The Journal of Experimental Biology 214, 88-97

doi:10.1242/jeb.046813

RESEARCH ARTICLE

State-dependent physiological maintenance in a long-lived ectotherm, the painted turtle (*Chrysemys picta*)

Lisa Schwanz[1,2,*], Daniel A. Warner[1], Suzanne McGaugh[1], Roberta Di Terlizzi[3,4] and Anne Bronikowski[1]

[1]Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, IA 50011, USA, [2]School of Marine and Tropical Biology, James Cook University, Townsville, QLD 4811, Australia, [3]College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA and [4]School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

*Author for correspondence (lisa.schwanz@gmail.com)

Accepted 13 October 2010

SUMMARY

Energy allocation among somatic maintenance, reproduction and growth varies not only among species, but among individuals according to states such as age, sex and season. Little research has been conducted on the somatic (physiological) maintenance of long-lived organisms, particularly ectotherms such as reptiles. In this study, we examined sex differences and age- and season-related variation in immune function and DNA repair efficiency in a long-lived reptile, the painted turtle (*Chrysemys picta*). Immune components tended to be depressed during hibernation, in winter, compared with autumn or spring. Increased heterophil count during hibernation provided the only support for winter immunoenhancement. In juvenile and adult turtles, we found little evidence for senescence in physiological maintenance, consistent with predictions for long-lived organisms. Among immune components, swelling in response to phytohemagglutinin (PHA) and control injection increased with age, whereas basophil count decreased with age. Hatchling turtles had reduced basophil counts and natural antibodies, indicative of an immature immune system, but demonstrated higher DNA repair efficiency than older turtles. Reproductively mature turtles had reduced lymphocytes compared with juvenile turtles in the spring, presumably driven by a trade-off between maintenance and reproduction. Sex had little influence on physiological maintenance. These results suggest that components of physiological maintenance are modulated differentially according to individual state and highlight the need for more research on the multiple components of physiological maintenance in animals of variable states.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/1/88/DC1

Key words: somatic investment, reproductive value, phenotypic plasticity, comet analysis, immune function.

INTRODUCTION

Physiological maintenance, such as immune function and cellular repair, can be costly metabolically (e.g. Ots et al., 2001; Freitak et al., 2003; Martin et al., 2003; Demas, 2004; Derting and Virk, 2005; Martin et al., 2008) (but see Nilsson et al., 2007). In addition, the costs associated with physiological (somatic) maintenance are often observed through phenotypic trade-offs with reproduction or performance (e.g. Metcalfe and Monaghan, 2001; Zuk and Stoehr, 2002; Alonso-Alvarez et al., 2004a; Alonso-Alvarez et al., 2007; Martin et al., 2006; Martin et al., 2008; Metcalfe and Alonso-Alvarez, 2010). Owing to these apparent costs and trade-offs, it is often assumed that maximal investment in maintenance is not necessarily optimal (Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002; Martin et al., 2008). Plasticity in physiological maintenance may provide a mechanism for maximizing fitness when optimal resource allocation depends on individual or environmental states, such as age, sex, size or season (McNamara and Houston, 1996; Love et al., 2008; Martin et al., 2008). Despite the theoretical importance of state-dependent variation in physiological maintenance, there is a relative paucity of information on the topic.

Examining state-dependent resource allocation tests key components of evolutionary theory. The reproductive value of an individual (expected future reproductive success) should predict the degree to which individuals invest in maintenance (Williams, 1966). For example, senescence – the reduction in condition and performance with increasing age – should evolve when reproductive value declines with age (Williams, 1957; Kirkwood and Rose, 1991) and is thought to be explained at the proximate level by the accumulation of oxidative damage resulting from metabolic processes (free radical theory of aging) (Harman, 1956; Harman, 1981; Beckman and Ames, 1998; Finkel and Holbrook, 2000; Robert et al., 2007). Furthermore, if one sex has reduced longevity, then that sex is also likely to invest less in maintenance and senesce faster than the other sex (Williams, 1957; Zuk and Stoehr, 2002). In addition, seasonal changes in physiological maintenance are expected as organisms shift resources away from maintenance when it competes with more immediate demands (e.g. during reproduction), and shift resources to maintenance when it is a priority (Zuk and Stoehr, 2002; Alonso-Alvarez et al., 2004a; Nelson and Demas, 1996; Martin et al., 2008; Metcalfe and Alonso-Alvarez, 2010). For example, winter immune enhancement often occurs in endothermic vertebrates as anticipatory compensation for the immunosuppressive effects of stress associated with increased energetic demands (Nelson and Demas, 1996; Bilbo et al., 2002; Baillie and Prendergast, 2008). Thus, sex, age and season may be important state variables that influence how maintenance decisions are optimized across the lifespan.

Physiological maintenance has been widely studied in birds, mammals and invertebrate model systems, but much less is known about maintenance in ectothermic vertebrates [e.g. reptiles (Saad and El Ridi, 1988; Patnaik, 1994; Nelson and Demas, 1996; Zapata et al., 1992; Muñoz and de la Fuente, 2004; Madsen et al., 2007; Martin et al., 2008; Pitol et al., 2008; Les et al., 2009; Paitz et al., 2009; Sparkman and Palacios, 2009; Zimmerman et al., 2010)]. Ectothermic vertebrates have traits that suggest physiological maintenance patterns should deviate from those seen in previously studied endothermic vertebrates. For example, many reptiles are extremely long-lived and have increased reproductive output with age in adult females (as a result of indeterminate growth), suggesting that senescence should be minimal in these taxa and reduced in females compared with males (Patnaik, 1994; Congdon et al., 2003; Madsen et al., 2007; Bronikowski, 2008) (but see Congdon et al., 2001). Senescence in reproductive output appears to be nonexistent in long-lived reptiles (Congdon et al., 2001; Congdon et al., 2003; Sparkman et al., 2007). However, age-dependent physiological maintenance in long-lived taxa or in reptiles is largely unknown (Patnaik, 1994; Madsen et al., 2007; Robert et al., 2007; Bronikowski, 2008; Sparkman and Palacios, 2009). In addition, ectotherms experience torpor in temperate winters and aestivate in hot summers, which leads to reduced metabolism and movement, suggesting that maintenance should be lower either over the winter or summer, respectively, compared with other seasons. Although little is known about seasonal variation in physiological maintenance in hibernating animals (Prendergast et al., 2002; Martin et al., 2008), immune data from reptiles suggest that immune function is generally depressed over winter and potentially enhanced during autumn (Muñoz and de la Fuente, 2004; Nelson and Demas, 1996) (reviewed by Zapata et al., 1992).

We measured multiple components of physiological maintenance in a long-lived reptile, the painted turtle (*Chrysemys picta* Schneider), across age, sex and season (in autumn, during hibernation and in spring). We maintained wild painted turtles in the lab from autumn to spring and measured several innate and specific immune components that are expressed constitutively (white blood cells, natural antibodies and complement proteins) (Lee, 2006), as well as the degree of parasitism by hemogregarine protozoans. In addition, we included a one-time measure of induced immune function [inflammation in response to *in vivo* phytohemagglutinin (PHA) injection] and measures of DNA repair efficiency to assess a different component of physiological maintenance. Immune function and resistance to cellular damage (DNA repair) are intrinsically linked through the production of free radicals and the presence of circulating antioxidants (von Schantz et al., 1999; Alonso-Alvarez et al., 2004b; Bertrand et al., 2006), yet are rarely measured together. All of these measures were made across the two sexes and a large range of body sizes, which serves as a good indicator of age in painted turtles (Wilbur, 1975a) (F. Janzen, personal communication).

We predicted modest to no decline in immune function or DNA repair with increasing turtle size, as little senescence was anticipated (Bronikowski, 2008; Ungvari et al., 2008). We also predicted that immune function would be lowest in hatchlings because of immaturity of the immune system (Palacios et al., 2009; Sparkman and Palacios, 2009). With regards to sex differences, we predicted that males should show reduced levels of physiological maintenance (immune function and DNA repair) compared with females, given their reduced longevity (Congdon et al., 2003). Finally, across seasons, we predicted that physiological maintenance would be reduced during winter hibernation compared with autumn and spring, similar to other reptiles (Zapata et al., 1992).

