Immunosenescence and its influence on reproduction in a long-lived vertebrate

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Summary Statement:

This study finds differences in immune function of painted turtles across ages, sexes, and

sampling date, and finds some evidence of a trade-off between reproductive effort and immune

function with age.

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Abstract

Immunosenescence is a well-known phenomenon in mammal systems, but its relevance in other long-lived vertebrates is less understood. Further, the influence of age and reproductive effort on immune function in long-lived species can be challenging to assess, as long-term data are scarce and it is often difficult to sample the oldest age classes. We used the painted turtle (*Chrysemys picta*) to test hypotheses of immunosenescence and a trade-off between reproductive output and immune function in a population of a long-lived vertebrate that has been monitored for over 30 years. These long-term data are utilized to employ a unique approach of aging turtles with mark-recapture data and population-specific growth modeling to obtain more accurate estimates of age. We analyzed natural antibodies, lysis ability, and bactericidal competence in 126 individuals from 1 to 33 years of age captured during May and June in 2011. Older turtles exhibited greater natural antibody levels than young individuals across sexes. Young females with large clutches exhibited greater lysis ability, while older females with large clutches had decreased lysis ability, suggesting a trade-off between reproductive output and immune function conditional upon age. However, bactericidal competence increased later in the nesting season for older females. Our study rejects the hypothesis of immunosenescence in a long-lived turtle, despite evidence of actuarial and reproductive senescence in this population. Additionally, we detected mixed evidence for a trade-off between reproduction and immune health.

Introduction

Vertebrate aging is most commonly characterized by declining reproduction and survival with advancing age. Immunosenescence, the progressive dysregulation of the immune system with age in vertebrates, is a potential mechanism by which senescence shapes population demography. Immune defenses are energetically costly, but reduced immune system function lowers defenses to parasitism and disease, which could decrease survivorship, reproductive ability or parental care in older individuals (Schmid-Hempel, 2003; Palacios et al., 2011b). Immunosenescence has been well-studied in humans, where impacts of advancing age have been found in both the innate and adaptive branches of the human immune system (Pawelec et al., 2010). The complex relationship between age and immune function in other vertebrates is less understood, in part due to the difficulty of maintaining long-term datasets to reliably age long-lived vertebrates and obtain confident representation of the oldest age classes where senescence patterns are often most pronounced (e.g. Reed et al., 2008; Warner et al., 2016).

The decline of immune function with age in wild vertebrates is hypothesized to be dependent on the life history of an organism. Long-lived species may invest more in adaptive immune responses, which take longer to develop than innate responses yet are more effective against repeated pathogen exposure, while short-lived species rely on less energetically expensive innate immune responses (Lochmiller and Deerenberg, 2000; Martin II et al., 2006; Lee et al., 2008). Both immune system branches can senesce at different rates, and this rate of senescence may be influenced by life history (e.g. Zimmerman et al., 2013b). Reproductive status and frequency also are predicted to influence immune function, such that increased reproductive investment occurs at the expense of immune function (e.g. Palacios and Bronikowski, 2017; Leivesley et al., 2019). Age may mediate the trade-off between reproduction

and immune function, and older and younger individuals may express different immune responses to increased reproductive output. For example, the terminal investment hypothesis suggests that older individuals may respond differently to immune challenge than younger individuals, such that older individuals invest heavily in reproduction at the expense of immune function as their likelihood of future reproductive bouts decreases (e.g. Hanssen, 2006).

There are many gaps in our understanding of immunosenescence in reptiles. Many reptile species have long lifespans (30-70 years for turtles and crocodilians), grow past reproductive maturity, and increase reproductive effort with size (Congdon et al., 2001; Wilkinson et al., 2016; reviewed in Hoekstra et al., 2020). These traits suggest that immunosenescence may not manifest in the same manner in reptiles when compared to other vertebrates, such as mammals or birds, and may even be absent due to selective pressures on maintaining immune function with increased reproduction (e.g. Sparkman et al., 2007). Alternatively, reptile immune systems may experience trade-offs throughout their lives that prioritize either the less costly innate or relatively expensive acquired immune branches (Lee, 2006), dependent upon the age, sex, season, or reproductive status of the individual. In reptiles, unlike mammals, the adaptive immune system responds more slowly and does not exhibit robust response during repeated pathogen exposure (Zimmerman et al., 2010a). Thus, innate immune function is hypothesized to be critical to overall immune health. As individuals age, the innate immune system may be prioritized over the energetically expensive adaptive immune system, and this may result in the maintenance or even increase in function of the innate immune system with age, in contrast to aging mammals (Zimmerman et al., 2010a; Zimmerman et al., 2013b).

To increase our understanding of immunosenescence patterns and the influence of reproduction, sex, and sampling date on immune function in a long-lived reptile, we studied the

painted turtle (*Chrysemys picta*, Schneider 1783). Painted turtles are a widespread species throughout North America, and exhibit lifespans greater than 55 years in some populations (Congdon et al., 2003). While one study has assessed the immune function of painted turtles during the fall, winter, and spring (Schwanz et al., 2011), and another assessed the immune status of female painted turtles during the active nesting season (Refsnider et al., 2015), the variation in immune function between the sexes as they age is unknown. In this study we sought to quantify the relationships among age, sex, reproductive output, and the innate immune system during the nesting season (May – June) as an integrative approach to understanding immunosenescence in painted turtles. Specifically, we assessed three innate immune measures - natural antibodies, lysis ability, and bactericidal competence of plasma - across age, sex, and date during the nesting season in a well-studied population in eastern Illinois in the United States. This population of painted turtles has been studied for over 30 years, and represents a rare resource for studying senescence in reptiles. This population displays increasing adult mortality with advancing age in both males and females (Reinke et al., 2019), and older females have lower fitness than younger adult females (i.e., they produce eggs with reduced hatching success) (Warner et al., 2016). We hypothesized that painted turtles would demonstrate immunosenescence, consistent with this recent evidence of actuarial and female reproductive senescence in this population, and that the impact would be particularly pronounced in the oldest individuals. Additionally, we tested the hypothesis that reproductive effort negatively affects immune function, independent of age. This study therefore gives the first insight into the potential trade-offs between reproduction and immune system maintenance in turtles.

Methods

Field Methods and Measures

The sampling for this study took place at a long-term research site, Thomson Causeway Recreation Area (TCRA), in Thomson, Illinois, United States. Painted turtle handling and sample collection were permitted by the United States Army Corps of Engineers, the United States Fish and Wildlife Service, and the Illinois Department of Natural Resources. This research was performed in accordance with the Iowa State University IACUC guidelines (12-03-5570-J). In May and June 2011, the nesting season for painted turtles in this population, we trapped painted turtles using fyke traps. We marked individuals caught for the first time (N = 33) with a unique identifier and estimated age when possible using plastral scute rings (Moll, 1973). For individuals that were first captured and marked in previous years (N = 93), we noted age based on plastral scute rings and previous capture histories. We recorded six measurements of size for each turtle: curved carapace length and width, straight carapace length and width, and plastron length and width. We drew 200μl of whole blood from the caudal vein for immune measures. Immediately following the blood draw, we separated the whole blood into plasma and red blood cells through centrifugation, and the plasma was placed into a separate tube and frozen in liquid nitrogen before storage at -80°C. We monitored nesting female painted turtles and processed them in a similar manner to trapped turtles. To measure reproductive output, we excavated nests and recorded clutch size for each nesting female. The 126 painted turtles we sampled for this study include reproductively mature males (≥ 3 yrs old, N = 39) and females (≥ 6 yrs old, N = 76) to 33 years old (see *Age Estimation*). Juveniles (N = 11) were also included to represent the early stages of life before maturation.

