no effect of *in ovo* estradiol treatment on any of our immune measures despite using doses that are known to influence other phenotypic parameters during development and varying the timing of dosing across development. Our results suggest that maternally derived estradiol does not affect the development of the immune system in *T. scripta*.

Introduction

Conditions experienced during embryonic development can produce effects that persist throughout an individual's lifespan. In oviparous vertebrates, maternally derived substances such as carotenoids (Blount et al., 2001), antibodies (Grindstaff et al., 2003), and steroids (Groothius and Schwabl, 2008; Groothius et al., 2005b) have all been shown to affect offspring development. Important processes such as growth, behavior, and immune function are all potentially subject to the influence of maternally derived substances (Groothius et al., 2005b). Because of their ability to influence phenotypic variation, these substances are often studied by evolutionary biologists as a means by which females might be able to adjust offspring traits to prevailing conditions in an adaptive manner.

Recently, many of these types of studies have examined the effect of yolk steroids on immune system development. Most research in this area addresses the role of androgens. Androgen receptors have been identified in various lymphoid tissues including the thymus and the bursa of Fabricius in birds (Gasc and Stumpf, 1981), and the bone marrow of other vertebrates (Klein, 2004) but are not found on immunocompetent cells. In chickens, the thymus and the bursa become actively involved in the development of immunity starting with the embryonic stages, making them potential targets for androgens (Navara and Mendonca, 2008). Though androgens are generally considered to be immunosuppressors, the effects are much more complicated. Studies examining the role of androgen exposure early in development have reported both positive and negative effects on immune function. For example, a number of studies in birds have shown responses across branches of immunity to be decreased (Muller et al., 2005; Navara et al., 2005; Sandell et al., 2009), while others have found either no change in immune function (Pitala et al., 2009; Tschirren et al., 2005) or increased immunity (Clairardin et al., 2011; Navara et al., 2006). Prenatal administration of testosterone in black-headed gulls resulted in an attenuation of both cellular and humoral immunity (Groothuis et al., 2005a; Muller et al., 2005), and, in at least one case, the immunosuppression was linked to increased nestling growth (Groothuis et al., 2005a), suggesting a tradeoff between growth and immune function. In reptiles, prenatal exposure to testosterone led to increased growth rate in the common lizard (*Lacerta vivipara*), but at the cost of increased susceptibility to ectoparasites (Uller and Olsson, 2003).

Studies have also examined the immunomodulatory effects of glucocorticoids in oviparous species. In adults, chronic exposure to corticosterone often suppresses immunity by decreasing immune cell numbers and function, and increasing immunosuppressive mechanisms (Dhabhar, 2009). Negative effects of corticosterone treatment on developing immune responses have been reported as well (Rubolini et al., 2005). However, the effects of *in ovo* corticosterone treatment may be dependent on factors such as sex and postnatal environment. A study in European starlings (*Sturnus vulgaris*) found

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that increasing corticosterone in freshly laid eggs resulted in increased response to the mitogen phytohemagglutinin (PHA) in males, but a decreased response in females when there was normal maternal provisioning. However, when maternal provisioning was limited by clipping the wings of the mothers, corticosterone treatment resulted in a decreased PHA response in males, while treatment had no effect in females (Love and Williams, 2008). This study highlights the complexity of steroid-immune interactions.

While the focus on testosterone and glucocorticoids has revealed a diverse array of outcomes of early steroid exposure on the development of the immune system, there is a deficit in our understanding of the effects of estrogens on the developing immune system. In adults, estrogen receptors are found on numerous components of the immune system, including B cells, T cells, natural killer cells, and macrophages, as well as on lymphoid tissues (Klein, 2004; Lang, 2004; Nalbandian and Kovats, 2005). Estrogens generally enhance immunity by promoting immunoglobulin production and stimulating T cell expansion, but can also depress some cell-mediated responses (Salem, 2004). There are limited studies on the effects of estrogens early in development, and the available data suggest that the interactions between estrogens and the immune system are also quite complex. In chickens, 17β-estradiol treatment early in embryonic development resulted in a dose-dependent increase in primary and secondary antibody responses. Later treatment, however, resulted in suppressed antibody responses (Kondo et al., 2004). Another study in chickens found no effect of estradiol treatment on bursa growth at low doses, but at high doses growth of the bursa was inhibited (Norton and Wira, 1977).

