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Characterization of innate immune activity in *Phrynops geoffroanus* (Testudines: Chelidae)

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ABSTRACT. The innate immune activity of the freshwater turtle *Phrynops geoffroanus* (Schweigger, 1812) was investigated, using a sheep-red-blood cell hemolysis assay. The time- and concentration-dependent hemolytic activity of the turtle plasma was low compared to that reported for other reptiles. However the plasma of *P. geoffroanus* exhibited higher activity at elevated temperatures, resulting in temperature-dependent hemolysis. The sensitivity of turtle plasma to temperature could be interpreted as a mechanism by which freshwater turtles use basking behavior to elevate body temperature, thus enhancing the innate immune response. However, we cannot discard the possibility that environmental contaminants could be affecting the turtle's immune response, since the animals in this investigation were captured in a polluted watercourse.

KEY WORDS. Chelonian; immunology; plasma.

Defensive strategies used by the immune system are mainly divided into two distinct but interrelated types of response: acquired immunity and innate immunity. The innate immune response accounts for a substantial portion of the immune system and acts as an initial defense mechanism against microbial growth shortly after infection occurs (MERCHANT *et al.* 2003). The serum complement system, an important portion of the innate immune system, is an ancient mechanism of innate immunity, and is composed of proteins that can be activated to initiate the inflammatory response (DALMASSO *et al.* 1989, MERCHANT & BRITTON 2006).

The innate immune response of reptiles has been addressed in the literature (KAWAGUCHI *et al.* 1978, KOPPENHEFFER 1987, WORK *et al.* 2001, KELLER *et al.* 2005, MERCHANT *et al.* 2005, FREEDBERG *et al.* 2008). It can be measured by several techniques such as lysozyme activity (KELLER *et al.* 2006), mitogen-induced lymphocyte proliferation (WORK *et al.* 2001, KELLER *et al.* 2005, 2006) and hemolysis of sheep-red-blood cells (SRBCs) (MERCHANT & BRITTON 2006, MERCHANT *et al.* 2006a, FREEDBERG *et al.* 2008).

The SRBCs hemolysis assay, used for humans in the clinical setting (LEVINE *et al.* 1953) has recently been adapted to measure the innate immune activity of crocodylians (MERCHANT & BRITTON 2006, MERCHANT *et al.* 2006a) and turtles (FREEDBERG *et al.* 2008). It is a rapid and inexpensive method that is based on the hemolytic disruption of the SRBCs by the immunological pro-

teins in the reptile serum (MERCHANT *et al.* 2006a). The reptile serum does not distinguish the SRBCs from a pathogenic microbe and SRBC are used because they have been shown to elicit a strong immune response from animal serum (ARYA & GOEL 1992, MERCHANT *et al.* 2006a). The hemolytic activity is measured at 540 nm as the hemoglobin is released from the hemolyzed SRBCs (MERCHANT *et al.* 2006a).

The study of immune systems in wildlife has acquired major importance since many diseases can be secondary to a compromised immune system (DUNCAN *et al.* 1975, CRAY *et al.* 2001), and environmental contamination has been associated with increased incidence of immunosuppression in birds, reptiles and marine mammals (ROSS *et al.* 1996, RUIZ *et al.* 2002, KELLER *et al.* 2006).

Phrynops geoffroanus (Schweigger, 1812) is the freshwater turtle with the widest geographical distribution in South America, ranging from the Colombian Amazon to southern Brazil and northern Argentina, living in rivers, streams, and lakes (PRITCHARD & TREBBAU 1984, ERNST & BARBOUR 1989). It is a common species in polluted urban rivers (SOUZA & ABE 2000, BRITES & RANTIN 2004).

This investigation is an evaluation of the innate immune response of *P. geoffroanus*, using the hemolysis of SRBCs assay, and examines the possible relationships between the results and the biology of the turtle.

MATERIAL AND METHODS

Field work was carried out in Piracicaba River basin, in the state of São Paulo, southeastern Brazil. It is a highly developed basin (12,400 km²), with industries and high population densities (KRUSCHE *et al.* 1997, MARTINELLI *et al.* 1999). The main industrial activities in the basin include textile, paper and pulp, sugar and ethanol, metallurgic, food crops, tanning, chemical and fuel refineries (CETESB 2001). The resulting elevated domestic and industrial sewage loads are responsible for the decrease in water quality, causing profound changes in the river's basin (MARTINELLI *et al.* 1999, DANIEL *et al.* 2002).