MATERIALS AND METHODS

Study organism and animal husbandry

Painted turtles mature at about 4 (males) and 6 (females) years of age at our study site (F. Janzen, personal communication). Maximum longevity for northern populations of painted turtles appears to be near 40–50 years, with males having lower survivorship than females, although mean longevity may be considerably shorter (~15–20 years) (Wilbur, 1975b; Congdon et al., 2003) (F. Janzen, personal communication). Painted turtles spend the winter (approximately November to March) in a state of hibernation, probably buried in the mud beneath shallow water, emerging to bask in the sun on occasional warm days (Ernst and Lovich, 2009). Mating may occur during the spring and autumn (Gibbons, 1968; Gist et al., 1990). Females begin depositing yolk in eggs in the autumn prior to hibernation (September to October) and complete yolk deposition in the spring following emergence (Congdon and Tinkle, 1982). Second or third nests produced by females during a nesting season are probably provisioned during the spring (Congdon and Tinkle, 1982). Nesting occurs between mid-May and July of every year. Hatching occurs in August and September, but hatchlings remain in their nests, where they overwinter before emerging and entering the water in the following spring.

On 5–13 October 2007, juvenile (N=10; 8 females, 2 males) and adult (N=26; 9 females, 17 males) painted turtles were trapped or captured by hand at the Thomson Causeway Recreation Area near Thomson, Illinois, USA (41°57′ N, 90°07′ W). After capture, turtles were measured (plastron length, PL), weighed and individually marked with unique numbers painted on their carapaces. Juvenile or adult status was based on plastron length [PL; adult females >130 mm (F. Janzen, personal communication) adult males >80 mm) (Moll, 1973)]. Sex was unambiguously identified based on length of the foreclaws and the position of cloaca on the tail relative to the edge of the carapace. Turtles were transported to Iowa State University where they were randomly distributed between two aquatic pools. The pools were kept in a room at ~20°C with a 12 h:12 h light:dark cycle, and reptile food (Tetrafauna ReptoMin; Blacksburg, VA, USA) was provided three times per week. The turtles remained under these conditions until 10 December 2007, when they were randomly distributed among four water-filled plastic boxes and placed into a cold room set at 4°C for hibernation during the rest of winter; this temperature mimics those that turtles would naturally experience during winter (Ernst, 1972). Turtles were removed from hibernation conditions on 13 March 2008, housed individually in glass aquaria (37.85 l) under identical environmental conditions as in the autumn and remeasured (PL and weight) on 5 May 2008. Turtles were housed individually in spring. All juvenile and adult turtles were released at their location of capture in May 2008. In addition, hatchling turtles were collected from their nests at our field site on 15 September 2007. Because hatchling painted turtles overwinter in their nests, these hatchlings were housed with their known clutch-mates in plastic containers in laboratory incubators until blood sampling (see below).

Blood sampling

Juveniles and adults

Blood samples were collected from each individual prior to hibernation, during mid-hibernation, and in the spring immediately after turtles were taken out of hibernation (Table 1). For the mid-hibernation blood draw, it was necessary to place the turtles in warm water prior to bleeding (19–29 h) in order to increase blood circulation and allow sufficient blood draw. Although this sudden temperature change may represent a physiological stress for the

Table 1. Sampling schedule used in the experiment

Turtle age class	Autumn	Winter	Spring
Adults and juveniles	Blood components, agglutination and lysis (19–27 Nov 2007)	Blood components, agglutination and lysis (19 Feb 2008)	Blood components, agglutination and lysis (26–27 Mar 2008)
	DNA repair (12 Nov–8 Dec 2007)		PHA challenge (22–25 Apr 2008)
Hatchlings	Blood components, agglutination and lysis (28–29 Nov 2007)		
	DNA repair (28–29 Nov 2007)		

animals, it allowed us to control the temperature at which we collected blood. In addition, it represents an ecologically relevant stress, as hibernating turtles briefly emerge to bask on sufficiently warm days during February and March (Ernst and Lovich, 2009) and must experience any physiological stress associated with these behaviors. Approximately 0.5–1.0 ml of blood was drawn from the caudal vein. Samples were immediately put in heparinized tubes on ice. A portion of the whole blood sample was used for counts of blood cells and intracellular parasites (see below) at the Iowa State University Veterinary School. From the remaining blood, plasma was separated using Plasma Separator Tubes (BD Microtainer® 365958; Franklin Lakes, NJ, USA) and frozen at –80°C for future agglutination and lysis immune assays (see below). In the autumn, an additional drop of blood was drawn from each turtle for analyses of DNA damage and repair efficiency (see below).

Hatchlings

Blood samples were also taken from hatchling turtles to evaluate variation among age classes in blood cell components (compared with juveniles and adults), agglutination and lysis ability, parasitism and DNA repair efficiency. In the autumn (Table 1), twenty hatchlings (two siblings each from 10 nests) were decapitated, and exsanguinated. Blood was pooled among clutch mates to ensure adequate volumes for all assays.

DNA damage and repair

We measured DNA repair efficiency as an indicator of cellular maintenance using single-cell gel electrophoresis (i.e. comet assay) (Tice, 1995) on red blood cells (which are nucleated in reptiles). Blood drawn from each turtle in the autumn (or pooled for sibling hatchlings) was diluted 10,000-fold in PBS, and mixed with low melt agarose (LMA) (25 µl of diluted blood with 375 µl of 1.5% LMA). Each LMA–cell solution was divided into five 150 µl aliquots and immediately transferred to Trevigen slides, which had a base level of 1% agarose already applied, and gently covered with a coverslip to ensure an even distribution of the LMA–cell layer. An additional 1% agarose layer was applied to the slides after the lower layers had solidified. Two slides for each individual were assigned randomly to one of five treatment groups: baseline (treatment 1), UV exposure (treatment 2), UV exposure + repair time (treatment 3), hydrogen peroxide (H_2O_2) exposure (treatment 4), and H_2O_2 exposure + repair time (treatment 5). Both UV exposure (during basking) and oxidative agents (e.g. H_2O_2; from metabolic processes) represent potential damaging agents for turtles. Baseline slides (treatment 1) were immersed in lysis buffer immediately for a baseline measure of DNA damage in erythrocytes. The slides in treatments 2 and 3 were subjected to 312 nm UV light for 5 min. Cells in treatment 2 were lysed immediately following UV exposure, and those in treatment 3 were allowed 5 min to repair DNA damage at room temperature (approximately 22°C) and then lysed. Similarly, slides in treatments 4 and 5 were immersed in 200 µmol l^{-1} H_2O_2 in Hanks' balance salt solution (HBSS) for 15 min. Cells in both treatment 4 and treatment 5 were washed three times with plain HBSS. Cells in treatment 4 were lysed immediately after the HBSS washes, and cells in treatment 5 were allowed to repair DNA damage for 1 h at room temperature and then lysed. Electrophoresis was performed in electrophoresis buffer (300 mmol l^{-1} NaOH, 1 mmol l^{-1} EDTA, pH 13) at 25 V and 300 mA for 20 min. After electrophoresis, the slides were neutralized and the DNA was fixed by incubation in 100% ethanol for 10 min and air-dried. Slides were stained with SYBR Green for image analysis. Fluorescence images were evaluated immediately after staining, using comet analysis software to calculate the percentage of damaged DNA, based on the size of the comet tail (greater comet tails indicate greater DNA damage; Viscomet, Impuls Imaging GmbH, Buchloe, Germany).