In order to further understand the effects of estradiol on the development of the immune system, we examined the effects of experimentally increased estrogen in red-eared slider turtles (*Trachemys scripta*). Red-eared sliders make an excellent model system for this type of study for several reasons. First is the substantial amount of natural variation in yolk estradiol levels, where the concentration of yolk estradiol in a female's second clutch of the nesting season is approximately 10-fold higher than the concentration in her first clutch of the season (Paitz and Bowden, 2009). Second is the relatively detailed understanding we have of how yolk estradiol is processed by the developing embryo. In *T. scripta*, concentrations of estradiol in the yolk decline to undetectable levels during the first 15 days of development (Paitz and Bowden, 2009). This decline is the result of estradiol being metabolized to various estrogen sulfates early in development (Paitz and Bowden, 2008, 2011; Paitz et al., 2012). Importantly, estrogen sulfates have been shown to be biologically active in *T. scripta* embryos where the administration of estradiol-3-sulfate to eggs at oviposition results in the increased production of female hatchlings (Paitz and Bowden, 2011). At present, we do not know if estrogen sulfates affect other phenotypic parameters. This mechanistic understanding of how *T. scripta* embryos process yolk estradiol facilitates our interpretation of how *in ovo* estradiol might influence the development of the immune system in this species. Finally, estrogens are known to have demonstrable effects on development in *T. scripta*; administration of estrogens to developing embryos can alter sex determination in this species (Crews et al., 1991).

We conducted a series of studies to examine whether embryonic exposure to exogenous estradiol altered the immune responsiveness of *T. scripta* hatchlings. Because of the limited amount of information on the effects of estrogens on the development of the immune system, we based our predictions on the reported effects of estrogens on adults. Though there are no studies that examine the effect of estrogens on bacterial killing capacity, estrogen can increase macrophage phagocytosis and natural killer cell activity (Klein, 2004). Estrogens can also shift the T helper response from a TH1 response, which is associated with strong cell-mediated immunity, to a TH2 response, which is associated with humoral immunity, by inhibiting TH1 cytokines and increasing production of TH2 cytokines (Salem, 2004). This shift in cytokines along with suppression of antigen-presenting cell function in response to estrogen results in a decreased delayed type hypersensitivity (DTH) response (Salem et al., 2000). Estrogens affect the humoral response by causing the hyperactivity of B cells, which increases the number of plasma cells and the amount of antibodies produced by each cell (Ahmed et al., 1999). To address our question, we used three common tests of immune responsiveness in an attempt to capture variation throughout the different branches of the immune system: plasma bacterial killing capacity, DTH test response, and total immunoglobulin (Ig) levels. Bacterial killing capacity measures components of the innate immune system including complement. The DTH test stimulates T cells which then recruit other innate and adaptive immune components. Total Ig levels are used to assess humoral immunity. Known antibody isotypes in turtles include IgM and IgY, but it is unknown if they have other isotypes such as IgA or IgD (Zimmerman et al., 2010a). To measure total Ig levels, we used an anti-light chain antibody that is capable of detecting antibodies of any isotype (Zimmerman et al., 2010b). Thus, based on this information, we predicted that estrogen treatment would increase bactericidal killing capacity and total Ig while decreasing the DTH response.

Materials and methods

Egg collection and treatment

Clutches of eggs were collected during May and June of 2008, 2009, and 2010 ($n = 10$ clutches per year) from Banner Marsh State Fish and Wildlife Area, IL (IDNR permits NH08.2084, NH09.2084, NH10.2084). We used eggs from freshly laid natural nests or from induced oviposition of gravid females in the laboratory using an injection of oxytocin (Ewert and Legler, 1978; Paitz and Bowden, 2009). All eggs were rinsed and gently dried to remove any debris prior to being randomized for use in the experiment. Clutch sized vary from to 7 to 17 eggs, with an average clutch size of 10 eggs. Whole clutches were divided so that they were represented across all treatment groups (control and estradiol treatments) within a year.

In 2008 and 2009, all eggs were treated within 24 hours of collection. In 2008 only, eggs were collected at two points across the laying season and likely represented both first and second clutches from females, although we did not collect more than one clutch from any given female. We designate these clutches as early and late. For both years, eggs designated for the estradiol treatments were given one of three doses (0.1 μg, 1.0 μg, or 10.0 μg estradiol in 95% ethanol) as a 5 μl bolus topically. This method of dosing has been previously used to administer steroids in red-eared sliders, and both the 1.0 and 10.0 μg doses are sufficient to induce sex reversal at male-producing temperatures (Crews et al., 1991; Wibbels et al., 1991). Eggs designated as controls were given a 5 μl bolus of 95% ethanol.