Age Estimation

Aging estimates of reptiles in immune studies have often relied on size as a proxy for age due to the difficulty of long-term monitoring of individuals across time and the general correlation between reptile age and size (e.g. Palacios et al., 2013; Zimmerman et al., 2017). While this proxy may be appropriate for general patterns of aging and immune function, growth curves can be sex-specific, and in painted turtles, growth slows markedly at advanced ages, which complicates age estimation (Hoekstra et al., 2018). We incorporate new methods for aging the set of painted turtles in this study for whom age was unknown (35 out of 126). These new methods combine long-term capture history data and growth models to more accurately estimate individual ages for these cases.

We considered turtles to be of known age if they had visible, countable plastral scute rings at first capture, and these individuals were given an age equivalent to the number of scute rings (Moll, 1973). To calculate age in the study year (2011) for individuals of known age, we added the number of years that had elapsed since first known-age capture. The age distribution of known-aged individuals in this study is shown in Figure 1 (N = 91). A recent investigation of painted turtle growth found that sex-specific von Bertalanffy growth models (VBGF) best describe the relationship between growth and age of individuals of known age in the population of turtles (Hoekstra et al., 2018). Thus, for the turtles we captured that had no recapture history, or whose initial capture age was questionable, we used a sex-specific VBGF to estimate their age at first capture, and added subsequent elapsed years since first capture (N = 35; Fig. 1). While the ages of several of the older turtles in this study were estimated, the maximum age estimated in this study (33) is reasonable given the reported known ages of turtles from this population

(Hoekstra et al., 2018) and the recent recapture of female turtles in 2019 with known ages of 31 and 34 years of age and multiple males with known ages greater than 15. Additionally, the distribution of ages in this study is consistent with the distribution of ages reported in this population for males and females (Hoekstra et al., 2018).

Immune Measures

Natural antibodies and lysis

Natural antibodies and lysis ability are proposed to be the first line of defense against pathogens in vertebrates (reviewed in Ochsenbein and Zinkernagel, 2000), and these aspects of innate immune function have been studied in many reptile species (e.g. western terrestrial garter snake, Sparkman and Palacios, 2009; painted turtle, Schwanz et al., 2011; water python, Ujvari and Madsen, 2011; desert tortoise, Sandmeier et al., 2012). In the fall of 2011, we completed all measures of immune function on plasma samples. To assess natural antibody levels and lysis ability of plasma, we used a haemolysis-haemagglutination assay adapted from Matson et al. (2005) for use in painted turtles (Schwanz et al., 2011; Refsnider et al., 2015). Agglutination in this assay should be representative of natural antibody levels, which are abundant in a closely related turtle species, the red-eared slider turtle (Matson et al., 2005; Zimmerman et al., 2013a). Lysis may reflect activity of natural antibodies and the activation of the complement system through natural antibodies, but the complement system may also be initiated by an alternative pathway involving cleavage of the C3 complement protein thioester, as has been found in snapping turtles (Baker et al., 2019). We deviated from the protocol used in painted turtles by using rabbit red blood cells (RRBC) instead of sheep red blood cells because haemolysis is not consistently observed with sheep red blood cells. We added 10µL phosphate-buffered saline

(PBS) to each well of a 96-well plate and added 10µL positive control diluted 1:16 in PBS (anti-rabbit antibodies, Fisher Scientific; row 1), 10µL plasma (rows 2-7), or 10µL PBS (row 8) to the first column. We serially diluted two-fold across two 96-well plates (columns 2-24). Finally, we added 10µL of washed 3% RRBC in Alsever's anticoagulant (HemoStat Laboratories, Dixon, CA, USA) to each well. We incubated plates for 120 min at 26ºC. Following incubation, we removed plates and tilted them for ease of scoring. After 20 minutes of resting the plate at an angle, we scored agglutination titers as $-\log_2(1/D)$. D represents the highest dilution at which agglutination occurred, and we gave half scores if titers appeared intermediate. As we added PBS to the first column of the first plate for each run, the titer of the first column was scored as 2. We returned plates to the incubator at 26ºC until 24hr after the initial plate incubation start time and scored lysis immediately following this incubation in the same manner as agglutination. We ran all plates with the positive and negative control and samples in duplicate where possible, with exception of plasma-limited samples (N = 10). We used only one bottle of RRBC in Alsever's anticoagulant for this assay, which was sourced from a single rabbit (HemoStat Laboratories, personal communication), to prevent any deviations in reactivity to RRBC across plasma samples. Agglutination and lysis ability were measured for 117 individuals. Higher titers for agglutination indicate greater abundance of natural antibodies in the plasma sample, as high titers are an indication that natural antibodies are at high concentrations even in increasingly diluted plasma (Matson et al., 2005). Similarly, high titers for lysis indicate the plasma is able to lyse RRBCs even at more dilute concentrations (Matson et al., 2005). Thus, increased natural antibody levels and lysis ability predict increased immune function.

Bactericidal Competence of Plasma

We assessed bactericidal competence of plasma by quantifying its ability to kill or inhibit growth of *Escherichia coli* using a method modified from Matson et al. (2006) and Palacios et al. (2011a) for use in painted turtles (Refsnider et al., 2015). We performed the assay in the same manner as Refsnider et al. (2015), with a few deviations noted here. We reconstituted a pellet of lyphophilized *Escherichia coli* (Microbiologics, ATCC#8739) using 40ml 1X PBS, and we further diluted the stock solution to a working concentration of 1:40, which produced approximately 150 colony-forming bacteria per 10µl. We performed three control reactions by adding 10µl of the bacterial working solution to 100µl PBS. Sample reactions consisted of 10µl plasma, 90µl warm PBS, and 10µl *E. coli* working solution, and both control and sample reactions were incubated for 20 minutes at 28ºC. We plated the sample reactions and the three control reactions in duplicate using 50µl aliquots on 4% tryptic soy agar and incubated the plates overnight at 28ºC. For each sample and the controls, we averaged bacterial colony counts between the duplicates, and we divided this average by the average number of colonies on the control plates. This value was subtracted from 1 and multiplied by 100 to obtain percentage bacteria killed or inhibited from growth. Bactericidal competence was assessed in 105 individuals. Increased bactericidal competence predicts increased immune function.