Blood cells and intracellular parasites

Blood cell counts (red and white blood cells) were examined, by the College of Veterinary Medicine at Iowa State University, as general indicators of health and immune function (Beldomenico et al., 2008; Davis et al., 2008). Although the functions of leukocytes (white blood cells) in reptiles are poorly understood, their role in innate and adaptive immunity appears to be similar to that in mammals (Jacobson, 2007). Lymphocytes (B cells and T cells) form the basis of adaptive (specific) immunity, proliferating in response to antigens to generate a specific humoral or cell-mediated response. The remaining leucocytes function primarily in innate immunity. Heterophils form a first line of defense by phagocytizing foreign substances and exhibiting microbicidal activity, and, in mammals (i.e. neutrophils), have very short lifespans, so requiring constant generation (Tizard, 2004). Monocytes (macrophages) phagocytize foreign particles and play a role in granuloma formation and activation of adaptive immunity. Eosinophils have many functions, including phagocytic and microbicidal activity, and basophils release histamines to trigger inflammation.

The packed cell volume (PCV) was obtained by microhematocrit centrifugation. Total white blood cell count was determined by mixing whole blood in a Becton Dickinson Unopette®, with 1% buffered ammonium oxalate, and examining the sample in an improved Neubauer hemacytometer (Rosskopf, 1982). Differential counts of white blood cells were performed on blood smears by counting 200 white blood cells.

In addition, we measured parasitic infection by vector-borne, protozoans of the hemogregarine family (phylum Apicomplexa). These parasites infect red blood cells and have the potential to cause anemia (Schall et al., 1982; Jacobson, 2007), but typically do not cause clinical disease in reptiles (Campbell and Ellis, 2007). The prevalence of hemogregarines was evaluated during the blood smear review and scored as 0–3, depending on abundance of parasites in the smear (0=no infection). Hemogregarines were grossly identified as sausage-shaped intracytoplasmic gametocytes in erythrocytes. Specifically, these gametocytes distort the host cell by creating a bulge in the cytoplasm and lack the refractile pigment granules found in the gametocytes of *Plasmodium* spp.

Agglutination and lysis assay

Plasma contains natural antibodies (NAbs) and complement proteins that are expressed constitutively and function to agglutinate and lyse foreign cells as part of innate humoral immunity (Matson et al., 2005). We assessed agglutination and lysis ability of turtle blood plasma against foreign red blood cells (RBC) as a measure of constitutive immunocompetence.

Plasma samples from non-hatchlings in all three seasons and for autumn hatchlings were thawed and analyzed together between 29 April and 1 May 2008 for agglutination and lysis ability against foreign red blood cells. To measure agglutination and lysis of plasma, 25 µl of 0.01 mol l^{-1} Dulbecco's PBS were added to all wells in a 96-well round bottom plate (Corning Costar 3795; Lowell, MA, USA). Plasma samples (25 µl; rows 1–7) or positive control (row 8; rabbit anti-sheep RBC serum, MP Biomedical 55800; Solon, OH, USA) were mixed with the PBS in column 1. Samples were serially diluted 1:2 for columns 2–11. Column 12 contained only PBS and served as a negative control. To all wells, we added 25 µl 1% sheep RBCs (in Alsever's anticoagulant; Hemostat SBA050; Dixon, CA, USA). Fresh sheep RBC suspensions were prepared daily as described by Matson et al. (Matson et al., 2005). Addition of sheep RBCs to each well effectively halved all plasma dilutions, with column 1 having a final plasma dilution of 1:4.

Plates were dry incubated at 25°C for 90 min, then tilted 45 deg on their long axis for 20 min to aid visualization. Agglutination titres were scored, and then the plates were set aside at room temperature for an additional 70 min before scoring lysis titres. No lysis was observed in our samples, so only agglutination titres were analyzed. Agglutination titres were scored as $\log_2(D)$–1, where D is the final dilution of plasma where agglutination has occurred (i.e. column 1 has a titre of 1). Half scores were recorded when agglutination appeared to terminate between two dilutions. Samples were given an agglutination score of zero when no agglutination was observed. Except for five occasions, due to sample exhaustion, samples were run in duplicate on separate plates and titres were averaged.

PHA challenge

As an additional measure of immune function, we measured phytohemagglutinin (PHA)-induced localized skin swelling response of turtles. PHA typically triggers local infiltration by all types of leukocytes and recruitment of lymphocytes to initiate a cell-mediated response, and thus swelling can represent both an innate response as well as an adaptive response (Martin et al., 2006).

Swelling response to PHA (see details below), was conducted on non-hatchling turtles in the spring. For PHA challenges, 10 µl of 10 mg ml^{-1} PHA (Sigma L9017; St Louis, MO, USA) in 0.01 mol l^{-1} cell-culture grade PBS was injected into the webbing between the fourth and fifth digit (first scale above non-scaled webbing) on a randomly chosen hindfoot of each turtle. PBS (10 µl) was injected into the webbing of the other foot of each turtle as a within-individual control. The webbing of each foot was measured in duplicate to the nearest 0.01 mm by a single researcher (L.E.S.) with digital calipers at four times: prior to injection and at 6 h, 24 h and 48 h post-injection. The average of the duplicate measurements was used as the foot web thickness at each of these four times.

Statistical analysis

DNA damage and repair efficiency

To evaluate differences among ages and between sexes in baseline DNA damage and repair efficiency in the autumn, we used two sets of mixed model analyses of variance (ANOVA). In all analyses, the percentage of DNA in the comet tail was used as the dependent variable. In the first model, age class (hatchling, juvenile, adult), treatment (baseline, exposure, exposure + repair) and their interaction were included as independent variables in a mixed model ANOVA. Sex was not included here because of unequal representation of the sexes within age classes and sex was unknown for the hatchlings. Plastron length was not used as a covariate in this analysis because it is reflected in the age class categories. Because of multiple measures of DNA damage for each turtle–treatment combination (e.g. an average of 43.5 cells were analyzed per individual–treatment), individual identification nested within treatment was included as a random effect in the model.

In the second model, independent variables included sex, treatment (baseline, exposure, exposure + repair) and their interactions. Hatchlings were not included in this model because their sex was not identified. To account for potential variation due to age (size), plastron length (PL) was included as a covariate; initial analyses indicated that interactions between the main effects with PL were not significant, thus the final model included PL as a covariate without interaction terms. Individual identification nested within treatment was included as a random effect in the model. Both models were run twice, once each for baseline, exposure and exposure + repair treatments for UV light and H_2O_2.

DNA repair efficiency was evaluated as the difference in DNA damage between exposure and exposure + repair values divided by the baseline damage. Because DNA damage was measured in many cells within each turtle and treatment, mean values were calculated for each individual and used as a dependent variable in two sets of ANOVAs (with either age or sex as the independent variable). These models were run twice, once for exposure to UV and once for exposure to H_2O_2.

Immune function and parasitic infection

Differences in blood cell components among age classes (hatchling, juvenile, adult) in the autumn were tested with ANOVA (when data appeared normal by visual inspection) or Kruskal–Wallis ANCOVA tests (when data appeared non-normal). Across the three seasons, blood cell components in non-hatchlings were analyzed with mixed-effect models, with season, PL, sex and all two-way interactions entered as fixed effects, and turtle ID entered as a random effect to account for repeated measures. To examine hemogregarine infection across seasons, hemogregarine infection category was treated as a continuous variable and season, sex, PL and all two-way interactions were entered into a linear regression model with turtle ID as a random effect. We examined linear correlation coefficients among blood cell components and hemogregarine infection among individuals and across seasons to assess relationships between each independent component (supplementary material Table S1, S2).

Agglutination titres were analyzed with ANOVA to quantify differences between age classes (hatchlings, juveniles, adults) in autumn. Across seasons, the occurrence of zero mean titres (no immune response detectable) was examined for seasonal differences in non-hatchlings using likelihood ratio tests. To examine seasonal, PL and sex differences in agglutination titres, a linear mixed effect model was used with mean titre as the response variable, and season, PL and age class as fixed effects and turtle ID as a random effect.

To analyze foot web swelling response to PHA in the spring, we first tested for individual response over time to control injections and to PHA injections separately by fitting models of foot web thickness with the predictors of time (0, 6, 24 and 48 h), sex, plastron length and the two-way interactions with time, and turtle ID entered as a random effect. To test whether the response to PHA was greater than that to the control and influenced by biological variables, we analyzed

the peak swelling response in both feet at 24 h (24-h swelling=24-h thickness – pre-injection thickness). Treatment (control or PHA injection), PL, sex, treatment×PL and treatment×sex were entered as predictors with turtle ID entered as a random effect.

