Immune Activity, but Not Physiological Stress, Differs between the Sexes during the Nesting Season in Painted Turtles

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Immune Activity, but Not Physiological Stress, Differs between the Sexes during the Nesting Season in Painted Turtles

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ABSTRACT.—Energetically expensive life-history events often require energy trade-offs with day-to-day maintenance activities, particularly when such life-history events are also physiologically stressful. To understand how an energetically expensive life-history event affects physiological stress and day-to-day maintenance, we compared stress levels and immune activity in male and female Midland Painted Turtles (*Chrysemys picta picta*, hereafter Painted Turtles), during the nesting season. We captured adult Painted Turtles during the nesting season and quantified baseline physiological stress levels (by determining ratios of circulating leukocytes) and immune activity (by measuring the skin-swelling response to a phytohemagglutinin challenge). We predicted that females would exhibit higher physiological stress levels and decreased immune activity compared to males attributable to the energetic demands and stressful conditions that reproduction places on females, but not males, in this species. Contrary to our predictions, we found that the sexes did not differ in physiological stress levels and that females demonstrated greater immune activity than males during the nesting season. Our results agree with a growing body of literature suggesting that immune function in Painted Turtles is not negatively correlated with physiological stress levels, as is common in other vertebrate taxa. Instead, female turtles may demonstrate enhanced immune activity during the nesting season to counter the increased infection risk they may experience as they come into contact with new individuals and environments during overland travel to nesting sites.

Life-history theory predicts, and empirical evidence supports, that trade-offs exist between allocation of resources to day-to-day growth and maintenance activities compared to more infrequent but expensive reproductive efforts (e.g., Metcalfe and Monaghan, 2001; Alonso-Alvarez et al., 2004; Martin et al., 2008). A consequence of such trade-offs is that the high energy demand of certain life-history events, such as reproduction, overwintering, or molting, may require the temporary suppression of other day-to-day functions, such as growth or immune activity (e.g., Nelson and Demas, 1996; Nordling et al., 1998; Hanssen et al., 2005). For example, immune activity is suppressed during hibernation in turtles (Schwanz et al., 2011) and during molt in House Sparrows (Martin, 2005). Moreover, trade-offs in resource allocation also explain why energetically expensive life-history events are separated temporally throughout the annual cycle (e.g., migratory birds typically molt between bouts of reproduction and migration; Barta et al., 2008).

In addition to the constraints placed by energy limitation on how an individual allocates resources, physiological stress also can affect the prioritization of expensive life-history events compared to day-to-day maintenance activities (Landys et al., 2006; Angelier and Wingfield, 2013). In vertebrates, hormones such as glucocorticoids are released in response to acute stressors such as an attack by a predator, and the sudden elevation of glucocorticoids is advantageous in concentrating energy toward activities essential for survival (such as escape behavior; Moore et al., 1991). Chronic elevation of glucocorticoid stress hormones such as corticosterone, however, can lead to allostatic overload and result in negative physiological effects such as depressed immune function (McEwen and Wingfield, 2003; Millet et al., 2007). If certain life-history events, such as migration or reproduction, are both energetically expensive and physiologically stressful (e.g., entailing increased exposure to predators, travel through inhospitable habitat or inclement weather, etc.), we might expect that during such events,

individuals incur a substantial cost manifesting in decline of body condition, depressed immune function, and even reduced survival (e.g., Moore et al., 2000; Romero and Wikelski, 2001; Jessop et al., 2013). Indeed, immune function is lowest and disease prevalence is highest in birds and mammals during the breeding season (Nelson and Demas, 1996). To cope with such physiological challenges brought on by predictable life-history events, animals may circulate higher baseline levels of corticosterone, a phenomena described by the preparative hypothesis (Eikenaar et al., 2012). This "buffering" against predictably challenging events by increasing circulating glucocorticoids may explain the seasonal pattern of corticosterone levels seen in many reptiles, where peak corticosterone levels coincide with the breeding season (Romero, 2002). The physiological costs incurred during challenging life-history events have also been demonstrated by experimentally increasing the magnitude of the predictably stressful event. For example, when researchers experimentally increased brood sizes, male Pied Flycatchers (*Ficedula hypoleuca*) engaged in feeding nestlings demonstrated an increase in physiological stress levels, and their nestlings showed higher physiological stress resulting in a concomitant decrease in immune activity compared to nestlings in smaller broods (Ilmonen et al., 2003). Similarly, during the reproductive season, Galápagos Marine Iguanas (*Amblyrhynchus cristatus*) at sites with recently introduced predators demonstrated higher physiological stress levels compared to iguanas from sites without predators (Berger et al., 2007).