Statistical Analyses

We performed the following statistical analyses in SAS v. 9.4 (SAS Institute, Cary, NC). We used a threshold of three standard deviations from the mean for outliers in the immune variables and subsequently removed two outliers from the agglutination results. As there were occasional negative percentages of bactericidal competence due to minor variations in bacterial

density from the working stock solution, we removed all negative percentages of bactericidal competence from the dataset if the percentage was less than -10% (N = 12), and we adjusted percentages between 0% and -10% to zero (N = 7) to represent no bactericidal competence. Thus, final sample sizes for the following models were 115 for natural antibody levels, 117 for lysis ability, and 93 for bactericidal competence. We assessed normality for natural antibodies, lysis, and bactericidal competence and found that while natural antibodies and lysis were normally distributed, bactericidal competence was not. Transformations failed to achieve normality, thus we analyzed untransformed bactericidal competence. We z-transformed age for each sex grouping with mean zero and unit variance (hereafter "zAge") for male, female, and juvenile painted turtles, as painted turtles in this population exhibit different distributions of age (female mean age ± SD = 14 ± 6.39; male = 8.36 ± 3.26; juvenile = 2.22 ± 0.77). Juveniles, while not a sex class, were thus treated as a separate sex for the models, as z-transforming age removed the correlation between sex and age, and juveniles display characteristics separate from sexually mature adults.

We assessed the relationships between zAge, sex, and day of year and the dependent variables - plasma natural antibodies, lysis ability, and bactericidal competence - using a general linear model. We included all two-way interactions between independent variables in original models and removed interactions that were non-significant ($\alpha = 0.10$). The original models for each dependent variable took the form:

$$Y = \mu + zAge + Sex + Day + (Sex \times Day) + (Sex \times zAge) + (zAge \times Day) + \varepsilon$$

For all final models, we removed interaction terms if they were non-significant (Pr > 0.1), and all interactions were non-significant for these models including all individuals.

We assessed the relationship between female reproductive output and the immune measures by including clutch size and its interactions with age, sex, and day of year in models that initially took the form:

$$Y = \mu + \text{Clutch Size} + \text{Age} + \text{Day} + (\text{Clutch Size} \times \text{Day}) + (\text{Clutch Size} \times \text{Age})$$
$$+ (\text{Age} \times \text{Day}) + \varepsilon$$

This model only assessed reproductive females in our dataset for which we have clutch size data (N = 62, ages 6-33). As only females were included in this model, corrections for sex-specific age distributions are not needed, and thus we use age in years instead of zAge. For the agglutination model, we removed all interactions, as they were non-significant (Pr > 0.1). Some interactions were significant for the female lysis and bactericidal competence models, and were thus included in the final models. We discuss model results in terms of least squares means (LS mean) ± SEM. We created all figures with ggplot2 (Wickham, 2016) in R (R Core Team, 2017). To visualize significant interactions among continuous variables from each model, we calculated minimum, maximum, and quartiles of continuous variables and calculated LS means estimates at these values from the model. We then plotted these LS means estimates ± SEM.

Finally, we assessed the influence of the oldest turtles for which we have estimated ages from sex-specific VBGF, rather than known ages, on model estimates (Fig. 1). For individuals with an estimated age (N = 35), we replaced the age we estimated from sex-specific VBGF with the minimum age possible given the number of years elapsed since first capture and age at sexual

maturity (3 yr. for males, 6 yr. for females). This is a conservative estimate of age for individuals whose age was not known. We found that the model results using minimum age did not differ from the models including ages estimated from sex-specific VBGF, and thus we used the dataset including known ages and estimated ages from sex-specific VBGF.

Results

There were no significant correlations among the immune variables: natural antibodies, lysis ability, and bactericidal competence of plasma. Level of natural antibodies and lysis ability of the plasma samples were not correlated (Pearson's r = -0.09, Pr = 0.30), and bactericidal competence was not correlated with either natural antibodies or lysis ability (Pearson's r < 0.2, Pr > 0.05).

The results of models of the relationships between zAge, sex, and day of year and the dependent variables across all individuals included in this study are summarized in Table 1. The full model of natural antibody levels versus zAge, sex, and day indicated significant effects of all three variables on natural antibodies (Table 1). Natural antibodies were significantly different between the sexes, with females displaying greater average agglutination titers, or greater natural antibody levels, than males (female LS mean ± SEM = 18.31 ± 0.32, male 16.74 ± 0.41, Pr = 0.0039), and juveniles having intermediate titers between the two sexes (17.82 ± 0.76). To assess the relationship of zAge and day of year on natural antibodies, we calculated residuals from the model excluding zAge or excluding day, respectively, and assessed the regression of residuals on the dependent variable. Natural antibodies increased with age (Fig. 2) and decreased with sampling date (Y = –0.06x + 9.64; R^2 = 0.04). The results for lysis ability indicated a marginally significant difference in lysis ability between sex groupings, with males having the highest lysis

ability (3.78 ± 0.20), juveniles the lowest (2.91 ± 0.38), and females having intermediate lysis ability (3.27 ± 0.16; Table 1). There were no significant effects of zAge, sex, or day of year on bactericidal competence (Pr > 0.3, Table 1).

The models testing the relationship between female reproductive output and the immune measures are summarized in Table 2. For the subset of female individuals for which we had clutch size, age, day of year, nor clutch size significantly explained natural antibody levels (all Pr > 0.1, Table 2). Lysis ability was affected by an interaction of clutch size and age (Pr = 0.01, Table 2). As clutch size increases, lysis ability also increases for all ages except the oldest females (Fig. 3). For the oldest quartile of females (ages > 18), in contrast to all other ages, lysis ability decreased with increasing clutch size. Clutch size interacted with day and age interacted with day to affect bactericidal competence of plasma (Table 2). For most of the female nesting season, bactericidal competence was of similar magnitude across clutch sizes. Late in the nesting season, however, females with larger clutches had increased bactericidal competence. Bactericidal competence decreased with day of year for most ages except the oldest females, whose bactericidal competence increased with day (Fig. 4).

Discussion

We found that contrary to our predictions, painted turtles did not show evidence of immunosenescence, despite both males (Reinke et al., 2019) and females (Warner et al., 2016) exhibiting actuarial senescence (increasing mortality with increasing adult age) in this population. In contrast, we found that the oldest animals within each sex category had increased circulating natural antibodies (Fig. 2). There were, however, age-related impacts on female immune function. The oldest reproductive females had decreasing lysis ability with increasing reproductive effort (Fig. 3). However, these same old females had increasing bactericidal

competence across the nesting season (Fig. 4), in contrast to all other age classes of females. Thus, the evidence for a cost of reproduction is mixed at best.