All statistical analyses were completed with JMP v. 7 or SAS v. 9.1 (SAS Institue, Inc.). Significant effects in linear models were examined for pairwise differences using Tukey's *post-hoc* comparison, with the exception of the occurrence of zero mean titres in the agglutination assay, for which *post-hoc* pairwise *P*-values were Bonferroni-corrected. Values reported in the text are mean ± 1 s.d., whereas values presented in figures are least square means ± 1 s.e.m.

RESULTS

We collected data from 10 hatchling clutches, 16 non-hatchling females (PL=117±41; range 62–172) and 19 non-hatchling males (PL=120±26; range 70–157). Plastron length did not change between autumn and spring measurement, so spring plastron length was used in all analyses.

DNA damage and repair

Hatchlings showed a greater degree of DNA repair efficiency (for UV exposure: $F_{2,28}$=3.9, P=0.030) than juveniles and adults, despite their higher baseline levels of damage (Fig. 1, Table 2). Interestingly, the amount of DNA damage induced by UV was much greater in juvenile turtles than in adults. However, after a period of 5 min to repair, DNA damage in adult erythrocytes increased to a level similar to that of juveniles (Fig. 1B,C), which shows that damage continued to accumulate after exposure to UV in adults but not in the other age classes. DNA damage induced by H_2O_2 and levels of repair after H_2O_2 exposure did not differ significantly among age classes. Our data suggest that in hatchlings there may be repair to DNA damage caused by exposure to H_2O_2 (Fig. 1D), but caution is needed because we successfully measured DNA repair in only one individual hatchling. Repair efficiency of DNA exposed to H_2O_2 did not differ among age classes ($F_{2,12}$=0.3, P=0.775).

Non-hatchling male and female turtles did not differ in their baseline damage of erythrocyte DNA, the amount of damage induced by UV or H_2O_2 exposure, or DNA repair efficiency (UV: $F_{1,22}$=0.1, P=0.778; H_2O_2: $F_{1,12}$=0.3, P=0.591; Table 2; Fig. 2). However, regardless of sex, the amount of DNA damage caused by H_2O_2 was greater than that caused by UV exposure (Figs 1, 2). For non-hatchling turtles, plastron length was not related to levels of DNA damage or repair efficiency as indicated by non-significant interaction terms in the full model.

Blood cells and intracellular parasites

Hatchlings had lower basophils counts compared with juveniles and adults (Table 3). Age classes did not differ in any other blood cell component in autumn. Across non-hatchling samples over three seasons, basophil counts were lower in larger (greater PL) individuals. Plastron length had an interactive effect with season on lymphocytes, because of a negative relationship between lymphocytes and PL in the spring only. When using age class in place of PL in the lymphocyte model, the season by age interaction persisted [P(season)<0.0001, P(age)=0.22, P(sex)=0.07, P(season×age)=0.02, P(season × sex)=0.10, P(sex × age)=0.63; N=92]. Juveniles in the spring had higher lymphocyte levels than either age class in any other season, and males tended to have higher levels of lymphoctyes compared with females, particularly in the spring (Fig. 3H).

Seasonal differences were seen in PCV (trend for highest values in the autumn), heterophils (elevated during hibernation), lymphocytes (elevated in the spring compared with hibernation) and basophils (highest values in the autumn; Table 4, Fig. 3). Because seasonal patterns in circulating heterophils and lymphocytes were the converse of each other, we also examined the heterophil to lymphocyte (H:L) ratio, which typically increases following stress (Davis et al., 2008). H:L ratios were dramatically increased during hibernation (10.55±1.66; LSM ± s.e.m.) compared with autumn (1.14±1.64) or spring (3.40±1.62; Table 4), suggesting that the animals were undergoing physiological stress during hibernation. Males had higher measures of PCV, white blood cells (WBC) and basophils than females when accounting for PL.

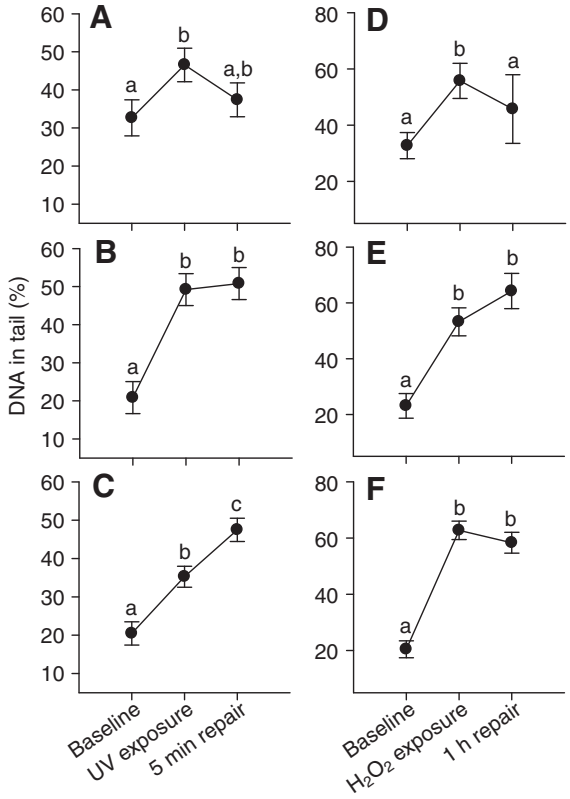

Fig. 1. Percentage of DNA present in the comet tail for hatchlings (A,D), juveniles (B,E) and adults (C,F) (LSM ± s.e.m.). Each panel shows baseline values alongside values recorded immediately after exposure to UV (A–C) or H_2O_2 (D–F) and after a time delay following exposure during which repair was anticipated. A higher percentage DNA in the tail indicates greater DNA damage. Lowercase letters denote statistically significant differences between treatments. In D, s.e.m. for the 1-h repair means are from the mixed-model ANOVA based on the multiple measurements made on the single hatchling sampled in that treatment.

Thirteen of 33 non-hatchlings had hemogregarines present in red blood cells, but none were found in hatchlings. Among non-hatchlings (Fig. 4), hemogregarine infection showed no seasonal effects, and was related positively to PL (P(season)=0.65, P(PL)=0.008, P(sex)=0.20, P(season × PL)=0.97, P(season × sex)=0.54, P(sex × PL)=0.37; N=96). Within individuals, infection intensity in one season was strongly correlated with infection intensity in the other seasons (supplementary material Table S1).

In mixed effect models across season, neither PCV nor lymphocyte counts were related to hemogregarine infection category [PCV: P(hemogregarine category)=0.75, P(season)=0.65, P(hemogregarine category × season)=0.08, N=95; lymphocytes:

Table 2. Statistical results of mixed-effect models of DNA damage

Effect	UV exposure	H_2O_2 exposure
Age class model including hatchling data		
Age	$F_{2,4142}$=2.35, P=0.0954	$F_{2,3140}$=0.11, P=0.8924
Treatment	$F_{2,96}$=24.58, **P<0.0001**	$F_{2,63}$=42.76, **P<0.0001**
Age × treatment	$F_{4,4142}$=3.82, **P=0.0042**	$F_{4,3140}$=2.21, P=0.0659
Size and sex model excluding hatchling data		
Sex	$F_{1,3161}$=1.53, P=0.2126	$F_{1,2668}$=0.93, P=0.3339
Treatment	$F_{2,75}$=32.98, **P<0.0001**	$F_{2,54}$=65.09, **P<0.0001**
Sex × treatment	$F_{2,3161}$=1.26, P=0.2827	$F_{2,2668}$=0.02, P=0.9827
Plastron length	$F_{1,3161}$=0.61, P=0.4363	$F_{1,2668}$=0.28, P=0.5962

DNA damage was measured for three treatments: baseline, immediately following exposure to a stressor (UV or H_2O_2), and after repair time.
Bold type indicates statistically significant results.

P(hemoproteus category)=0.29, P(season)=0.02, P(hemogregarine category × season)=0.72, N=95], despite the potential for infection to lead to anemia and trigger cell-mediated immunity.

