Behavioral Ecology
doi:10.1093/beheco/ari091
Advance Access publication 12 October 2005

Age, parasites, and condition affect humoral immune response in tropical pythons

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Mounting an immune response has been suggested to be physiologically costly because of metabolic requirements of immune cells specifically and upregulation of the immune system in general. We investigated such costs in free-living water pythons (*Liasis fuscus*), immunized with a harmless antigen, keyhole limpet hemocyanin. In the present study, we analyze the independent effects of age, blood parasite load, and body condition on the ability to mount a humoral immune response (level of antibody production to novel antigens). Python humoral immune response decreased with increasing body length/age, decreased with increasing blood parasite load, and decreased with declining body condition. The results suggest an energetic trade-off between immunocompetence and other energetically costly processes. *Key words:* age, condition, humoral immune response, parasites, python. *[Behav Ecol 17:20–24 (2006)]*

Studies of immune function in wild vertebrate populations were virtually nonexistent until about 15 years ago (Schmid-Hempel, 2003). The lack of such work was most likely due to the fact that studies on host-parasites interactions, until the early 1980s, had been neglected by most evolutionary biologists. However, the seminal paper by Hamilton and Zuk (1982) stressed the importance of including effects of parasitemia in studies of vertebrate evolution. This publication redirected subsequent studies to focus on parasite-host interactions and incorporating immunological processes into vertebrate evolutionary biology (reviewed by Schmid-Hempel, 2003; Schmid-Hempel and Ebert, 2003; Zuk and Stoehr, 2002).

Because individual organisms are likely to be subjected to attack by an array of diverse pathogens throughout their life span, the benefits of immunological response mechanisms are obvious (Boots and Bowers, 2004). As a result, in the last decade, a number of studies have examined how resistance to pathogens is associated with fitness and, in turn, how investment into pathogen resistance may compromise investment in other traits such as survival and reproduction (Bonneaud et al., 2003; Hanssen et al., 2004; Hasselquist et al., 2001; Kilpimaa et al., 2004; Lochmiller et al., 1993; Moret and Schmid-Hempel, 2000; Norris and Evans, 2000; Råberg et al., 2000). Although the cost of maintaining immune defense in the absence of infection is still a matter of debate (Klasing, 1998; Kraaijeveld and Godfray, 1997; Webster and Woolhouse, 1999), mounting an immune response has been suggested to be energetically costly because of the metabolic requirements of immune cells and the upregulation of the immune system (Demas et al., 1997; Lochmiller and Deerenberg, 2000). Thus, mounting an immune response results in cost-benefit trade-offs with other nutrient-demanding processes such as reproduction, growth, and thermoregulation (Lochmiller and Deerenberg, 2000).

In two previous studies, we demonstrated that all examined water pythons (*Liasis fuscus*) in our study area, situated in the Northern Territory of Australia, were infected with a hematozoan parasite (*Hepatozoon* sp.) (Ujvari et al., 2004) and that the infection reduced python growth rate, nutritional status, reproductive output, and juvenile survival (Madsen et al., 2005). In the present study, we explore the independent effects of body length/age, parasite load, and nutritional status on water python ability to mount a humoral immune response to a harmless antigen, keyhole limpet hemocyanin (KLH).

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Received 28 February 2005; revised 21 July 2005; accepted 1 September 2005.

MATERIALS AND METHODS

Study species

Water pythons are large (up to 3 m), nonvenomous snakes widely distributed across tropical Australia (Cogger, 1992). The results in the present paper are based on 81 pythons collected in September 2003 at our study site, the Fogg Dam conservation reserve, situated in the lower reaches of the Adelaide River floodplain 60 km south east of Darwin in the Northern Territory of Australia. The snakes were captured at night by spotlighting (on foot or from a slow-moving vehicle). Body length (to the nearest ±1.0 cm), mass (to the nearest ±1.0 g), and sex were recorded. The pythons were kept in separate cages during the immunization period (25 days) and were provided with water and heating in order to be able to achieve optimal body temperatures. However, no food was provided as pythons do not require to be fed when kept in captivity for such a short period as used in the present study (Secor, 2003).

Although the water pythons at our study site show considerable among-individual variation in annual growth rate, python body size still constitutes a robust predictor of python age, i.e., large snakes are older than small snakes (Madsen and Shine, 2000). The body length of the snakes used in the present study ranged from 81 to 202 cm corresponding to an age of 6 months and >15 years, respectively.