In 2010, eggs were treated during the middle of development (day 20–21 post-oviposition). Eggs were divided into two groups: 10.0 μg estradiol in 95% ethanol or 95% ethanol only. Prior to dosing, eggs were placed in an incubator set at 31 °C and monitored as described below.

Once dosing was complete, eggs were placed into plastic boxes containing moistened vermiculite (approximately −150 kPa) and all boxes were placed into an incubator set at 31 °C, a temperature that produces all female hatchlings in this species. Eggs were checked at least twice per week, water replaced as needed, and boxes rotated to minimize any temperature variation within the incubator. Upon pipping (initial breach of the eggshell) eggs were transferred into individually labeled containers that were housed within plastic boxes to maintain humidity. These boxes were held at room temperature. Mass was recorded 10 days after hatching to the nearest (0.1 g).

Hatchlings were monitored daily and maintained following standard husbandry protocols. At approximately 10 weeks post-hatch,

plastron length (to the nearest mm) was collected and the DTH test was performed on all hatchlings (see methods below). A blood sample was collected to assess either bacteria killing capacity (2008) or total Ig levels (2009). All hatchlings were euthanized following blood collection. This research was conducted under IACUC protocols 01-2008 and 01-2009.

Bactericidal assay

The bactericidal assay measured the ability of hatchling plasma to kill the bacteria *Escherichia coli* (Paitz et al., 2010; Tieleman et al., 2005). Five microliters of plasma was mixed with a 10 μl aliquot containing 200 colony forming units of the bacteria *Escherichia coli* (ATCC #8739; Microbiologics, St. Cloud, MN) in PBS, and 100 μl of CO_2 independent media enriched with 4 mM L-glutamine and 5% fetal bovine serum. Samples were incubated for 30 minutes at 31 °C, and 50 μl was plated onto agar plates and incubated overnight at 37 °C. The number of colonies present on these plates was compared to control plates grown only with *E. coli* in media and the proportion of bacteria killed was determined.

ELISA

Total Ig levels were determined using an enzyme-linked immunosorbent assay (ELISA) (Zimmerman et al., 2010b). Polystyrene 96-well plates were coated with 100 μl/well of a 20 μg/ml solution of unlabeled mouse anti-turtle light chain antibody (HL 673; University of Florida Hybridoma Facility) dissolved in PBS, then incubated overnight at 4 °C. Wells were washed three times for 3 minutes with 200 μl per well of PBS-1% BSA-0.05% Tween buffer (PBS-T) to remove excess antibody and block the plate. Plasma samples were diluted 1:300 in 100 μl PBS-T in each well. The standard curve was determined using 25 μg purified turtle Ig making eight twofold serial dilutions. Turtle Ig was purified using a Melon gel IgG spin purification kit (Pierce) following the manufacturer's directions. The plates were incubated at room temperature for 1 hour, and then washed as before. One hundred microliters of a 1:500 dilution of biotinylated mouse anti-turtle antibody was added to each well, and plates were incubated and washed as before. Biotin conjugates were prepared using standard methods. Briefly, N-hydroxysuccinimidobiotin (NHS biotin; H1759 Sigma Aldrich) was dissolved in dimethylsulfoxide (DMSO; at 4 mg/ml). Twenty-five microliters of this solution was added to the purified antibody (1 mg) and allowed to sit for 2 hours at room temperature. The mixture was dialyzed overnight to remove unconjugated biotin and stored at 4 °C with sodium azide. One hundred microliters of a 1:1000 dilution of Streptavidin-HRP was added to each well, and then incubated and washed as before. Plates were washed once 100 μl/well with ddH2O before adding 100 μl/well of ABTS powder dissolved in ABTS buffer solution. The optical density was determined after 20 minutes using a Powerwave 340 plate reader (BioTech Inc) with a 405-nm wavelength.

DTH test

The DTH test measured the immune response to the novel mitogen phytohemmaglutinin (PHA; 61764, Sigma Aldrich) (Les et al., 2009; Paitz et al., 2010). A priming injection of 20 μl of a 10 mg PHA/1 ml PBS solution was given intraperitoneally. The thickness of the front right foot was measured to the nearest 0.01 mm using a digital thickness gauge. The challenge dose was injected 48 hours later into the right foot. The thickness of the foot was measured, as before, 12 hours later. Swelling response was calculated as the difference between the pre-injection and the post-injection measurements.