Agglutination assay

Agglutination titres were significantly different across the three age groups in the autumn (N=41, F=4.56, P=0.02; adult, 2.4±1.2; juvenile, 3.0±1.0; hatchling, 1.2±0.9), indicating differential activity by natural antibodies among age classes. Hatchlings (N=6) had significantly lower titres compared with juveniles (N=9; P<0.05), but not compared with adults (N=26; P>0.05). Juveniles and adults did not differ significantly (P>0.05).

Over the three sampling seasons, for juvenile and adult samples only, adults had a significantly greater number of zero-mean titres (i.e. no visible natural antibody response to sheep RBC) compared with juveniles (χ^2=13.6, P=0.001). Autumn samples contained significantly fewer zero titres (0 of 36 samples) than hibernation (5 of 31 samples, P=0.01, all pairwise P-values reported post-Bonferroni correction) and spring samples (8 of 32 samples, P=0.001; hibernation *vs* spring samples, P=1). In a mixed effect model including zero-mean scores, there were no significant two-way interaction effects, so these interactions were removed from the ANCOVA. Agglutination titres were not predicted by sex (P=0.93) or plastron length (P=0.52), but varied significantly across season (P<0.0001; Fig. 5A). Plasma samples taken during hibernation (N=31, 1.2±1.3) and spring (N=32, 0.9±1.0) had significantly lower agglutination titres than those taken in the autumn (N=35, 2.5±1.2; autumn *vs* hibernation and autumn *vs* spring, P<0.05; hibernation *vs* spring, P>0.05). The results were similar in a model with zero mean scores excluded [P(season)<0.0001, P(PL)=0.73, P(sex)=0.58, N=85; autumn *vs* hibernation and autumn *vs* spring, P<0.05; hibernation *vs* spring, P>0.05]. Thus, natural antibodies appear to be expressed at higher levels in the autumn than in winter and spring, but do not vary according to sex and age between juveniles and adults.

PHA

Foot web thickness increased over time in response to both injection with the control (PBS alone) and to injection with PHA (Table 5;

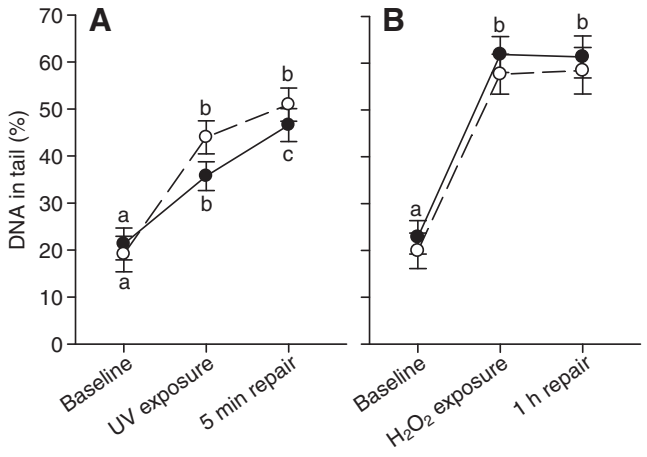

Fig. 2. Percentage of DNA present in the comet tail for males (solid lines and filled circles) and females (dashed lines and open circles; juveniles and adults are combined). Each panel shows baseline values alongside values recorded immediately after exposure to UV (A) or H_2O_2 (B) and after a time delay following exposure, during which time repair was anticipated. A higher percentage DNA in the tail indicates greater DNA damage. Lowercase letters denote statistically significant differences between treatments. LSM ± s.e.m. are reported.

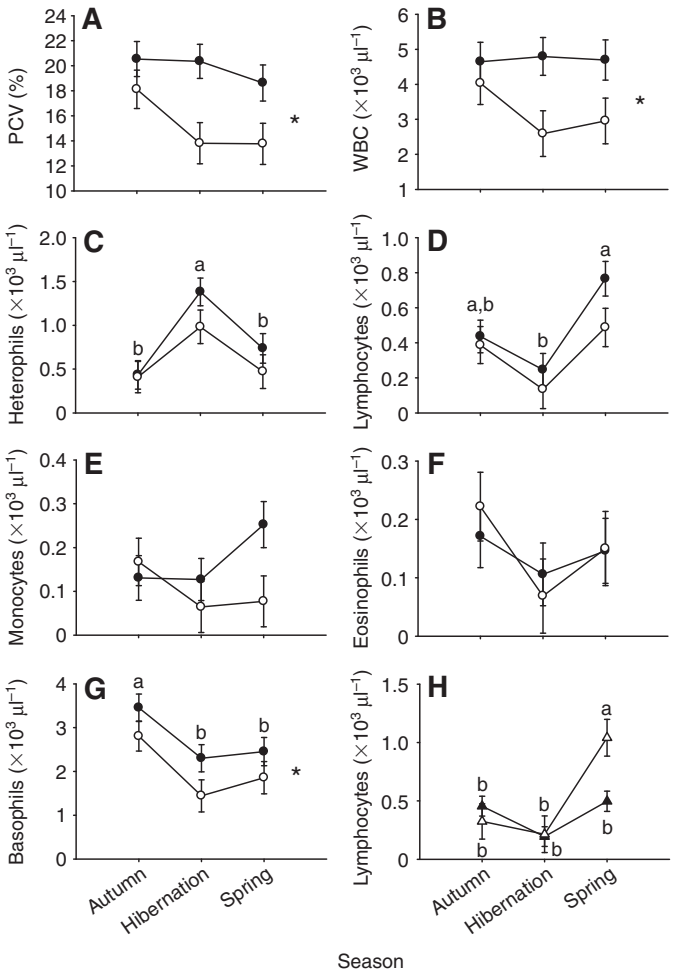

Fig. 3. Blood cell components (LSM ± s.e.m.) as a function of season and sex (A–G; males, filled circles; females, open circles) and age and season (H; adults, closed triangles; juveniles, open triangles). Asterisks on right of panels indicate significant differences between the sexes. Letters indicate statistically significantly different seasons. Letters in H indicate statistically significant differences between age × season means. PCV, packed cell volume; WBC, white blood cells.

Table 3. Blood cell components from autumn samples of the three age classes

	Hatchlings (*N*=10)	Juveniles (*N*=8)	Adults (*N*=25)	Test statistic	*P*
PCV (%)	16.6±6.6	19.0±6.1	19.6±6.0	F=0.86	0.43
WBC ($\times10^3\,\mu l^{-1}$)	3.74±2.87	4.32±1.29	4.38±1.76	F=0.38	0.69
Heterophils ($\times10^3\,\mu l^{-1}$)	0.69±1.16	0.30±0.17	0.46±0.50	χ^2=0.48	0.79
Lymphocytes ($\times10^3\,\mu l^{-1}$)	0.79±1.01	0.30±0.35	0.57±0.71	χ^2=1.66	0.44
Monocytes ($\times10^3\,\mu l^{-1}$)	0.63±1.67	0.13±0.12	0.19±0.25	χ^2=0.10	0.95
Eosinophils ($\times10^3\,\mu l^{-1}$)	0.04±0.09	0.10±0.10	0.23±0.28	χ^2=4.49	0.11
Basophils ($\times10^3\,\mu l^{-1}$)	1.59±1.53	3.48±1.31	3.05±1.36	F=5.14	**0.01**

PCV, packed cell volume; WBC, white blood cells.
Values are means ± 1 s.d.
Bold type indicates statistically significant results.

Fig. 5B). Swelling in response to PHA peaked 24-h post-injection and declined by 48 h. Larger turtles had thicker foot webs that tended to increase at a greater rate with time. Twenty-four hour swelling (24-hour thickness – pre-injection thickness) was significantly greater following PHA injection compared with control injection (one-tailed P=0.034) and was greater for larger turtles. However, there was no interaction between PL and treatment, indicating that the increased swelling response in larger turtles was due to injection alone and not to a PHA-specific response (i.e. seen in control injections as well).