Detection of parasites and parasite life cycle

Blood parasites were examined by placing a drop of blood, obtained by cutting off approximately 2 mm of the snakes' tail tip directly onto a glass slide and smearing it with a second slide to produce a one-cell-thick blood layer. The blood smears were air-dried, fixed in methanol, and stained with

Giemsa. Each slide was examined under oil immersion (×100). A total of 2000 red blood cells were analyzed per slide, and infection intensity was quantified as number of infected erythrocytes. To determine whether a single blood sample provided reliable data on parasite load, 15 pythons were sampled twice within a month. The two parasite counts were highly significantly correlated ($r = .99$, $p = .0001$, df = 14), strongly suggesting that the infection level from a single count yields a robust measure of *Hepatozoon* infection level. The morphological similarity among the observed parasites suggests that all belonged to the genus *Hepatozoon*, which was subsequently confirmed by molecular (polymerase chain reaction [PCR]) analysis (Ujvari et al., 2004). This group of protozoa has a heteroxenous life cycle involving merogony and gamogony within the vertebrate host and sporogony within an invertebrate vector (Telford, 1984). Anopheline and culicine mosquitoes, ixodid ticks, and phlebotomine sand flies have all been shown to constitute experimental vectors (Telford, 1984).

Immunization

To measure humoral immunocompetence, pythons were immunized with a harmless, protein antigen, KLH (Sigma Chemical Co., St. Louis, Missouri, USA). KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*), and it is therefore highly unlikely that the pythons have been exposed to this antigen. KLH was used because it generates a robust antigenic response in animals, without causing any adverse reactions such as inflammation (Demas et al., 1997). KLH was emulsified with complete Freund's adjuvant (Sigma Chemical Co.), and 100 µl of the emulsion, containing 100 µg KLH, was injected subcutaneously into the pythons. A preimmunization blood sample (200 µl) was collected prior to immunization, and a primary immunization sample was collected on day 14. On the same day, a second booster immunization of 100 µg KLH emulsified with 50 µl of incomplete Freund's adjuvant was injected into the snakes, and a third blood sample was taken on day 24. Serum was separated by centrifugation and stored at −20°C.

Humoral immune response

Serum concentrations of anti-KLH antibodies were determined using an enzyme-linked immunosorbent assay (ELISA). Ninety-six–well ELISA plates (Corning, Acton, Massachusetts, USA) were coated with 0.5 mg/ml KLH dissolved in sodium bicarbonate buffer and incubated overnight at 4°C. Plates were then washed 5× (ImmunoWash, Model 1575, Bio-Rad Laboratories, Inc., Hercules, California, USA) with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), blocked with 200 µl per well 0.5% nonfat dry milk in PBS-T overnight at 4°C, and after incubation washed 5× with PBS-T. Python serum samples were diluted 1:100, 1:600, 1:3600, and 1:21,600 with PBS-T, and 50 µl was added in duplicate to the wells. Positive controls, pooled serum from all pythons similarly diluted with PBS-T, and negative controls (blanks) from non-immunized pythons were also added in duplicate to each plate. The plates were sealed, incubated at 37°C for 1 h, and then washed 5× with PBS-T. A total of 50 µl secondary rabbit anti-python antibody diluted 1:8000 (Chemicon International, Inc., Temecula, California, USA) was added to the wells, and plates were sealed and incubated at 37°C for 1 h. Plates were again washed with PBS-T, and 50 µl commercial peroxidase-conjugated affinitypure goat anti-rabbit immunoglobulin G diluted 1:5000 (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) was added to the plates. The plates were sealed, incubated, and washed as above. Fifty microliters of 2,2′-azino-bis (3-ethylbenthiazoline-6-sulforic acid) buffer solution (Roche, Co., Basel, Switzerland) was added to each well. Plates were protected from light during the enzyme substrate reaction. The optical density (OD) of the wells was determined using a plate reader equipped with a 405-nm-wavelength filter (Microplate reader, model 680, Bio-Rad Laboratories, Inc.). In order to minimize intra- and interassay variability, the concentration of each sample was expressed relative to the positive control OD, and the mean OD from each set of duplicate wells was calculated using Bio-Rad Microplate Manager Software Version 5.2.1.