Twenty four adult turtles (16 males and 8 females) were captured in March 2008 in a region of the Piracicaba River known as Monte Alegre (22°41'75"S, 47°33'58"W), located in the central area of the basin, in the suburban area of the municipality of Piracicaba. We captured turtles by active search (LAGUEUX *et al.* 1995) using a motor boat and hand dip nets. They were kept in plastic containers and brought to the laboratory. Turtles were housed in an indoor pen and blood was collected from the external jugular vein (ROGERS & BOOTH 2004) one day after capture, using a 25 ga needle and a 5 ml syringe. Each blood sample was placed in lithium-heparin tubes, and then pooled. The samples were centrifuged (4000 xg for 20 minutes), and the plasma was collected and frozen until time for use. We used turtle plasma instead of serum because of the higher total volume of fluid obtained with plasma. Turtles were released back into their natural environment after the assay was completed. The animals were captured under Brazilian Environmental Agency (Ibama) license (Proc. 02010.000005/05-61).

Whole blood, obtained from healthy sheep reared at the campus of University of São Paulo, was treated with heparin to prevent coagulation. The blood was centrifuged at 3000 xg and the plasma discarded. The sheep red blood cells (SRBCs) were resuspended in phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 3000 xg. After one more PBS resuspension and centrifuge, the SRBCs were diluted to 2% (v/v) with PBS. The turtle plasma was thawed at room temperature and used for analysis. We evaluated the concentration-, time- and temperature-dependence of turtle plasma to unsensitized SRBCs as previously described (MERCHANT *et al.* 2006a).

The turtle plasma was diluted to different titers using PBS. One ml of turtle plasma, at different titer dilutions, was incubated at room temperature with 1 ml of 2% unsensitized SRBCs (diluted in PBS). The samples were incubated for 60 minutes, except during the kinetic study, and centrifuged at 2000 xg for 20 minutes. Two hundred µl of the resulting supernatant was transferred to a 96-well microtiter plate and the optical density was measured in a standard spectrophotometer at 540 nm to evaluate the percentage of maximum hemolysis or maximum activity. For the kinetic study, 20 ml of turtle plasma (100%) was mixed, at room temperature, with 20 ml of 2% unsensitized

SRBCs (diluted in PBS) and 1 ml aliquots were removed and centrifuged (2000 xg) at various time points. The temperature-dependent study was conducted by preincubating aliquots of PBS and turtle plasma (100%) at specific temperatures for 20 min. The serum samples SRBCs were then mixed and incubated for 60 min at the respective temperatures as previously described (MERCHANT & BRITTON 2006, MERCHANT *et al.* 2006a).

For each SRBC hemolysis assay, two controls were used (the controls contained only turtle plasma and PBS). These control samples were used as a comparison for all other samples, and to observe any spontaneous hemolysis of SRBCs in the absence of turtle plasma. Each sample was analyzed in quadruplicate so that valid statistical results could be obtained. All results represent the means ± standard deviations of four independent determinants. Data were subjected to analysis of variance to determine the statistical differences between groups.

RESULTS

The data in figure 1 shows the concentration-dependent hemolysis of unsensitized SRBCs by plasma from *P. Geoffroanus*. Incubation of increasing concentrations of turtle plasma with SRBCs *in vitro* did not increase hemolytic activity at concentrations lower than 80% (v/v). However, a small amount of hemolysis was observed at 90% turtle plasma, which increased to only 25% ($p < 0.01$) at 100% plasma.

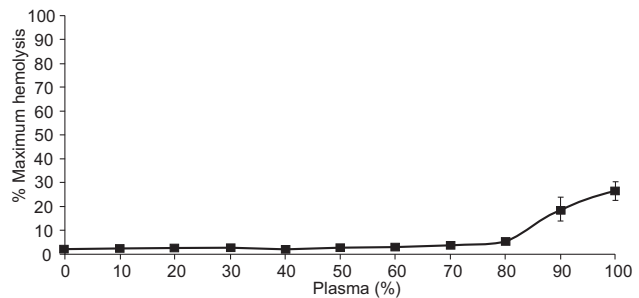


Figure 1. Concentration-dependent hemolysis of SRBCs by freshwater turtle *P. Geoffroanus*. SRBCs were incubated with different concentrations of the plasma from *P. Geoffroanus* for 60 minutes. The samples were centrifuged and the optical densities of the supernatants were measured at 540 nm. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations.