DISCUSSION

Age effects: development and senescence

The effect of age on physiological maintenance in the long-lived painted turtle varied among components, which may reflect the relative cost and benefit of development of each component across age (e.g. Sparkman and Palacios, 2009). As predicted, hatchlings had reduced immune function compared with non-hatchlings in some components (NAbs and basophils). Reduced immunocompetence in young individuals has been shown in a few bird species (reviewed by Hausmann et al., 2005), and is thought to be due to a costly and protracted development of the immune system. Recent research on painted turtles has revealed that hatchling immune function depends on incubation temperatures (Les et al., 2009), thus, variation among the measures of immune function employed in the current study may reflect effects of the nest environment on development of the immune system.

Compared with studies in short-lived endotherms (Hausmann et al., 2005; Palacios et al., 2007), we found little evidence of senescence in physiological maintenance in our long-lived turtle, supporting the general prediction that senescence in long-lived animals should be minimal. Only basophil and lymphocyte counts decreased with age in non-hatchlings. The relationship in basophils was weak, and the decrease in lymphocytes occurred only in the spring, apparently driven by a difference between non-reproductive juveniles and reproductive adults. This suggests that a trade-off may occur between spring reproductive effort and lymphocyte generation in adults.

The ability to mount swelling in response to injection with a foreign antigen showed no senescence. Instead, swelling after injection (even with control substances) increased with size. Swelling in response to PHA in birds has been shown to be associated with local recruitment of many types of leukocytes, with heterophils showing the strongest correlations to swelling response (Martin et al., 2006). We found no correlation among individuals in the degree of PHA-induced swelling and counts of any type of circulating white blood cell (supplementary material Table S3), suggesting that circulating levels of leukocytes are not indicative of the ability to mount a swelling response to a foreign antigen. The degree of infection with one type of pathogen, intracellular hemogregarine parasites, increased with age. Although we found no correlations between immune function and hemogregarine infection, we cannot rule out the possibility of hemogregarines or unmeasured pathogens contributing to age-related immune patterns. The absence of lysis and any age effect on agglutination in response to foreign antigens in painted turtles contrasts with research in other reptiles, which has demonstrated robust lytic activity against foreign antigens [crocodiles (Merchant and Britton, 2006); garter snakes (Sparkman and Palacios, 2009)] and increasing antibody activity with age [water pythons (Madsen et al., 2007); red-eared sliders (Zimmerman et al., 2010)]. It is possible that similar patterns may occur in painted turtles during the main active season (summer), if immune activity is more robust at that time.

Owing to the natural exposure of DNA to damaging agents such as UV radiation (during basking) and reactive oxygen species (produced by metabolic activity), mechanisms exist in many organisms to repair DNA damage (Finkel and Holbrook, 2000). In our study, hatchling painted turtles had a much higher ability to repair DNA damage caused by exposure to UV compared with non-hatchlings. Given the high rate of cell division and growth in young

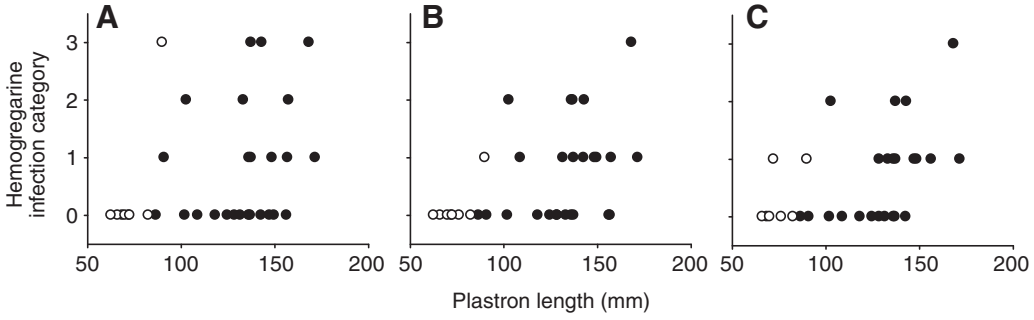

Fig. 4. Hemogregarine infection category as a function of plastron length for juveniles (open circles) and adults (filled circles) in (A) autumn, (B) hibernation and (C) spring.

Table 4. Blood cell components and heterophil to lymphocyte ratio (H:L) for non-hatchling turtles collected across three seasons

	Predictors					
Response	Season	PL	Sex	Season × PL	Season × sex	PL × sex
PCV	0.07	0.41	**0.003** (M>F)	0.86	0.31	0.34
WBC	0.47	0.23	**0.009** (M>F)	0.40	0.34	0.82
Heterophils	**<0.0001**	0.14	0.16	0.13	0.52	0.27
Lymphocytes*	**0.0002**	0.22	0.11	**0.02**	0.49	0.97
Monocytes†	0.34	0.25	0.09	0.66	0.42	0.70
Eosinophils	0.52	0.68	0.76	0.61	0.48	0.46
Basophils‡	**0.0002**	**0.03** (–)	**0.04** (M>F)	0.39	0.90	0.84
H:L§	**0.0005**	0.15	0.17	0.31	0.63	0.85

P-values are presented for the predictors entered into separate mixed-effect models of different blood cell response variables. Entries in parentheses indicate the direction of the effect. In all models, turtle ID was entered as a random effect. The data include 95 samples of 35 individuals. PL, plastron length; PCV, packed cell volume; WBC, white blood cells.

*One outlier removed for each season (different individual each time).

†One outlier removed from spring.

‡One outlier removed from hibernation.

§89 samples of 35 individuals.

Bold type indicates statistically significant results.

animals, high DNA repair efficiency is probably advantageous during this phase of life (Metcalfe and Alonso-Alvarez, 2010). Importantly, juvenile and adult animals were unable to repair damage at all, a result in contrast to other studies of reptilian DNA repair (Robert et al., 2007; Bronikowski, 2008; Robert and Bronikowski, 2010). This complete lack of repair in non-hatchlings suggests that senescence is not the appropriate interpretation of the age-class pattern. It is possible that painted turtles, being longer-lived animals than those previously studied, have evolved reduced generation of oxidative stress agents in adults (Robert et al., 2007; Ungvari et al., 2008), and so have not faced selection for substantial repair mechanisms (Cohen et al., 2008). In addition, different cell types may respond differently to damage. Further studies on different cell populations with differing longevity (e.g. erythrocytes *vs* granulocytes) are warranted.

Sex effects

Sex had little effect on any measures of physiological maintenance, and where it did, males had heightened levels of immune components. This provides no support for the prediction that males would show reduced physiological maintenance as a correlate of reduced longevity. We also saw no significant season by sex interactions in the different immune measures. Whereas immune function and cellular maintenance are generally depressed by reproductive effort (Zuk and Stoehr, 2002; Alonso-Alvarez et al., 2004a; Martin et al., 2006; Martin et al., 2008), sex differences in these measures are not consistently found (e.g. Zuk and Stoehr, 2002; Hausmann et al., 2005; Bertrand et al., 2006; Martin et al., 2006; Beldomenico et al., 2008; Zimmerman et al., 2010). Instead, sex, season, reproductive state, prior pathogen exposure, and recent stress may interact to produce complicated patterns in immune function between the sexes, making it difficult to predict sex differences over more limited sampling efforts (Beldomenico et al., 2008; Davis et al., 2008).