Immunosenescence

Reptiles represent an important vertebrate group in our understanding of senescence, as most exhibit growth throughout their lifetimes and demonstrate increasing reproductive output with age. However, our results and the results of the few studies of immunosenescence in non-avian reptiles are varied (reviewed in Vogel et al., 2018). For example, in populations of terrestrial garter snake (*Thamnophis elegans*), both natural antibodies and lysis ability decreased in older snakes (Sparkman and Palacios, 2009), while in water pythons (*Liasis fuscus*), natural antibodies increased with age (Ujvari and Madsen, 2011). In Dickerson's collared lizards, immune response to a mitogen, phytohaemagglutinin, declined with age (Plasman et al., 2019). In red-eared slider turtles (*Trachemys scripta*) and painted turtles, results are similarly inconsistent. When using plastron length as a proxy for age, larger adult red-eared slider turtles exhibited increased natural antibody levels when compared to smaller adults (Zimmerman et al., 2010b; Zimmerman et al., 2013a). However, bactericidal competence declined with age, as did a number of adaptive immune measures: specific antibody responses to lipopolysaccharide challenge and B-cell function were maintained with age, while white blood cell count declined with age (Zimmerman et al., 2010b; Zimmerman et al., 2013b; Zimmerman et al., 2017). In painted turtles, one study found increased swelling response and decreased basophil counts in larger individuals, but no change in natural antibody level with size (Schwanz et al., 2011). Our results, however, indicate that older painted turtles had higher levels of natural antibodies than younger individuals within each sex (males, females, and juveniles). This is likely due to

increased sample sizes in this study, a more confident age estimate, and a different sampling season, which can impact natural antibody levels (Schwanz et al., 2011).

There are multiple explanations for the inconsistency across measures in immunosenescence results among reptiles, including our study. First, reptiles may not follow the classic expectation of immunosenescence due to their life-history traits, such as indeterminate growth and, in the case of some chelonians, extreme lifespans compared to other vertebrates (e.g. Galapagos tortoises). Body size is positively correlated with reproductive output in many reptiles, which may allow for the evolution of decreased or negligible immunosenescence in long-lived species such as turtles (reviewed in Hoekstra et al., 2020). Our results are mostly in agreement with the maintenance of immune function with age. However, there is evidence of immunosenescence in certain immune measures in both our study and in other reptiles (reviewed in Vogel et al., 2018). Another explanation for inconsistency in immunosenescence results is that immune components are likely to respond differently to age. The measures in this study, natural antibody levels, bactericidal competence, and lysis ability, are responses of the innate immune branch rather than the specific, adaptive immune branch of the humoral immune system in reptiles (though natural antibodies may function as an innate and adaptive response, Vogel et al., 2018). In reptiles, adaptive humoral immune responses take longer to reach peak antibody concentrations after infection and exhibit no increased binding affinity to antigens upon secondary infection, in contrast to mammals (Zimmerman et al., 2010a). Thus, innate responses should be strongly selectively maintained with age in reptile immune systems as a first line of defense to compensate for less robust adaptive immune responses (Zimmerman et al., 2013a). Natural antibodies have been shown to increase with age in many reptiles, while components of the adaptive immune system displayed decreased responses in older individuals, consistent with

a trade-off between innate and adaptive immune branches with age (Ujvari and Madsen, 2011; Zimmerman et al., 2013b).

Sex-specific immune function

We identified sex-specific differences in adult painted turtles in this study, such that males have lower natural antibody levels than females. This finding is unique, as other studies of turtle natural antibodies have found no difference between the sexes (Zimmerman et al., 2010b; Schwanz et al., 2011; Zimmerman et al., 2013a; Zimmerman et al., 2013b). Sex-specific differences in vertebrate immune function are common and are explained by the action of androgens and behavioral differences between males and females (Klein, 2000). Males often exhibit reduced immune function, particularly in polygynous species, and experimental studies suggest that testosterone is a suppressive agent (reviewed in Foo et al., 2017). Behavioral differences in intraspecific interactions and habitat choice may subject males to greater infection pressures than females, which may also suppress male immune function (Klein, 2000). Painted turtle males are polygynous, have differing parasite loads and basking behavior than females in some populations, and demonstrate increasing testosterone beginning in mid-May with a fall peak (Esch and Gibbons, 1967; Licht et al., 1985; Ernst and Lovich, 2009). These characteristics, and the intermediate natural antibody levels found in juvenile painted turtles in this study, support a role for increased testosterone and behavioral differences in suppressing male painted turtle immune function. However, lysis ability was greater in male than female painted turtles, though the finding was only marginally significant, and bactericidal competence did not indicate sex-specific differences, which suggests that certain immune components may not be impacted by testosterone or behavioral differences, or that our study may not have captured sex-specific

immune differences due to limited seasonal sampling. Indeed, in the turtle *Mauremys caspica*, there were sex-specific seasonal differences in multiple innate and adaptive immune measures (Muñoz et al., 2000).

Effects of day of year on immune function

Seasonality of immune function has been documented across vertebrates, and is predicted by cyclical activity of steroid hormones and reproduction, resource availability, and temperature changes through the year (Martin et al., 2008; Moeller et al., 2013). In ectothermic vertebrates, temperature is particularly important to immune function, and multiple studies have found changes to immune function during or just following winter dormancy in reptiles (Muñoz et al., 2000; Schwanz et al., 2011; Sandmeier et al., 2016). Sampling for this study took place in May and June, which correspond with heightened female activity associated with nesting behaviors, increased basking behavior, and male movement to find mates and new territories (Ernst and Lovich, 2009). Additionally, these months represent a marked increase in temperature. In 2011, the average maximum May temperature was 21.5°C in Thomson, IL, and the average maximum June temperature was 26.8°C (Thornton et al., 2017). A study of a Michigan population of painted turtles found that adults had increased parasite loads in warmer temperatures compared to cooler temperatures (Esch and Gibbons, 1967), and parasite pressures may also differ with temperature in this population.

While this sampling period is relatively short for understanding seasonal patterns in immune function, we still detect associations between sampling day and immune measures. Natural antibodies decreased with day of year, which may be a function of a decrease in immune health from female reproductive energy costs and an increase in male testosterone. There was

also a significant interaction between age and sampling day for females with clutch information, such that all but the oldest females exhibit declines in bactericidal competence later in the nesting season (Fig. 4). However, bactericidal competence across all individuals did not correlate with day in this study, which differs from seasonal changes detected in other turtle species. Adult red-eared slider turtles from a population in Illinois demonstrated a marked drop in bactericidal competence in late June, while Mojave desert tortoises (*Goperhus agassizii*) increase bactericidal competence from spring through fall (Zimmerman et al., 2010b; Goessling et al., 2016; Sandmeier et al., 2016). The different findings of seasonal impacts on bactericidal competence in other turtles versus this study may be predicted by trade-offs in energetic balance and the seasonal life cycles of parasites that are different between species and may invoke distinct immune responses to season across both species and immune components (Martin et al., 2008; Martinez-Bakker and Helm, 2015).