To understand how an energetically expensive life-history event affects physiological stress and day-to-day maintenance, we compared stress levels and immune activity in male and female Painted Turtles during the nesting season. Although Painted Turtles mate throughout the year and females store sperm until fertilization (Pearse et al., 2001), nesting is restricted to a few weeks in May and June, when females emerge 2–3 times from their wetland habitat to deposit a clutch of eggs on land (Ernst and Lovich, 2009; Refsnider and Janzen, 2012). Nesting is an energetically expensive process, during which a female turtle travels over land to a suitable nest site and then

spends an hour or more constructing a nest and depositing eggs (Ernst and Lovich, 2009). Moreover, nesting is also likely to be physiologically stressful because it increases females' exposure to risks such as predation and thermal stress (e.g., Spencer, 2002; Angilletta et al., 2009). Importantly, however, only female Painted Turtles incur the costs associated with nesting, because males do not engage in concentrated, nesting-related travel or incur the associated risks.

We captured male and female Painted Turtles during the nesting season and quantified baseline physiological stress levels and immune activity. We predicted that females would exhibit higher physiological stress levels and decreased immune activity compared to males, because of the energetic demands and stressful conditions that reproduction places on females, but not males, in this species.

MATERIALS AND METHODS

Trapping.—We captured adult Midland Painted Turtles (*Chrysemys picta picta*) during June 2016 at Ottawa National Wildlife Refuge (Ottawa County, Ohio). We placed basking traps and hoop nets baited with corn and sardines in oil in wetland and canal habitat at six sites concentrated around Crane Creek and checked and rebaited traps every 1–3 days (Tran et al., 2007). We individually marked each turtle by filing unique combinations of notches in the marginal scutes (Cagle, 1939), and we used front claw length and the location of the cloacal opening in relation to the carapace to ascertain sex. We palpated females to determine whether they were gravid in case gravidity affected stress levels or immune function. Turtles were then transported to the University of Toledo, where they were maintained in captivity for 48 h during the phytohemagglutinen (PHA) challenge (see below). Following the PHA challenge, all turtles were returned to Ottawa National Wildlife Refuge and released at the site of capture.

Heterophil: Lymphocyte Ratios.—Physiological stress levels often are estimated by quantifying corticosterone concentration in plasma; however, corticosterone levels are known to increase within minutes of a stressful event such as capture (Cash et al., 1997; Romero and Reed, 2005), making corticosterone an inaccurate measure of baseline stress levels in situations such as ours where study animals may remain in traps for an unknown period of time that could range from minutes to hours. An alternative method for estimating physiological stress is to examine circulating leukocyte profiles and specifically to quantify the ratio of heterophils to lymphocytes (H:L) in blood (Davis et al., 2008). Heterophils (equivalent to neutrophils in mammals) and lymphocytes are two of five types of leukocytes, and both play important roles in immune activity by multiplying in response to infection, inflammation, and stress. In particular, elevated levels of glucocorticoid hormones, such as corticosterone, lead to an increased production of heterophils, thereby increasing the H:L ratio in blood. The positive correlation between glucocorticoid concentrations and H:L ratios in a variety of taxa validates the use of circulating leukocyte profiles in estimating baseline levels of physiological stress (Davis et al., 2008). Importantly, in contrast with corticosterone concentrations that can rise immediately upon handling by a researcher, H:L ratios in reptiles may take hours to days to increase in response to a stressful event, allowing for a more accurate assessment of an individual's baseline level of physiological stress (Davis et al., 2008).

Immediately after we collected blood from a turtle, we used one drop of blood to make a blood smear on a glass slide. Slides were allowed to air dry in the field and were subsequently stained with Stat-Quick Wright Giemsa Stain (ENG Scientific, Inc., Clinton, NJ). To quantify circulating leukocyte profiles for each individual, we counted 200 blood cells consecutively (excluding erythrocytes and thrombocytes) from each blood smear and identified each leukocyte as a heterophil, eosinophil, basophil, lymphocyte, or monocyte (Kassab et al., 2009; Javanbakht et al., 2013).

Phytohemagglutinin Challenge.—To quantify immune activity, we measured the localized skin-swelling response of turtles to phytohemagglutinin (PHA). PHA triggers infiltration by leucocytes at the site of injection, resulting in a localized skin-swelling response that typically increases quickly, peaks, and then more gradually returns to normal. Importantly, the PHA challenge measures immune activity but not immunocompetency or immune functioning per se (Martin et al., 2006). We used a 28-gauge syringe to inject 10 µL of 10 mg ml^{-1} PHA (L9017, Sigma-Aldrich Corp., St. Louis, MO) in 0.01M phosphate-buffered saline (PBS) into the webbing between the fourth and fifth toe on a random hind foot of each turtle (Schwanz et al., 2011). We also injected 10 µL of PBS into the webbing of the other hind foot as a control. We used digital calipers to measure the thickness of the foot webbing at the injection sites to the nearest 0.01 mm at four time points: immediately prior to injection and then at 6 h, 24 h, and 48 h after injection. We measured each foot twice at each time point and averaged the two measurements to obtain our final measurement of foot web thickness at each of the four time points.