Statistical analysis

Bacterial killing capacity was arcsine square root transformed, DTH responses were square root transformed, total Ig levels were log transformed, and mass was squared prior to statistical analysis. Only data from 2008 included early and late season clutches. Bacterial killing capacity from 2008 included season as a factor and clutch as the random factor nested in season. Including season in 2008 DTH did not result in significant estrogen treatment effects, so DTH data from 2008 and 2009 were combined into a single mixed model ANOVA in order to increase the sample size. Year was included in the analysis and clutch was nested in year. Total Ig levels from 2009 and DTH data from 2010 were analyzed in separate mixed model ANOVAs with clutch as the random factor. All interactions were tested in each ANOVA. Plastron length did not significantly vary between groups, and did not significantly contribute to the variance of the response variable when added as a covariate, and thus was removed from the models. Mass varied significantly between treatment groups in 2008 and so was included in models that included data from 2008. Mass at hatching did not vary in other years and did not significantly contribute to the variance of the response variable when added as a covariate, and thus was removed from the models from 2009 and 2010. All statistical analyses were done using SAS v. 9.2 (SAS Institute, Cary, NC, USA).

Results

Estradiol treatment did not significantly affect bacterial killing capacity of hatchling plasma to *E. coli* ($F_{3,\ 31.33} = 0.41$; $p = 0.75$; Fig. 1). Across all groups average killing capacity ranged from 19% to 31% (Fig. 1). In addition, early and late clutches did not significantly vary in killing capacity ($F_{1,\ 9.72} = 1.0$; $p = 0.34$). There was no effect of estradiol treatment on total Ig concentrations ($F_{3,\ 33} = 0.78$; $p = 0.51$; Fig. 2), although the average Ig level was highest in the 10.0 μg treatment group. For the DTH responses, there was a significant effect of clutch ($F_{12,\ 44.03} = 1.96$; $p = 0.042$) and a significant year by dose interaction ($F_{3,\ 55.39} = 3.12$; $p = 0.032$). Post-hoc analysis showed that the controls in 2009 were significantly lower than the controls in 2008 (Fig. 3). The response of individuals given the 0.1 μg dose in 2008 was also significantly lower than those given the 1.0 μg dose, but this pattern was not seen in the 2009 data. Estradiol treatment later in development did not significantly affect the DTH response ($F_{1,\ 9.96} = 1.21$ $p = 0.29$; Fig. 4). Mass significantly varied between treatment groups in 2008 only (2008: $F_{13,\ 81}$, $p = 0.042$; 2009: $F_{3,\ 75} = 0.49$, $p = 0.69$; 2010: $F_{1,\ 79} = 0.02$, $p = 0.90$). For 2008 data, post hoc tests showed that the 0.1 μg group was significantly lighter than the 1 μg group ($p = 0.027$). Other treatment groups did not significantly vary from each other. Plastron length did not vary in any year (2008:

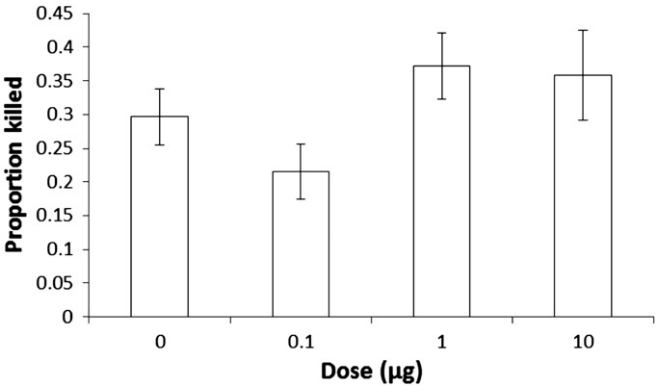

Fig. 1. Proportion of bacteria killed (±S.E.M.) by estrogen treatment in 2008. Bactericidal capacity of plasma was not affected by estrogen treatment.