Reproductive output and immune function

Reproduction represents a large energetic cost to females. Life-history theory predicts that individuals invest heavily in current reproduction only at the expense of future reproductive opportunities and self-maintenance (Roff, 2002). Thus, a trade-off between immune function and reproduction might be expected, such that increased reproductive effort results in a decline in immune function. This trade-off has been experimentally tested in brown anoles (*Anolis sagrei*), and females that were reproductively active displayed lower immune function than ovariectomized females (Cox et al., 2010). Painted turtles exhibit a positive relationship between size and egg mass, suggesting increased reproductive effort with age (Congdon et al., 2003; Warner et al., 2016). In this study, older females with larger clutches have a lower lysis ability

than younger females with large clutch sizes (Fig. 3). This is consistent with an age-dependent trade-off, as older females pay an increased cost to immune function with increased reproductive effort. While there is little known about the transfer of antibodies in turtles, in the desert tortoise, mothers transferred specific antibodies to their eggs and conferred resistance to a specific bacterial infection (Schunlacher et al., 1999). If innate immune function of mothers affects yolk provisioning in eggs, this may partially explain the reproductive senescence (reduced egg hatching success) reported for older females in this population (Warner et al., 2016).

In contrast, bactericidal competence of older females increased through the nesting season, while younger females exhibited a significant decrease (Fig. 4), again rejecting the hypothesis of immunosenescence. This increase in older females throughout the nesting season suggests that reproduction is not negatively impacting all aspects of immune function. Painted turtles have been commonly recorded laying second clutches in this population, and older females tend to lay second clutches more often than young females (Bowden et al., 2011). While this additional increased reproductive output may contribute to the lowered lysis ability for older females with large clutches, the increased bactericidal competence suggests that older females may be better able to respond to immune challenges later in the nesting season, when second clutches are laid. These results add to the growing research that suggests not all immune measures are equally influenced by age, reproduction, and season.

This study is a valuable contribution to our understanding of variation in immune function across age, sex, and reproductive effort in a long-lived reptile. Long-term studies of wild reptile populations allow the direct aging of individuals, which offers insight into senescent patterns that occur only in the oldest individuals. While immunosenescence was not supported in most measures of immune function in the painted turtle, we found interactions between sex, date,

and age that emphasize the importance of physiological state in immune function. Additionally, we report the first evidence of immunosenescence associated with reproductive output in older females in a turtle, which supports the life-history trade-off between reproduction and self-maintenance in this long-lived reptile.

Competing Interests

No competing interests declared.

Data Availability

Data will be made available in Iowa State University's Open Data Repository (hosted by

figshare), including results for all immune assays and SAS code used to perform statistical

analyses, upon acceptance of manuscript.

References

Baker, S., Kessler, E., Darville, L. and Merchant, M. (2019). Different mechanisms of serum complement activation in the plasma of common (*Chelydra serpentina*) and alligator (*Macrochelys temminckii*) snapping turtles. *PLoS One* **14**(6): e0217626.

Bowden, R. M., Paitz, R. T. and Janzen, F. J. (2011). The ontogeny of postmaturation resource allocation in turtles. *Physiol. Biochem. Zool.* **84**(2): 204-211.

Congdon, J. D., Nagle, R. D., Kinney, O. M. and van Loben Sels, R. C. (2001). Hypotheses of aging in a long-lived vertebrate, Blanding's turtle (*Emydoidea blandingii*). *Exp. Gerontol.* **36**(4-6): 813-827.

Congdon, J. D., Nagle, R. D., Kinney, O. M., van Loben Sels, R. C., Quinter, T. and Tinkle, D. W. (2003). Testing hypotheses of aging in long-lived painted turtles (*Chrysemys picta*). *Exp. Gerontol.* **38**(7): 765-772.

Cox, R. M., Parker, E. U., Cheney, D. M., Liebl, A. L., Martin, L. B. and Calsbeek, R. (2010). Experimental evidence for physiological costs underlying the trade-off between reproduction and survival. *Funct. Ecol.* **24**(6): 1262-1269.

Ernst, C. H. and Lovich, J. E. (2009). *Turtles of the United States and Canada*. Baltimore, USA: The Johns Hopkins University Press.

Esch, G. W. and Gibbons, J. W. (1967). Seasonal Incidence of Parasitism in the Painted Turtle, *Chrysemys picta marginata* Agassiz. *J. Parasitol.* **53**(4): 818-821.

Foo, Y. Z., Nakagawa, S., Rhodes, G. and Simmons, L. W. (2017). The effects of sex hormones on immune function: a meta-analysis. *Biol. Rev.* **92**(1): 551-571.

Goessling, J. M., Guyer, C. and Mendonca, M. T. (2016). Seasonal Acclimation of Constitutive Immunity in Gopher Tortoises *Gopherus polyphemus*. *Physiol. Biochem. Zool.* **89**(6): 487-497.

Hanssen, S. A. (2006). Costs of an immune challenge and terminal investment in a long-lived bird. *Ecology* **87**(10): 2440-2446.

Hoekstra, L. A., Weber, R. C., Bronikowski, A. M. and Janzen, F. J. (2018). Sex-specific growth, shape, and their impacts on life history of a long-lived vertebrate. *Evol. Ecol. Res.* **19**: 639–657.

Hoekstra, L. A., Schwartz, T. S., Sparkman, A. M., Miller, D. A. and Bronikowski, A. M. (2020). The untapped potential of reptile biodiversity for understanding how and why animals age. *Funct. Ecol.* **34**: 38-54.

Klein, S. L. (2000). Hormones and mating system affect sex and species differences in immune function among vertebrates. *Behav. Processes* **51**(1-3): 149-166.

Lee, K. A. (2006). Linking immune defenses and life history at the levels of the individual and the species. *Integr. Comp. Biol.* **46**(6): 1000-1015.

Lee, K. A., Wikelski, M., Robinson, W. D., Robinson, T. R. and Klasing, K. C. (2008). Constitutive immune defences correlate with life-history variables in tropical birds. *J. Anim. Ecol.* **77**(2): 356-363.

Leivesley, J. A., Bussiere, L. F., Pemberton, J. M., Pilkington, J. G., Wilson, K. and Hayward, A. D. (2019). Survival costs of reproduction are mediated by parasite infection in wild Soay sheep. *Ecol. Lett.* **22**: 1203-1213.

Licht, P., Breitenbach, G. L. and Congdon, J. D. (1985). Seasonal cycles in testicular activity, gonadotropin, and thyroxine in the painted turtle, *Chrysemys picta*, under natural conditions. *Gen. Comp. Endocrinol.* **59**(1): 130-139.

Lochmiller, R. L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**(1): 87-98.

Martin II, L. B., Hasselquist, D. and Wikelski, M. (2006). Investment in immune defense is linked to pace of life in house sparrows. *Oecologia* **147**(4): 565-575.

Martin, L. B., Weil, Z. M. and Nelson, R. J. (2008). Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philos. Trans. Royal Soc. B* **363**(1490): 321-339.

Martinez-Bakker, M. and Helm, B. (2015). The influence of biological rhythms on host-parasite interactions. *Trends Ecol. Evol.* **30**(6): 314-326.

Matson, K. D., Ricklefs, R. E. and Klasing, K. C. (2005). A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev. Comp. Immunol.* **29**(3): 275-286.

Matson, K. D., Tieleman, B. I. and Klasing, K. C. (2006). Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. *Physiol. Biochem. Zool.* **79**(3): 556-564.