Data Analysis.—We determined the mean proportion of circulating leukocytes made up by each of the five white blood cell types for males and females. We then used chi-square (χ^2) goodness-of-fit tests to compare H:L ratios between males and females.

To analyze skin-swelling response to PHA injection, we first tested individuals' responses over time by fitting mixed-effects models of foot web thickness separately for control and PHA injections. Time since injection, sex, and "time × sex" interaction were predictors, and individual identity was included as a random effect in both models. We also determined the peak swelling response in PHA-injected feet as thickness at 6 h minus thickness pre-injection. We then used a linear mixed model to test whether the skin-swelling response to PHA was greater than the response to the control injection and whether the response was influenced by sex. Treatment (control or PHA injection), sex, and "sex × treatment" interaction were predictors and individual identity was included as a random effect. We also compared both the peak swelling response at 6 h between males and females, and between gravid and non-gravid females, using t-tests. All statistical tests were conducted using R v.3.3.1 (R Core Team 2013), with α = 0.05.

RESULTS

During 7–28 June 2016, we captured 14 adult male and 20 adult female Painted Turtles during 385 trap-days. Sixteen of the 20 female turtles were gravid based on the presence of shelled eggs in the abdominal cavity. We assume the remaining four females also were reproductive during the study period, making them susceptible to the same seasonal stressors as the 20 gravid females; these four females were likely captured either between clutches or after completing nesting for the season.

TABLE 1. Circulating leukocyte profiles of male ($N = 14$) and female ($N = 20$) Painted Turtles captured at Ottawa National Wildlife Refuge, Ottawa County, Ohio, in June 2016. Values shown are mean proportions for each leukocyte type.

	Males	Females
Lymphocytes	0.578	0.596
Heterophils	0.239	0.248
Eosinophils	0.085	0.082
Basophils	0.067	0.082
Monocytes	0.031	0.027

Lymphocytes and heterophils made up the largest proportion of circulating leukocytes in Painted Turtles (Table 1). The H:L ratio did not differ between males (0.426) and females (0.422; $\chi^2 < 0.001$; $P > 0.05$) or between gravid (0.421) and non-gravid females (0.426; $\chi^2 < 0.01$; $P > 0.05$).

Foot web thickness increased over time in response to both the control and the PHA injection and was consistently greater in females than in males (Table 2). Peak skin-swelling was significantly greater in feet injected with PHA compared to control-injected feet ($F_{1,11} = 8.77$, $P = 0.013$). Females had a significantly greater peak skin-swelling response at 6 h than did males (males: 0.127 mm, females: 0.319 mm; $t_{14} = 3.13$, $P = 0.007$; Fig. 1); however, there were no differences at pre-injection, 24 h, or 48 h (all P-values > 0.05). The peak skin-swelling response did not differ between gravid and non-gravid females ($t_9 = -0.72$, $P > 0.05$).

DISCUSSION

We compared physiological stress and immune activity between male and female Painted Turtles during the nesting season. Because females, but not males, expend considerable energy to produce eggs, travel to a nest site, and construct a nest, we hypothesized that females would demonstrate higher physiological stress but lower immune activity than would males. Our results demonstrated the sexes did not differ in physiological stress as measured using H:L ratios. Furthermore, females exhibited higher immune activity, as measured by the skin-swelling response to a phytohemagglutinin challenge, than did males. We observed no differences in either H:L ratios or PHA-induced skin-swelling response between gravid and non-gravid females, although our sample size for non-gravid females was only four individuals.

Despite the considerable difference in reproductive effort expended by females compared to males during the nesting season, H:L ratios were nearly identical between the sexes. Contrary to our predictions, this result suggests that female Painted Turtles do not experience higher physiological stress levels during the nesting season than do males. Other studies on freshwater turtles have found that corticosterone levels were not significantly higher during the nesting season than at other times of the year (*Graptemys geographica*: Selman et al., 2012; *Chrysemys picta bellii*: Refsnider et al., 2015). For example, female Painted Turtles from sites with high levels of human disturbance did not have higher baseline corticosterone levels than females from less-disturbed sites during the nesting season (Polich, 2016). In combination, our study and others suggest that physiological stress is not substantially elevated in female freshwater turtles during the reproductive season. In contrast, other reptiles generally exhibit higher concentrations of corticosterone during the breeding season (*Sceloporus occidentalis*:

TABLE 2. Statistical results from mixed-effects models of foot web thickness following control and PHA injections, and model comparing peak swelling (6 h postinjection) in control- and PHA-injected feet of Painted Turtles. Individual identity was included as a random effect in all models. Asterisks indicate statistically significant differences at $\alpha = 0.05$.