Statistical analyses

Both ELISA absorbance values and parasite loads were highly skewed and therefore ln-transformed in order to conform to normality (Shapiro-Wilks's statistics, $p > .05$), before being submitted for statistical analysis. In order to explore the effects of immune response on python nutritional status, we calculated an index of body condition for each snake, using residual scores from a general linear regression of ln-transformed mass on body length (Bonnet et al., 1998; Weatherhead and Brown, 1996). Multiple regression analysis was used to assess the independent effects of body length/age, parasite load, and condition on python humoral response. We report two-tailed probabilities for all analyses.

RESULTS

The pythons responded to immunization by producing specific KLH antibodies (single-factor ANOVA with ln-transformed immune response as a factor, $F_{2,242} = 76.96$, $p = .0001$; Figure 1). However, a post hoc test did not reveal any difference between preimmunization and primary immune responses ($t = 1.14$, $p = .26$, df = 80, paired t-test; Figure 1). In a second post hoc test, we therefore compared the immune responses between preimmunization and secondary immunization, which revealed a significant difference in immune response ($t = 9.25$, $p = .0001$, df = 80, paired t-test; Figure 1). Mean secondary relative antibody titer was approximately five times higher compared to the preimmunization titer (Figure 1), clearly demonstrating that the pythons elicited a strong secondary

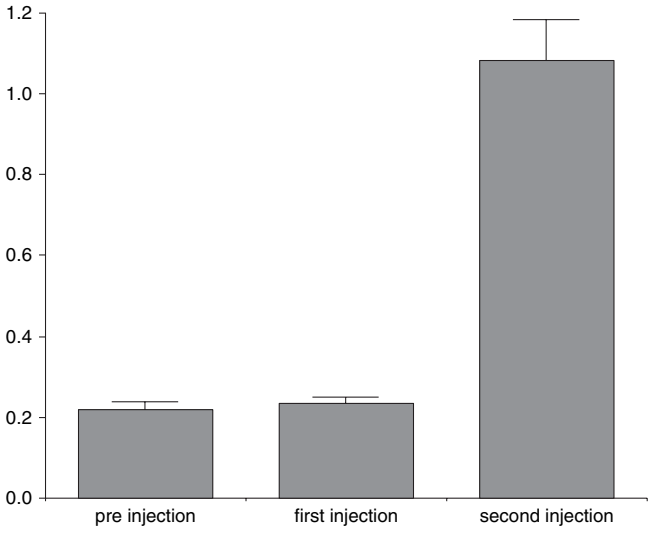

Figure 1
Mean antibody titer (OD) before antigen injection (control), 14 days after first injection, and 10 days after the second injection. Bars denote SEs.

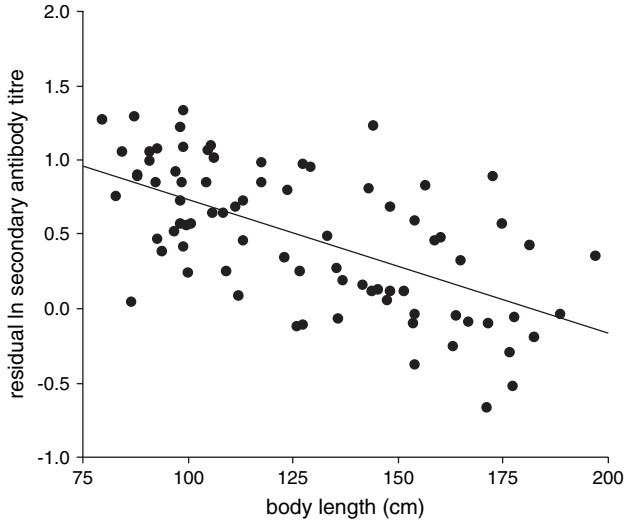

Figure 2
Relationship between python secondary antibody titer (ln-transformed) and body length. The figure is based on residuals derived from multiple regression analysis.