Moeller, K. T., Butler, M. W. and Denardo, D. F. (2013). The effect of hydration state and energy balance on innate immunity of a desert reptile. *Front. Zool.* **10**(1): 23.

Moll, E. (1973). Latitudinal and Intersubspecific Variation in Reproduction of the Painted Turtle, *Chrysemys picta*. *Herpetologica* **29**(4): 307-318.

Muñoz, F. J., Galván, A., Lerma, M. and De la Fuente, M. (2000). Seasonal changes in peripheral blood leukocyte functions of the turtle *Mauremys caspica* and their relationship with corticosterone, 17-beta-estradiol and testosterone serum levels. *Vet. Immunol. Immunopathol.* **77**(1-2): 27-42.

Ochsenbein, A. F. and Zinkernagel, R. M. (2000). Natural antibodies and complement link innate and acquired immunity. *Immunol. Today* **21**(12): 624-630.

Palacios, M. G. and Bronikowski, A. M. (2017). Immune variation during pregnancy suggests immune component-specific costs of reproduction in a viviparous snake with disparate life-history strategies. *J. Exp. Zool. Part A* **327**(8): 513-522.

Palacios, M. G., Sparkman, A. M. and Bronikowski, A. M. (2011a). Developmental plasticity of immune defence in two life-history ecotypes of the garter snake, *Thamnophis elegans* - a common-environment experiment. *J. Anim. Ecol.* **80**(2): 431-437.

Palacios, M. G., Cunnick, J. E. and Bronikowski, A. M. (2013). Complex interplay of body condition, life history, and prevailing environment shapes immune defenses of garter snakes in the wild. *Physiol. Biochem. Zool.* **86**(5): 547-558.

Palacios, M. G., Winkler, D. W., Klasing, K. C., Hasselquist, D. and Vleck, C. M. (2011b). Consequences of immune system aging in nature: a study of immunosenescence costs in free-living Tree Swallows. *Ecology* **92**(4): 952-966.

Pawelec, G., Larbi, A. and Derhovanessian, E. (2010). Senescence of the human immune system. *J. Comp. Pathol.* **142 Suppl 1**: S39-44.

Plasman, M., Sandoval-Zapotitla, E. and Torres, R. (2019). Immune response declines with age in a wild lizard. *Biol. J. Linn. Soc.* **In Press**.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Reed, T. E., Kruuk, L. E., Wanless, S., Frederiksen, M., Cunningham, E. J. and Harris, M. P. (2008). Reproductive senescence in a long-lived seabird: rates of decline in late-life performance are associated with varying costs of early reproduction. *Am. Nat.* **171**(2): E89-E101.

Refsnider, J. M., Palacios, M. G., Reding, D. M. and Bronikowski, A. M. (2015). Effects of a novel climate on stress response and immune function in painted turtles (*Chrysemys picta*). *J. Exp. Zool. Part A* **323**(3): 160-168.

Reinke, B. A., Hoekstra, L., Bronikowski, A. M., Janzen, F. J. and Miller, D. (2019). Joint estimation of growth and survival from mark-recapture data to improve estimates of senescence in wild populations. *Ecology* **In Press**.

Roff, D. A. (2002). *Life History Evolution*. Boston, USA: Sinauer Associates Inc.

Sandmeier, F. C., Horn, K. R. and Tracy, C. R. (2016). Temperature-independent, seasonal fluctuations in immune function of the Mojave Desert Tortoise (*Gopherus agassizii*). *Can. J. Zool.* **94**(8): 583-590.

Sandmeier, F. C., Tracy, C. R., Dupre, S. and Hunter, K. (2012). A trade-off between natural and acquired antibody production in a reptile: implications for long-term resistance to disease. *Biol Open* **1**(11): 1078-1082.

Schmid-Hempel, P. (2003). Variation in immune defence as a question of evolutionary ecology. *Proc. R. Soc. B.* **270**(1513): 357-366.

Schunlacher, I., Rostal, D. C., Yates, R. A., Brown, D. R., Jacobson, E. R. and Klein, P. A. (1999). Persistence of maternal antibodies against *Mycoplasma agassizii* in desert tortoise hatchlings. *Am. J. Vet. Res.* **60**: 826-831.

Schwanz, L., Warner, D. A., McGaugh, S., Di Terlizzi, R. and Bronikowski, A. (2011). State-dependent physiological maintenance in a long-lived ectotherm, the painted turtle (*Chrysemys picta*). *J. Exp. Biol.* **214**(Pt 1): 88-97.

Sparkman, A. M. and Palacios, M. G. (2009). A test of life-history theories of immune defence in two ecotypes of the garter snake, *Thamnophis elegans*. *J. Anim. Ecol.* **78**(6): 1242-1248.

Sparkman, A. M., Arnold, S. J. and Bronikowski, A. M. (2007). An empirical test of evolutionary theories for reproductive senescence and reproductive effort in the garter snake *Thamnophis elegans*. *Proc. R. Soc. B.* **274**(1612): 943-950.

Thornton, P. E., Thornton, M. M., Mayer, B. W., Wei, Y., Devarakonda, R., Vose, R. S. and Cook, R. B. (2017). *Daymet: Daily Surface Weather Data on a 1-km Grid for North America*. ORNL Distributed Active Archive Center Version 3. Retrieved October, 2019.

Ujvari, B. and Madsen, T. (2011). Do natural antibodies compensate for humoral immunosenescence in tropical pythons? *Funct. Ecol.* **25**(4): 813-817.

Vogel, L. A., Palackdharry, S., Zimmerman, L. M. and Bowden, R. M. (2018). Humoral Immune Function in Long-Lived Ectotherms, the Reptiles. In *Handbook of Immunosenescence* (ed. T. Fulop, C. Franceschi, K. Hirokawa and G. Pawelec), pp. 1-17. New York, USA: Springer.

Warner, D. A., Miller, D. A., Bronikowski, A. M. and Janzen, F. J. (2016). Decades of field data reveal that turtles senesce in the wild. *Proc. Natl. Acad. Sci. USA* **113**(23): 6502-6507.

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wilkinson, P. M., Rainwater, T. R., Woodward, A. R., Leone, E. H. and Carter, C. (2016). Determinate Growth and Reproductive Lifespan in the American Alligator (*Alligator mississippiensis*): Evidence from Long-term Recaptures. *Copeia* **104**(4): 843-852.

Zimmerman, L. M., Vogel, L. A. and Bowden, R. M. (2010a). Understanding the vertebrate immune system: insights from the reptilian perspective. *J. Exp. Biol.* **213**(5): 661-671.

Zimmerman, L. M., Bowden, R. M. and Vogel, L. A. (2013a). Red-Eared Slider Turtles Lack Response to Immunization with Keyhole Limpet Hemocyanin but Have High Levels of Natural Antibodies. *ISRN Zoology* **2013**: 1-7.

Zimmerman, L. M., Paitz, R. T., Vogel, L. A. and Bowden, R. M. (2010b). Variation in the seasonal patterns of innate and adaptive immunity in the red-eared slider (*Trachemys scripta*). *J. Exp. Biol.* **213**(9): 1477-1483.