Seasonal effects in immune function

Painted turtles showed marked seasonal fluctuations in immune function, consistent with findings on other reptiles (Zapata et al., 1992; Muñoz and de la Fuente, 2004; Zimmerman et al., 2010). Several components of immune function were reduced during hibernation and in the spring. Moreover, H:L ratios (indicative of stress) were increased during hibernation. These patterns could be due to an immune depression during hibernation and a delay in the animal in recovering function following emergence from hibernation, or could include spring depression associated with reproductive efforts (Zapata et al., 1992). Reduced levels of circulating white blood cells during the winter and increased lymphocytes in the spring have been recorded in other reptiles (Zapata et al., 1992; Muñoz and de la Fuente, 2004; Pitol et al., 2008). In interpreting our seasonal patterns, we must note that our hibernation measures (performed after a day of warming) approximate those of an animal that has been hibernating and has emerged for a day to bask, and does not necessarily reflect those of an animal currently in physiological torpor. Elevated stress

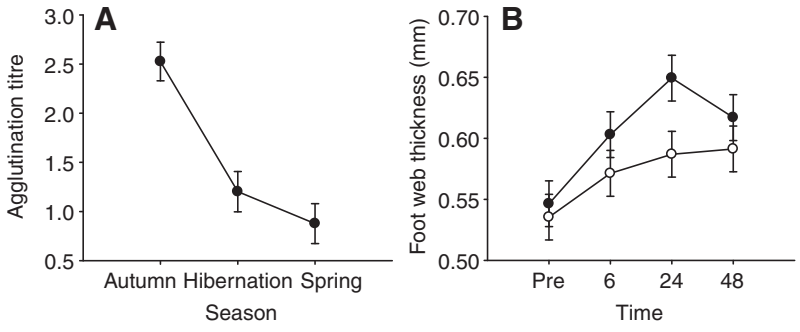

Fig. 5. (A) Seasonal agglutination titres for non-hatchling turtles (LSM ± s.e.m.). (B) Foot web thickness (LSM ± s.e.m.) over time after injection with PHA (filled circles) and with the vehicle control (open circles), measured during the spring.

Table 5. Statistical results from mixed-effect models of foot thickness following control and PHA injections and from mixed-effect model comparing 24-h swelling in control- and PHA-injected feet, within individuals

	d.f.	$d.f._{Den}$	*F*	*P*
Thickness (control injection)				
Time	3	96	3.9615	**0.0104**
Plastron length (PL)	1	32	90.4124	**<0.0001**
Sex	1	32	0.4883	0.4897
Time × PL	3	96	2.2562	0.0868
Time × sex	3	96	0.0942	0.9631
Thickness (PHA injection)				
Time	3	96	11.8505	**<0.0001**
PL	1	32	114.8518	**<0.0001**
Sex	1	32	0.1493	0.7018
Time × PL	3	96	2.2652	0.0858
Time × sex	3	96	0.7285	0.5374
24-h swelling				
Treatment	1	32	3.5581	**0.0684**
PL	1	32	6.3729	**0.0167**
Sex	1	32	0.0129	0.9104
Treatment × PL	1	32	0.2789	0.6011
Treatment × sex	1	32	0.2198	0.6424

d.f., degrees of freedom; $d.f._{Den}$, degrees of freedom denominator.
Turtle ID was entered as a random effect in each model.
N=35 individuals for all models.
Bold type indicates statistically significant results.

associated with a sudden change in body temperature, which is ecologically relevant for turtles during the winter, could alone lead to an alteration in the number of circulating leucocytes and in H:L values (Saad and El Ridi, 1988; Davis et al., 2008).

We found little evidence of winter immune enhancement in our turtles. Heterophil count was the only component of immune function that was elevated during hibernation (consistent with stress-induced elevation of H:L values) (Davis et al., 2008). Because heterophils typically serve as the first line of defense through phagocytosis of bacteria and other foreign agents, this pattern suggests that this front line is important during hibernation despite the relatively high costs (Jacobson, 2007). Alternatively, the speed with which heterophils can be generated may place value on their high expression during the brief warming of intermittent winter basking. In contrast to small endotherms, which often show winter immune enhancement (Nelson and Demas, 1996; Bilbo et al., 2002; Braille and Prendergast, 2008), ectotherms have much smaller energetic demands during the winter, remaining inactive and often in a state of torpor. The low temperatures, low food consumption, and small energy budgets of hibernating animals may reduce the need (i.e. decreased pathogen success) or ability to maintain or mount many types of host immune responses (Prendergast et al., 2002). Any of these mechanisms could contribute to reduced immune function during the winter. However, it is important to note that we cannot rule out the potential role of captivity or altered housing conditions in producing seasonal-like patterns (Davis et al., 2008).

Physiological maintenance across different immunological and non-immunological systems is not consistently determined by age, sex or season in the long-lived painted turtle. Rather, we find support for components being regulated differentially, perhaps in ways that optimize fitness while facing trade-offs among physiological systems and among maintenance, growth and reproduction (McNamara and Houston, 1996; Zuk and Stoher, 2002; Martin et al., 2008). The results from this study suggest that senescence in physiological maintenance in long-lived animals (particularly animals with indeterminate growth) may be minimal and additionally provide support for depressed immune function over winter in ectothermic animals.

ACKNOWLEDGEMENTS

We thank F. Janzen, M. G. Palacios and L. Martin for advice on the research and manuscript preparation. The manuscript was improved by comments from several anonymous reviewers. Funding for salaries and materials were provided by the National Science Foundation, including a Postdoctoral Fellowship in Biological Informatics (L.S.), Graduate Research Fellowship (S.McG.) and LTREB DEB-0640932 (D.A.W., awarded to F. Janzen). Animals were collected from the Thomson Causeway Recreation Area with permission from the US Army Corps of Engineers and the US Fish and Wildlife Service, All procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University.

REFERENCES

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B. and Sorci, G. (2004a). Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* **7**, 363-368.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. and Sorci, G. (2004b). An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *Am. Nat.* **164**, 651-659.

Alonso-Alvarez, C., Bertrand, S., Faivre, B. and Sorci, G. (2007). Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. *Funct. Ecol.* **21**, 873-879.

Baillie, S. R. and Prendergast, B. J. (2008). Photoperiodic regulation of behavioral responses to bacterial and viral mimetics: a test of the winter immunoenhancement hypothesis. *J. Biol. Rhythms* **23**, 81-90.

Beckman, K. B. and Ames, B. N. (1998). The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581.

Beldomenico, P. M., Telfer, S., Gebert, S., Lukomski, L., Bennett, M. and Begon, M. (2008). The dynamics of health in wild field vole populations: a haematological perspective. *J. Anim. Ecol.* **77**, 984-997.

Bertrand, S., Criscuolo, F., Faivre, B. and Sorci, G. (2006). Immune activation increases susceptibility to oxidative tissue damage in zebra finches. *Funct. Ecol.* **20**, 1022-1027.

Bilbo, S. D., Dhabhar, F. S., Viswanathan, K., Saul, A., Yellon, S. M. and Nelson, R. J. (2002). Short day lengths augment stress-induced leukocyte trafficking and stress-induced enhancement of skin immune function. *Proc. Natl. Acad. Sci. USA* **99**, 4067-4072.

Bronikowski, A. M. (2008). The evolution of aging phenotypes in snakes: a review and synthesis with new data. *Age* **30**, 169-176.

Campbell, T. W. and Ellis, C. K. (2007). *Avian and Exotic Animal Hematology and Cytology* Third edition. Ames, IA: Blackwell Publishing Professional.

Cohen, A. A., McGraw, K. J., Wiersma, P., Williams, J. B., Robinson, W. D., Robinson, T. R., Brawn, J. D. and Ricklefs, R. E. (2008). Interspecific associations between circulating antioxidant levels and life-history variation in birds. *Am. Nat.* **172**, 178-193.

Congdon, J. D. and Tinkle, D. W. (1982). Reproductive energetic of the painted turtles (*Chrysemys picta*). *Herpetologica* **38**, 228-237.

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**, 813-827.

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

Davis, A. K., Maney, D. L. and Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct. Ecol.* **22**, 760-772.

Demas, G. E. (2004). The energetic of immunity: a neuroendocrine link between energy balance and immune function. *Horm. Behav.* **45**, 173-180.

Derting, T. L. and Virk, M. K. (2005). Positive effects of testosterone and immunochallenge on energy allocation to reproductive organs. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **175**, 543-556.

Ernst, C. H. (1972). Temperature-activity relationship in the painted turtles, *Chrysemys picta. Copeia* **1972**, 217-222.

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

Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Freitak, D., Ots, I., Vanatoa, A. and Hörak, P. (2003). Immune response is energetically costly in white cabbage butterfly pupae. *Proc. R. Soc. Lond. B. Biol. Sci.* **270**, S220-S222.