	df	df_{den}	F	P
Thickness (control injection)				
Time	3	33	5.53	0.0034*
Sex	1	33	8.88	0.0054*
Time × sex	3	33	1.12	0.3568
Thickness (PHA injection)				
Time	3	42	25.08	<0.0001*
Sex	1	42	8.43	0.0059*
Time × sex	3	42	2.92	0.0448*
Peak swelling (6 h)				
Treatment	1	11	1.84	0.2024
Sex	1	11	8.77	0.0129*
Treatment × sex	1	11	5.90	0.0334*

Dunlap and Wingfield, 1995; *Crotalus atrox*: Taylor et al., 2004). Potentially, the lack of difference between the sexes in baseline physiological stress may be a physiological strategy to maximize resources available for reproduction in species where reproduction requires a substantial energy investment (Jessop, 2001). Importantly, our study did not assess the magnitude of turtles' response to a stressor such as capture and handling (e.g., Cash et al., 1997). If physiological stress in freshwater turtles is not elevated during the nesting season as a strategy to maximize resources available for reproduction, then we would predict that the stress response mounted by female turtles in response to capture and handling would be dampened compared to that of males.

In vertebrates, nonessential functions are generally depressed during physiologically stressful events such that more energy can be allocated to the "fight-or-flight" response. Generally, physiological stress leads to depressed immune function in vertebrates, and in many vertebrates, PHA-induced swelling, a response by the adaptive arm of the vertebrate immune system, is traded off with other costly physiological functions such as molting or breeding (Martin et al., 2006). Again, we observed a counterintuitive result in Painted Turtles: immune activity was greater in females during the nesting season than in males. There are several potential explanations for this result. First, previous research on Painted Turtles (Refsnider et al., 2015) suggests that physiological stress and function of the innate
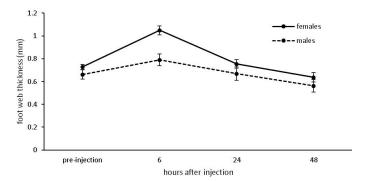

FIG. 1. Thickness of foot webbing before (pre-injection) and after injection (6–48 h) with PHA. Females demonstrated a significantly higher peak swelling response at 6 h than did males ($t_{14} = 3.13$, $P = 0.007$).

immune system are not strongly correlated in female Painted Turtles over the course of a year. Therefore, even if physiological stress levels during the nesting season were higher in females than in males, immune function in females likely would not be depressed compared to males. Our detection of similar baseline stress levels in males and females, but increased immune activity in females, suggests that physiological stress and activity of the adaptive immune system are also de-coupled in Painted Turtles. Second, during the nesting season, female turtles travel overland to a nesting site, which could increase their exposure to other individuals and different environments, either of which could increase infection risk and may, therefore, favor enhanced immune activity. Importantly, PHA-induced skin swelling should not be interpreted as a measure of immune competence but rather as an indicator of immune activity. In House Sparrows (*Passer domesticus*), for example, PHA-induced skin swelling is not a direct measure of T-cell–mediated immunity but rather a multifaceted index of cutaneous immune activity (Martin et al., 2006). Therefore, females' greater response to the PHA challenge compared to males may simply reflect an increase in overall immune activity without indicating increased immunocompetence during the nesting season. Finally, PHA-induced skin-swelling appears to be a relatively inexpensive response to mount and may not require a substantial trade-off with energy allocated to other activities (e.g., Hõrak et al., 2000). Alternatively, the response we observed in female turtles may be traded off with physiological functions other than breeding that we did not measure here, such as maintaining high body condition, wound-healing, or preventing oxidative stress.

We observed no differences in either H:L ratios or PHA-induced skin-swelling response between gravid and non-gravid females, although our sample size for non-gravid females was only four individuals. These four non-gravid females probably were captured either between clutches or after completing nesting for the season, as female Painted Turtles typically nest 2–3 times per season, ~2 weeks apart. Future studies should endeavor to compare reproductive versus nonreproductive female turtles during the nesting season to directly test how nesting affects baseline physiological stress levels, magnitude of the stress response, and immune function in female turtles.

Our study demonstrated that during the nesting season, an energetically expensive period for female but not for male Painted Turtles, the sexes did not differ in physiological stress but females demonstrated higher immune activity than males. Our results are in agreement with a growing body of literature suggesting that immune function in Painted Turtles is not negatively correlated with physiological stress levels, as is common in other vertebrate taxa (Selman et al., 2012; Refsnider et al., 2015; Polich, 2016). Instead, female turtles may demonstrate enhanced immune activity during the nesting season to counter the increased infection risk they may experience as they come into contact with new individuals and environments during overland travel to nesting sites.

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