Zimmerman, L. M., Carter, A. W., Bowden, R. M., Vogel, L. A. and White, C. (2017). Immunocompetence in a long-lived ectothermic vertebrate is temperature dependent but shows no decline in older adults. *Funct. Ecol.* **31**(7): 1383-1389.

Zimmerman, L. M., Clairardin, S. G., Paitz, R. T., Hicke, J. W., LaMagdeleine, K. A., Vogel, L. A. and Bowden, R. M. (2013b). Humoral immune responses are maintained with age in a long-lived ectotherm, the red-eared slider turtle. *J. Exp. Biol.* **216**(4): 633-640.

Table 1: Model results for relationships among zAge, sex, day, and immune variables across all painted turtles included in this study.

Source of Variation	Natural Antibodies	Lysis Ability	Bactericidal Competence
zAge	**Older > Younger (Fig. 2)**		
$F\ (df_n,\ df_d)$	**5.48 (1, 110)**	1.42 (1, 112)	0.02 (1, 86)
Pr > F	**0.021**	0.2358	0.8898
Sex	**Female > Male**	**Male > Female**	
$F\ (df_n,\ df_d)$	**4.36 (2, 110)**	**2.92 (2, 112)**	1.23 (2, 86)
Pr > F	**0.015**	**0.0582**	0.2968
Day	**Earlier > Later**		
$F\ (df_n,\ df_d)$	**5.15 (1, 110)**	0.39 (1, 112)	0.2 (1, 86)
Pr > F	**0.0252**	0.5338	0.6525

Significant (Pr < 0.05) and marginally significant (Pr < 0.10) effects are in bold and directionality of effect is indicated for highest order interaction. Interactions among variables were non-significant and removed from the final model. zAge is sex-specific z-transformed age; Day is day of year.

Table 2: Model results for relationships among clutch size, age, day, and immune variables for female painted turtles with clutch size information.

Source of Variation	Natural Antibodies	Lysis Ability	Bactericidal Competence
Clutch Size			
F (df_n, df_d)	0.01 (1, 50)	**10.23 (1, 50)**	**5.02 (1, 40)**
Pr > F	0.9218	**0.0024**	**0.0306**
Age	Older > Younger		
F (df_n, df_d)	2.76 (1, 50)	**7.10 (1, 50)**	**6.75 (1, 40)**
Pr > F	0.1026	**0.0103**	**0.013**
Day			
F (df_n, df_d)	0.50 (1, 50)	0.00 (1, 50)	1.35 (1, 40)
Pr > F	0.4808	0.9511	0.2514
Clutch Size × Day			
F (df_n, df_d)	—	—	**4.60 (1, 40)**
Pr > F	—	—	**0.0382**
Clutch Size × Age		Fig. 3	
F (df_n, df_d)	—	**7.09 (1, 50)**	—
Pr > F	—	**0.0104**	—
Age × Day			Fig. 4
F (df_n, df_d)	—	—	**6.67 (1, 40)**
Pr > F	—	—	**0.0136**

Significant effects (Pr < 0.05) are in bold, and directionality of effect is indicated for highest order interaction. Interactions not included in the table were non-significant and removed from the final model. Day is day of year.