The kinetic analysis of the hemolysis of SRBCs by *P. Geoffroanus* plasma is depicted in figure 2. The plasma showed almost no activity until 10 minutes of exposure to SRBCs. At 15 minutes, hemolysis increases to 8% of maximal activity, and increased ($p < 0.01$) further to only 20% in 30 minutes. Within 60 minutes of exposure to SRBCs, the hemolysis reached 50%.

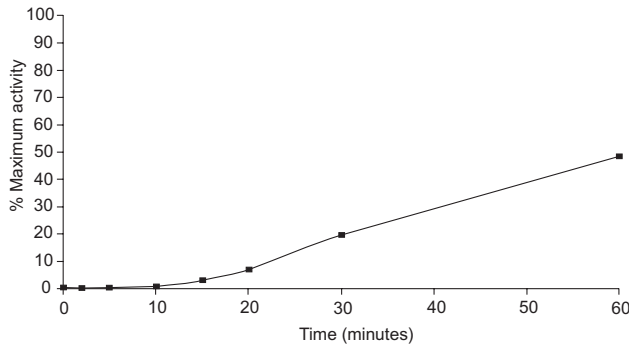


Figure 2. Kinetic analysis of plasma complement activity of the freshwater turtle *P. geoffroanus*. SRBCs were incubated in an equal volume of 100% of turtle plasma for various amounts of time. The samples were centrifuged and the optical densities of the supernatants were measured at 540 nm. Each sample was analyzed in quadruplicate and the results represent the mean \pm standard deviations.

Incubation of turtle plasma with SRBCs at different temperatures resulted in a temperature-dependent hemolysis (Fig. 3). Even at temperatures as low as 5–15°C, the turtle plasma exhibits more than 20% activity. The activity rises ($p < 0.01$) to about 62% at 20°C. At 30°C it reached 100% of maximal activity ($p < 0.01$), and increased to 110% at 35°C and exhibited 100% of maximal activity at 40°C.

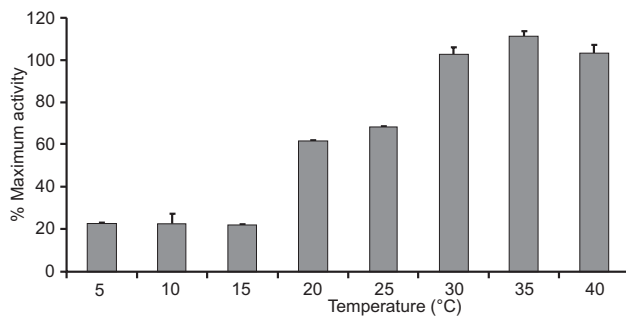


Figure 3. Temperature-dependent hemolysis of SRBCs by plasma of the freshwater turtle *P. geoffroanus*. SRBCs were incubated with 100% turtle plasma at different temperatures for 60 minutes. The samples were centrifuged and the optical densities of the supernatants were measured at 540 nm. Each sample was analyzed in quadruplicate and the results represent the mean \pm standard deviations.

DISCUSSION

The results obtained in this investigation demonstrate that the hemolysis of SRBCs technique, originally devised for crocodylians (MERCHANT & BRITTON 2006, MERCHANT *et al.* 2006a b), can be successfully used for the evaluation of the innate immune system of freshwater turtles.

According to the kinetic and concentration-dependent study, the plasma of *P. geoffroanus* exhibited low hemolytic activity. Investigations of the innate immune systems of the American alligator, *Alligator mississippiensis* (Daudin, 1801); the Australian freshwater, *Crocodylus johnstoni* (Kreff, 1873) and saltwater, *Crocodylus porosus* (Schneider, 1801), crocodiles; and the broad-snouted caiman, *Caiman latirostris* (Daudin, 1801), demonstrated rapid and concentration-dependent activity, resulting in 91% of maximal activity within five minutes for *A. mississippiensis* (MERCHANT *et al.* 2006a) and 88% of activity for the freshwater crocodile at 15 minutes (MERCHANT & BRITTON 2006). In this investigation, we observed only 50% of maximal activity for turtle plasma within 60 minutes (Fig. 2). The concentration-dependent hemolysis of SRBCs of 20% American alligator serum resulted in 90.4% of maximal activity (MERCHANT *et al.* 2006a). Turtle plasma showed less than 10% of activity with 80% of plasma concentration (Fig. 1).