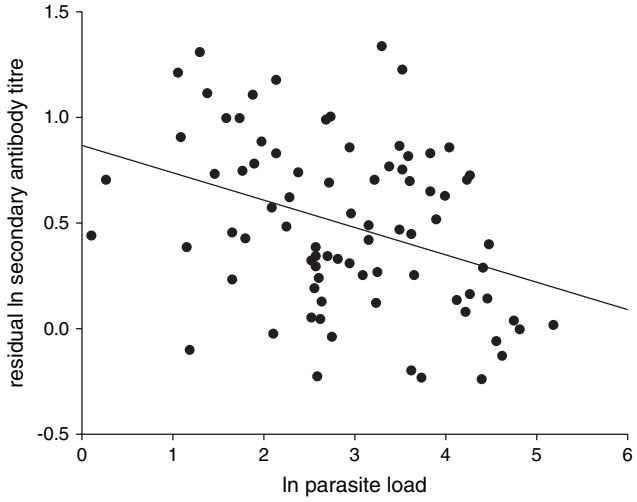

Figure 3
Python secondary antibody titer (ln-transformed) in relation to hematozoan parasite load (ln-transformed). The figure is based on residuals derived from multiple regression analysis.

immune response to the administered antigen. Due to the lack of primary immune response, we only used data from the secondary immunization in our subsequent analyses, where antibody response was defined as the antibody titer in the secondary response minus the antibody titer in the preimmunization sample.

No infected erythrocytes were detected in 2 of the 81 examined stained blood samples. In a previous study, no parasites were observed in 25 snakes (Ujvari et al., 2004). However, by employing *Hepatozoon*-specific primers, PCR amplification revealed that these snakes were indeed also infected by the parasite (Ujvari et al., 2004). Thus, the two snakes in the present study, in which no parasites were detected, were most likely also infected by *Hepatozoon* sp. Mean number of infected erythrocytes of the 81 examined snakes was 31.7 (SD = 37.3, range 0–173).

We used a multiple regression analysis to assess the independent effects of python body length/age, parasite load, and condition on humoral immune response. The overall model was highly significant ($F_{3,80} = 22.05$, $p = .0001$) with body length/age, parasite load, and condition all showing significant, independent contributions to immune response (body length: $F_1 = 46.33$, $p = .0001$; parasite load: $F_1 = 11.25$, $p = .0012$; and condition: $F_1 = 9.90$, $p = .0024$). None of the predictor interactions used in the model were significant (parasites × condition $p = .81$, parasites × body length $p = .88$, and condition × body length $p = .23$). The results from the analyses revealed that python secondary immune response decreased with increasing body length/age (Figure 2), decreased with increasing parasite load (Figure 3), and increased with increasing condition (Figure 4).

DISCUSSION

The lack of an increased immune response in pythons after the primary immunization is not surprising as antibodies produced by the primary antigenic challenge generally have a low average affinity, attain comparatively low titer levels, and have a short plateau phase (Roitt et al., 2001). However, the dramatic difference between the primary and secondary immune response clearly demonstrates that the pythons elicited a strong secondary response to the administered antigen (Figure 1).

A decline in immune function is characteristic of the aging process, resulting in increased susceptibility to infections and age-related changes in the adaptive immune system, including a reduction in clonal expansion and function of antigen-specific T and B cells (Miller, 1996). However, the molecular mechanisms underlying these processes are poorly understood (Johnson et al., 2002). Studies conducted on mice suggest that in aged individuals B cells undergo phenotypic and functional changes, and furthermore, bone marrow production of B cells declines sharply with age (Li et al., 2001; Weklser, 2000). Aging is also associated with a decreased diversity in antibody response, reflected in the loss of high-affinity antibodies (Weklser, 2000).

Although numerous studies have been conducted on the effect of aging in humans and laboratory animals (Ginaldi et al., 2001; Humphreys and Grencis, 2002; Renshaw et al.,

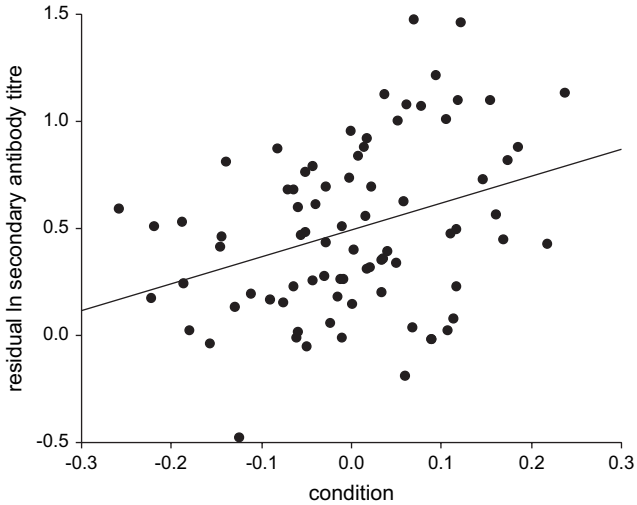

Figure 4
Relationship between secondary antibody titer (ln-transformed) and python condition scores (residuals from ln-transformed mass-length regression). The figure is based on residuals derived from multiple regression analysis.