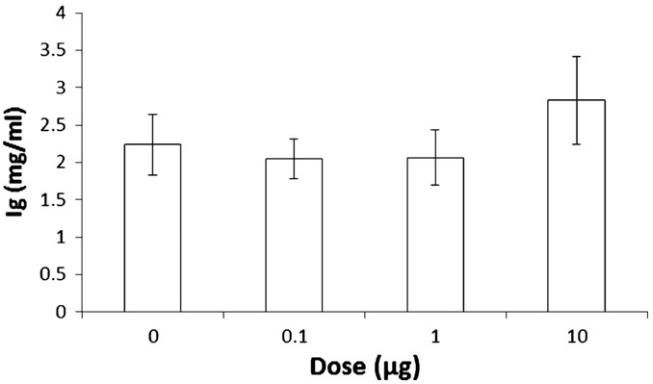

Fig. 2. Total Ig (±S.E.M.) for each estrogen treatment in 2009. Total Ig levels were not affected by estrogen treatment.

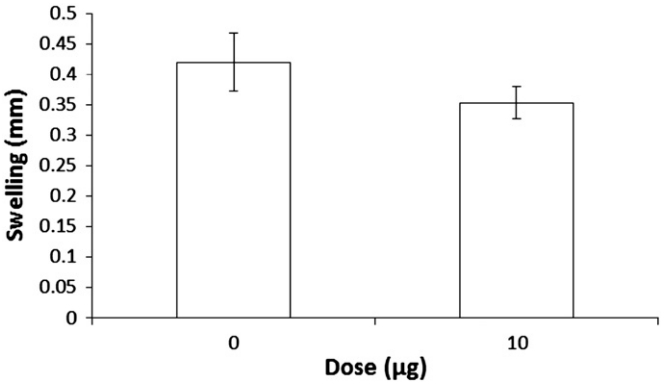

Fig. 4. Delayed-type hypersensitivity (DTH) response ±S.E.M. for each estrogen treatment in 2010. Eggs were dosed later in development compared to 2008 and 2009, near the middle third of development.

$F_{3,\ 81} = 2.24$, $p = 0.09$; 2009: $F_{3,\ 75} = 0.49$, $p = 0.69$; 2010: $F_{1,\ 79} = 3.72$, $p = 0.06$).

Discussion

In the present study, we set out to address whether exposure to estradiol during embryonic development affected the immune responses of hatchling turtles. We predicted that estradiol treatment would result in increased bactericidal activity and total Ig levels but a decreased DTH response. While we did find an effect of clutch on DTH responses and yearly variation in DTH responses in untreated individuals, we failed to detect any effect of estradiol treatment on any immune response that we measured. The finding of no effect with estradiol treatment is somewhat surprising given that early treatment with a variety of steroids has produced altered immune responses in other groups (see Navara and Mendonca, 2008). However our approach of using multiple doses, varying timing of steroid administration, and quantifying multiple branches of the immune system strongly suggests that maternal estradiol does not influence immune system development in *T. scripta*. We consider potential explanations of our findings below.

One possible explanation is that the early metabolism of estradiol may have prevented the interaction of estradiol with the developing immune system. However, we feel that this scenario is unlikely for several reasons. First, other steroids such as testosterone and corticosterone are also metabolized early in development (Paitz et al., 2011; von Engelhardt et al., 2009), and yet studies have found effects of these steroids on the development of the immune system. Second, steroid metabolites, including those of estrogen, have been shown to affect immune responses (Suitters et al., 1997). So, even though

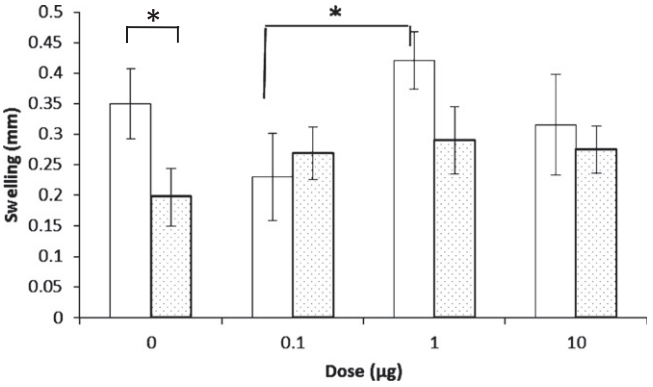

Fig. 3. Delayed-type hypersensitivity (DTH) response ±S.E.M. for each estrogen treatment. Asterisks represent significant differences after correcting for multiple post-hoc comparisons. Open bars represent 2008 data and stippled bars represent data from 2009.