Gibbons, J. W. (1968). Reproductive potential, activity, and cycles in the painted turtle, *Chrysemys picta. Ecology* **49**, 399-409.

Gist, D. H., Michaelson, J. A. and Jones, J. M. (1990). Autumn mating in the painted turtle, *Chrysemys picta. Herpetologica*, **46**, 331-336.

Harman, D. (1956). Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298-300.

Harman, D. (1981). The aging process. *Proc. Natl. Acad. Sci. USA* **78**, 7124-7128.

Hausmann, M. F., Winkler, D. W., Huntington, C. E., Vleck, D., Sanneman, C. E., Hanley, D. and Vleck, C. M. (2005). Cell-mediated immunosenescence in birds. *Oecologia* **145**, 270-275.

Jacobson, E. R. (2007). *Infectious Diseases and Pathology of Reptiles.* Boca Raton, FL: CRC Press.

Kirkwood, T. B. L. and Rose, M. R. (1991). Evolution of senescence: late survival sacrificed for reproduction. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **332**, 15-24.
Lee, K. A. (2006). Linking immune defense and life history at the levels of the individual and the species. *Integr. Comp. Biol.* **46**, 1000-1015.
Les, H. L., Paitz, R. T. and Bowden, R. M. (2009). Living at extremes: development at the edges of viable temperature under constant and fluctuating conditions. *Physiol. Biochem. Zool.* **82**, 105-112.
Love, O. P., Salvante, K. G., Dale, J. and Williams, T. D. (2008). Sex-specific variability in the immune system across life-history stages. *Am. Nat.* **172**, E99-E112.
Madsen, T., Ujvari, B., Nandakumar, K. S., Hasselquist, D. and Holmdahl, R. (2007). Do "infectious" prey select for high levels of natural antibodies in tropical pythons? *Evol. Ecol.* **21**, 271-279.
Martin, L. B., Scheuerlein, A. and Wikelski, M. (2003). Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc. R. Soc. Lond. B. Biol. Sci.* **270**, 153-158.
Martin, L. B., Han, P., Lewittes, J., Kuhlman, J. R., Klasing, K. C. and Wikelski, M. (2006). Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. *Funct. Ecol.* **20**, 290-299.
Martin, L. B., Weil, Z. M. and Nelson, R. J. (2008). Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **363**, 321-339.
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**, 275-286.
McNamara, J. M. and Houston, A. I. (1996). State-dependent life histories. *Nature* **380**, 215-221.
Merchant, M. and Britton, A. (2006). Characterization of serum complement activity of saltwater (*Crocodylus porosus*) and freshwater (*Crocodylus johnstoni*) crocodiles. *Comp. Biochem. Physiol.* **143A**, 488-493.
Metcalfe, N. B. and Monaghan, P. (2001). Compensation for a bad start: grow now, pay later? *Trends Ecol. Evol.* **16**, 254-260.
Metcalfe, N. B. and Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Funct. Ecol.* **24**, 984-996.
Moll, E. O. (1973). Latitudinal and intersubspecific variation in reproduction of the painted turtle, *Chrysemys picta. Herpetologica* **29**, 307-318.
Muñoz, F. J. and de la Fuente, M. (2004). Seasonal changes in lymphoid distribution of the turtle *Mauremys caspica. Copeia* **2004**, 178-183.
Nelson, R. J. and Demas, G. E. (1996). Seasonal changes in immune function. *Q. Rev. Biol.* **71**, 511-548.
Nilsson, J.-A., Granbom, M. and Råberg, L. (2007). Does the strength of an immune response reflect its energetic cost? *J. Avian Biol.* **38**, 488-494.
Ots, I., Kerimov, A. B., Ivankina, E. V., Ilyina, T. A. and Hõrak, P. (2001). Immune challenge affects basal metabolic activity in wintering great tits. *Proc. R. Soc. Lond. B. Biol. Sci.* **268**, 1175-1181.
Paitz, R. T., Gould, A. C., Holgersson, M. C. N. and Bowden, R. M. (2009). Temperature, phenotype, and the evolution of temperature-dependent sex determination: how do natural incubations compare to laboratory incubations? *J. Exp. Zool.* **314**, 86-93.
Palacios, M. G., Cunnick, J. E., Winkler, D. W. and Vleck, C. M. (2007). Immunosenescence in some but not all immune components in a free-living vertebrate, the tree swallow. *Proc. R. Soc. Lond. B. Biol. Sci.* **274**, 951-957.
Palacios, M. G., Cunnick, J. E., Vleck, D. and Vleck, C. M. (2009). Ontogeny of innate and adaptive immune defesnse components in free-living tree swallows, *Tachycineta bicolor. Dev. Comp. Immunol.* **33**, 456-463.
Patnaik, B. K. (1994). Ageing in reptiles. *Gerontology* **40**, 200-220.
Pitol, D. L., Issa, J. P. M., Caetano, F. H. and Lunardi, L. O. (2008). Radiographic stuffy of the seasonal distribution of leucocytes in turtles *Phrynops hilarii* (Chelona Chelidae). *Micron* **39**, 1381-1386.
Prendergast, B. J., Freeman, D. A., Zucker, I. and Nelson, R. J. (2002). Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R1054-R1062.
Robert, K. and Bronikowski, A. M. (2010). Evolution of senescence: physiological evolution in natural populations of the garter snake with divergent life history ecotypes. *Am. Nat.* **175**, 147-159.
Robert, K., Rossini, A. K. and Bronikowski, A. M. (2007). Testing the free radical theory of aging hypothesis: physiological differences in long lived and short lived Colubrid snakes. *Aging Cell* **6**, 395-404.
Rosskopf, W. J. (1982). Normal hemogram and blood chemistry values for California desert tortoises. *Vet. Med. Small Anim. Clin.* **77**, 85-87.
Saad, A. H. and El Ridi, R. (1988). Endogenous corticosteroids mediate seasonal cyclic changes in immunity in lizards. *Immunobiology* **177**, 390-403.
Schall, J. J., Bennett, A. F. and Putnam, R. W. (1982). Lizards infected with malaria: physiological and behavioral consequences. *Science* **217**, 1057-1059.
Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321.
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**, 1242-1248.
Sparkman, A., 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. Lond. B. Biol. Sci.* **274**, 943-950.
Tice, R. R. (1995). The single cell gel/comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In *Environmental Mutagenesis* (ed. D. H. Phillips and S. Venitt), pp. 315-339. Oxford: Bios Scientific.
Tizard, I. R. (2004). *Veterinary Immunology: an Introduction*, 7th edn. Elsevier.
Ungvari, Z., Krasnikov, B. F., Csiszar, A., Labinskyy, N., Mukhopadhyay, P., Pacher, P., Cooper, A. J. L., Podlutskaya, N., Austad, S. M. and Podlutsky, A. (2008). Testing hypotheses of aging in long-lived mice of the genus *Peromyscus*: association between longevity and mitochondrial stress resistance, ROS detoxification pathways, and DNA repair efficiency. *Age* **30**, 121-133.
von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. and Wittzell, H. (1999). Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond. B. Biol. Sci.* **266**, 1-12.
Wilbur, H. M. (1975a). A growth model for the turtle *Chrysemys picta. Copeia* **1975**, 337-343.
Wilbur, H. M. (1975b). The evolutionary and mathematical demography of the turtle *Chrysemys picta. Ecology* **56**, 64-77.
Williams, G. C. (1957). Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11**, 398-411.
Williams, G. C. (1966). Natural selection, the costs of reproduction, and a refinement of Lack's principle. *Am. Nat.* **100**, 687-690.
Zapata, A. G., Varas, A. and Torroba, M. (1992). Seasonal variations in the immune system of lower vertebrates. *Immunol. Today* **13**, 142-147.
Zimmerman, L. M., Paitz, R. T., Vogel, L. A. and Bowden, R. M. (2010). Variation in the seasonal patterns of innate and adaptive immunity in the red-eared slider (*Trachemys scripta*). *J. Exp. Biol.* **213**, 1477-1483.
Zuk, M. and Stoehr, A. M. (2002). Immune defense and host life history. *Am. Nat.* **160**, S9-S22.