It is possible that the concentration of serum complement proteins, responsible for the innate immune response (MERCHANT *et al.* 2005), is higher in crocodylians than in turtles because of the evolutionary selective pressure on crocodylians. Many members of the Crocodylia are territorial animals that are frequently injured during both intraspecies and interspecies aggression (LANG 1987, VERDADE 1992), and despite the environment they live in, rich in potentially pathogenic microorganisms, their severe wounds heal very rapidly and most often without infection (MERCHANT *et al.* 2006b).

The fact that the kinetic and concentration-dependent analysis demonstrated a low hemolytic activity does not mean that the innate immune response of *P. geoffroanus* is ineffective. The temperature-dependent study showed that at 5°C, turtle plasma exhibited 20% of maximal activity, much higher than crocodylians at the same temperature (MERCHANT & BRITTON 2006, MERCHANT *et al.* 2006a). Turtle plasma hemolysis activity continued to increase, at 20°C activity reached 60% of hemolysis and within 30°C it exhibited 100% of maximal activity (Fig. 3). The elevated activity of turtle plasma, at temperatures as low as 5°C, could be due to the fact that this species lives in tropical regions. This hypothesis is consistent with previous research that has demonstrated that at lower temperatures the innate immune activity of tropical crocodylians is higher than that of temperate species (MERCHANT & BRITTON 2006, MERCHANT *et al.* 2006a).

The fact that the plasma of *P. geoffroanus* showed a better *in vitro* activity associated with temperature rather than time- and concentration- dependence may reflect turtle's reliance on basking to activate their immune response. Basking, an ancestral and common behavior in freshwater turtles (BOYER 1965), has been suggested to act on thermoregulation (AUTH 1975, BOYER 1965, MOLL & LEGLER 1971, SPOTILA *et al.* 1990), by increasing physiological processes (CRAWFORD *et al.* 1983, HAMMOND *et al.* 1988). DODD (1988) suggested that ailing turtles use basking behavior to increase body temperature as a means to increase

the immune system's defenses. MERCHANT *et al.* (2007) demonstrated that LPS-treated American alligators exhibited higher internal body temperatures than uninfected animals, and that the febrile response was behavioral and could not be achieved in the absence of a thermal gradient. Investigation of turtle body temperature while basking demonstrated that *Chrysemys picta* (Schneider, 1783) cloacal temperatures ranged from 26.3 to 30.2°C (average 27.8) (BRATTSTROM 1965), *Trachemys scripta elegans* (Wied-Neuwied, 1839) from 27.2 to 38.0°C (average 30.6) and *Graptemys pseudogeographica* (Gray, 1831) ranged from 28.2 to 39.0°C (average 32.7) (BOYER 1965). These freshwater turtles' basking temperatures seem to fit with our data corresponding to the higher plasma activity levels of *P. geoffroanus* (Fig. 3). Future research should compare concentration- and time-dependent analysis at various temperatures to check if temperature also influences turtle plasma activity in such situations, since concentration- and time-dependent assays were conducted at room temperature in the present study.

Pollution and environmental contaminants have been suggested to suppress innate immunity in sea turtles (KELLER *et al.* 2006). Specimens of *P. geoffroanus* captured for this investigation inhabit the Piracicaba River, which suffers from industrial, agrochemical, and non-treated sewage influences (KRUSCHE *et al.* 1997, MARTINELLI *et al.* 1999). Even though the temperature-dependent results of this investigation showed high hemolysis activities, we cannot ignore the fact that environmental contaminants could have affected the innate immune responses of *P. geoffroanus*. FERRONATO *et al.* (2009) registered a diverse spectrum of potentially pathogenic bacteria in the oral cavity *P. geoffroanus* living in Piracicaba River, and healed boat propeller-induced lesions on the carapace of the animals were common (6 of 10 animals). However, none of the turtles captured had any signs of clinical disease (FERRONATO *et al.* 2009), which can be interpreted as additional evidence that these animals are not immunocompromised in such conditions. According to KELLER *et al.* (2006), the sensitivity to environmental contaminants can vary profoundly between species. Future research should compare the immune activity of *P. geoffroanus* between pristine and anthropogenically altered habitats.

The development of the Piracicaba River basin increased during the 1970's. As turtles are long-lived animals (GIBBONS 1987), the period of time *P. geoffroanus* has been exposed to anthropogenic disturbances such as boat propellers strikes and environmental contaminants is relatively short. Consequently, these factors may not be working as selective pressures to keep turtles with better immune responses alive. It is more likely that the turtles used in this investigation still depend on their ancestral immune system to resist against pathogens and contaminants.

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