estradiol is quickly metabolized in *T. scripta* eggs to estrogen sulfates (Paitz et al., 2012), these metabolites would still have the potential to influence immune development. Further, with our 2010 eggs, we attempted to bypass the early developmental window where estrogens are rapidly metabolized by dosing eggs during the middle third of development. It is unknown if exogenous estrogens would also be metabolized at this time point; however estrogen receptors are present, so it is possible that some estrogen could reach the receptors before being metabolized (Ramsey and Crews, 2007). In red-eared slider turtles, the majority of estrogen metabolites begin to reach the embryo just after the end of the temperature sensitive period, near the start of the last third of development (Paitz et al., 2012). A previous study also confirmed that estradiol is present in the embryo at this time (White and Thomas, 1992). Despite this, we saw no effects of estradiol treatment on the immune response.

So what else might explain our finding of no effect of estradiol treatment on immune responses? In mice, estrogen signaling has been shown to be necessary for normal development of the thymus and spleen (Erlandsson et al., 2001). However, not all doses of estradiol affect the normal development of immune related tissues. Indeed, several studies have reported a dose dependent effect of estrogen treatment. In chickens, low doses of estradiol, comparable to endogenous levels, had no effect on the bursa, while the highest dose which falls considerably out of physiological range substantially inhibited bursa growth (Norton and Wira, 1977). Follicles in the bursa, which are the site of B cell maturation, develop normally at low doses of estradiol, but are significantly reduced in both size and number at high doses outside of physiological range (Quinn et al., 2009). Likewise, in Japanese quail, only high doses of 17β-estradiol caused significant histological changes in structure and reduction in the number of lymphoid cells from the bursa (Razia et al., 2006). There may be a range of estrogen levels over which normal development of immune tissues occurs, and it is possible that our manipulations of estradiol may have fallen within this range. However, we feel that this scenario is unlikely given that we spanned two orders of magnitude with our treatments, and that at least some of our estradiol doses were above the normal physiological range—doses that are demonstrated to alter sex determination in this species (Crews et al., 1991).

Another possible factor that could account for our finding of no effect is the time at which our measurements were taken, and how that relates to the maturation of the immune system. The timing of the maturation of the immune system of reptiles varies widely by species. In the ocellated skink (*Chalcides ocellatus*) and the soft-shelled turtle (*Trionyx sinensis*), development of the thymus and spleen occurs during the final third of embryonic development (El Deeb and Saad, 1990). The garden lizard (*Calotes vesicolor*) can mount an antibody response to foreign antigens at hatch (Kanakambika and Muthukkaruppan, 1972), indicating a relatively mature immune system at hatching, while snapping

turtles (*Chelydra serpentina*) can only reject allografts after several months (Borysenko and Tulipan, 1973), indicating that their immune system takes longer to develop. Little information is available on the development of the immune response in red-eared sliders, and consequently, we had no previous studies on which to base our timing of immune measures. Thus, we could have collected our immune measures too early, before the immune system was fully mature, and thus were unable to detect small differences in immune responses that may become more pronounced as the immune system matures. Or, we could have measured responses too late, allowing immune responses that were depressed early after hatching time to catch up with other immune responses. However, other studies have detected post-hatch variation in immune measures following *in ovo* androgen treatment at or within a few days of oviposition (Navara et al., 2006; Sandell et al., 2009). Further, we used a variety of immune measures to assess multiple branches of the immune system, and we were able to detect differences in the DTH response among clutches (Fig. 2). Nonetheless, additional studies aimed at determining the timing of immune maturation would be very useful for understanding the trajectory of the development of immunity in this species and may help resolve when steroid-mediated effects, if present, are most likely to be detected.

One final factor that could account for our results is the fact that we assessed immune function in only female hatchlings. Previous research on maternal steroid effects indicates that some outcomes are sex specific (Love and Williams, 2008). Because our highest estradiol dose would have caused complete sex reversal, we could not have assessed immune responses in both sexes. At present, we cannot rule out the possibility that the lower doses, those closer to physiological levels, might affect immune function in males, but we found no evidence for such an effect in females.

In conclusion, we found no effects of estradiol treatment on immune system development in the red-eared slider despite using multiple doses, varying timing of steroid administration, and quantifying multiple branches of the immune system. While it is possible that other experimental approaches may allow us to detect effects of maternal estradiol on immune system development, our current findings suggest these effects may be subtle, if present at all. Further study is needed on the development and maturation of the immune system of sliders in order to understand the role estrogen and other steroids play in the immune system.

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