Figure 1: **Distribution of female and male painted turtle ages represented in this study.** This figure depicts a stacked histogram of female (N = 76) and male (N = 39) painted turtle ages, in years, of the known-aged individuals (N = 80 excluding juveniles) and the individuals for which age was estimated using sex-specific von Bertalanffy growth models (N = 35) developed from known-aged individuals collected from the same population as the turtles used in this study. The counts of individuals are shown on the y-axis, and age in years on the x-axis. Juveniles (N = 11) are not included in the figure, but were all of known age and ranged from 1 to 3 years of age.
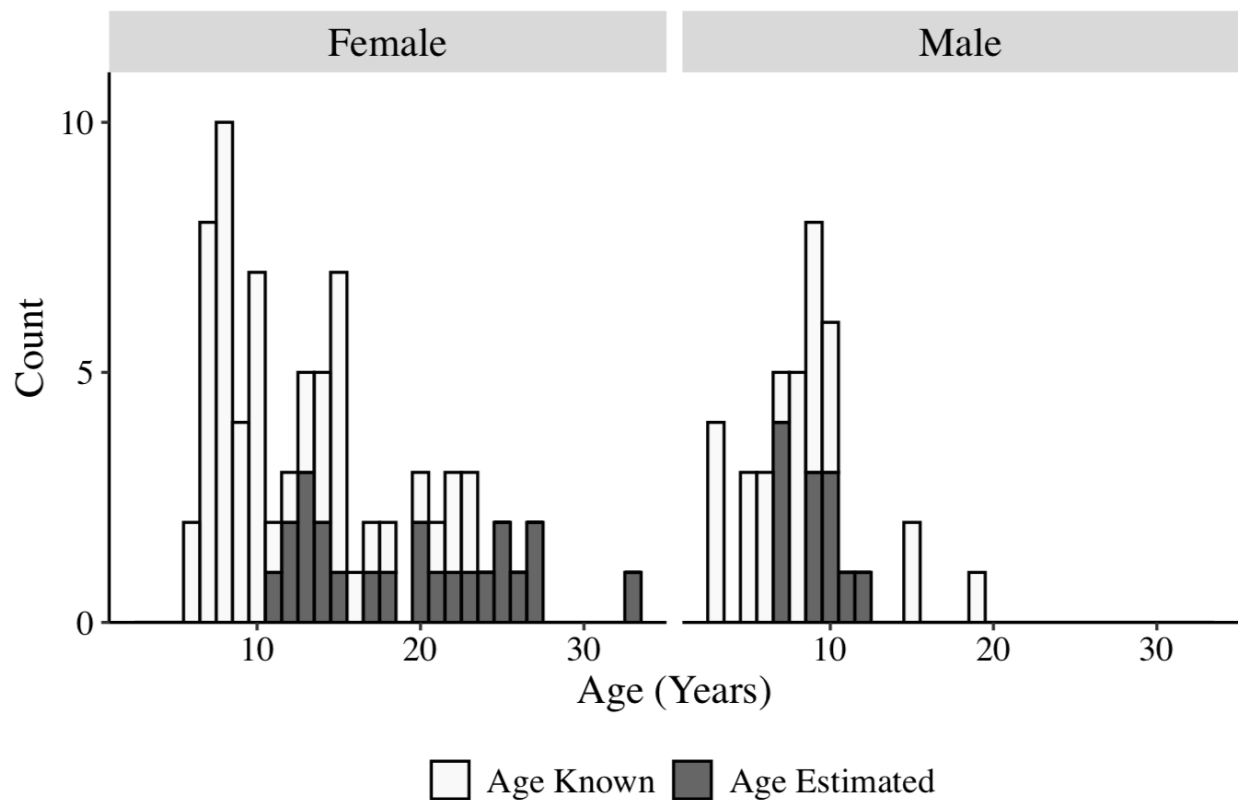
Figure 2: **Older painted turtles exhibit greater levels of natural antibodies than younger individuals across sexes (Pr = 0.021).** This figure depicts the relationship of z-transformed age (zAge) and the residuals of natural antibody titers in response to rabbit red blood cells across painted turtles measured in this study (N = 115). Residuals were calculated from the model excluding zAge. Equation of the line of best fit and R^2 shown on plot. Scale bars underneath x-axis represent the ages (in years) corresponding to the minimum, median, and maximum zAge shown in the plot for each sex.
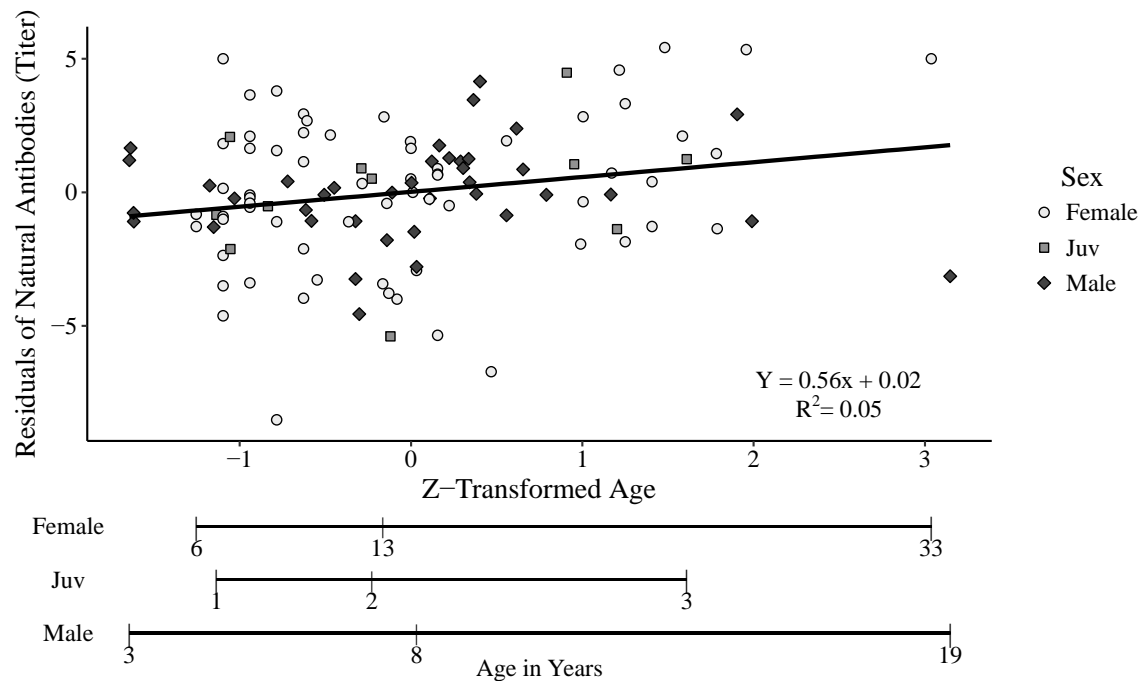
Figure 3: **Old female painted turtles show opposing lysis ability responses to small and large clutch sizes, suggesting different immune pressures of reproduction dependent on age.** This figure depicts the relationship of plasma ability to lyse rabbit red blood cells and age x clutch size interaction for females with clutch size information (N = 55). To visualize the relationships, age was split into quartiles, and five ages were selected to represent the range of ages in the data (minimum, first quartile, median, third quartile, and maximum age). Points represent least squares means ± SEM from the female lysis ability model in Table 2. When comparing the smallest (7) and largest (12-15) clutch sizes, the oldest females display a significant decrease in lysis ability with larger clutch sizes compared to those with small clutches (Pr < 0.05).
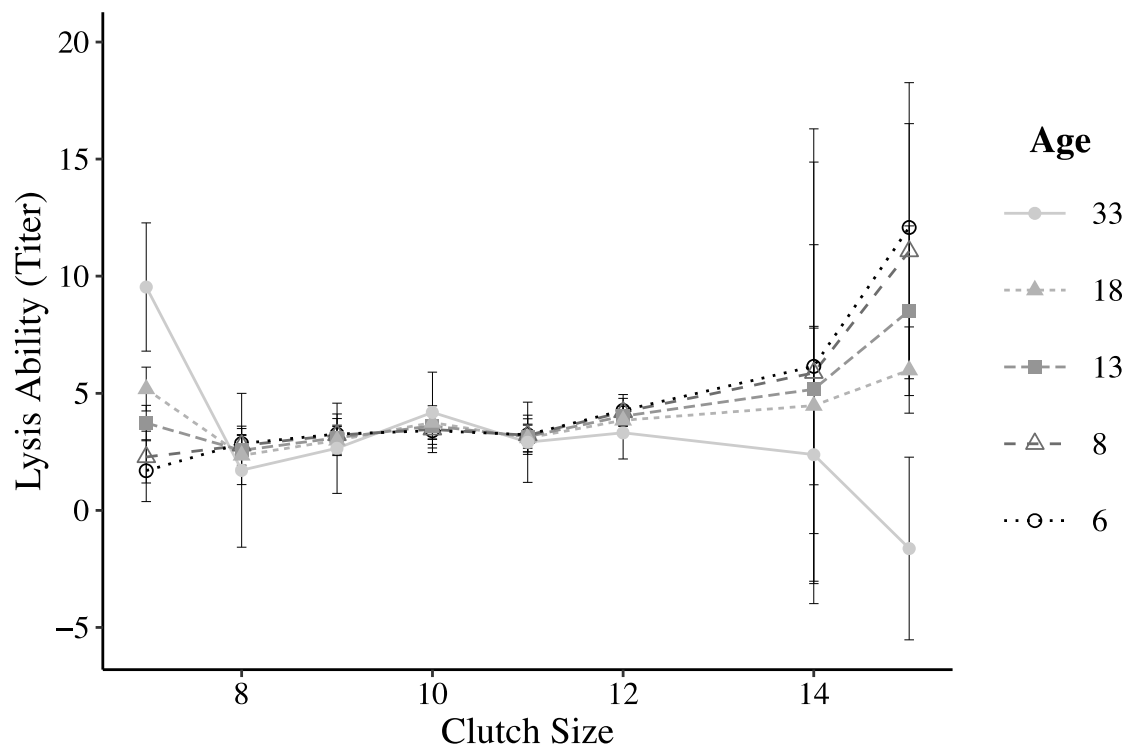
Figure 4: **Old female painted turtles increase bactericidal competence from the beginning to the end of the nesting season (Pr = 0.0093), while younger females (6-8 years old) show a significant decrease in bactericidal competence (Pr =0.0025).** This figure depicts the relationship of bactericidal competence of plasma and age x day interaction for females with clutch size information (N = 46). To visualize the relationships, day and age were split into quartiles, and five ages were selected to represent the range of ages in the data (minimum, first quartile, median, third quartile, and maximum age). The first quartile date (2 June) was not estimable and is not shown. Points represent least squares means ± SEM from the female bactericidal competence model in Table 2.