2002), very little is known about aging of the immune system in wild vertebrate populations. To our knowledge, effects of aging on immune function in the wild have only been studied in collared flycatchers (*Ficedula albicollis*), where old females showed markedly lower humoral immune response compared to younger birds (Cichon et al., 2003). Thus, our results mirror the results reported by Cichon et al. (2003), that is, a decline in humoral immune response with increasing age (Figure 2).

It has become increasingly clear that mounting an immune response against pathogens is associated with physiological costs, which in bumblebees (*Bombus terrestris*) and eiders (*Somateria mollissima*) may even impair subsequent survival (Hanssen et al., 2004; Moret and Schmid-Hempel, 2000). Thus, the age-dependent reduction in humoral immune response in the pythons may have been caused by age-related costs in mounting an immune response. Water pythons in our study area are long-lived, and most snakes will reach an age of >15 years (Madsen and Shine, 2000). However, the age-associated decline in immune function was evident even in small/young snakes (Figure 2). This begs the question, given that the humoral immune response decreases with increasing age, why most pythons have such a long life span?

Older pythons have most likely been exposed to a larger number of different antigens compared to younger snakes. Thus, older pythons may therefore possess a larger immunological experience and may have an enhanced ability to recognize and elicit an immune response to antigens exposed to when young. Furthermore, studies of human age-related differences in humoral immunity have uncovered a paradox: serum levels of immunoglobulins increase with age despite the dramatic decrease in B lymphocytes (Franceschi et al., 1995). Franceschi et al. (2000) proposed that the innate immune system may compensate for an age-related reduction in humoral immune function. We therefore suggest that innate immune functions may counteract the negative effects of the reduced humoral immune response in older pythons.

In an experimental approach to quantify effects of hematozoan parasitemia in blue tits (*Parus caeruleus*), Merino et al. (2000) detected a significant parasite-dependent deterioration in condition of control females but not in medicated birds, strongly suggesting that the parasites affected female nutritional status. In a previous study, we demonstrated that hematozoan parasite load also had a strong negative impact on python nutritional status (Madsen et al., 2005). In the present study, python humoral immune response was also affected by the nutritional status of the snakes, and pythons with low condition scores showed a lower humoral immune response compared to snakes with higher scores (Figure 3). An association between low nutritional status and a reduction in immune function has also been observed in yellow-legged gulls (*Larus cachinnans*, Alonso-Alvarez and Tella, 2001), praire voles (*Microtus ochrogaster*, Demas et al., 2003), and Siberian hamsters (*Phodopus sungorus*, Demas et al., 2003). Furthermore, our results also reveal that pythons with a higher parasite load exhibited reduced humoral immune response compared to snakes with lower infection levels (Figure 4), suggesting that the ability to mount an immune response is also affected by parasite infection level.

Thus, pythons suffering from high parasite infection levels and poor nutritional status showed a reduced humoral immune response, suggesting that mounting an immune response involved energetic costs. Indeed, mounting an immune response resulted in a substantial increase in energy consumption in both mice (Demas et al., 1997) and collared doves (*Streptopelia decaoto*; Eraud et al., 2005). The reduced immune response in pythons suffering from high parasite loads and low nutritional status could therefore be due to an energetic trade-off between immunocompetence and other energetically costly processes (Boots and Bowers, 2004; Lochmiller and Deerenberg, 2000; Nelson and Demas, 1996; Sheldon and Verhulst, 1996). Thus, our results suggest that pythons elicited a reduced humoral response when energetic costs may have surpassed the possible adaptive benefits. In conclusion, in spite of being long-lived, a reduction in immune response was evident even in young pythons, stressing our incomplete knowledge of the immunological and evolutionary significance of age-associated decline in humoral immune function. Furthermore, our results suggest that increased parasitemia and low nutritional status independently reduced python ability to mount a specific humoral immune response, suggesting that mounting an immune response to a novel antigen entails energetic costs.

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