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Comparative Biochemistry and Physiology, Part A 148 (2007) 921–925
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Febrile response to infection in the American alligator (*Alligator mississippiensis*)

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Received 23 May 2007; received in revised form 27 September 2007; accepted 27 September 2007
Available online 5 October 2007

Abstract

Temperature probes were inserted into the stomachs of juvenile American alligators (*Alligator mississippiensis*) maintained outdoors at ambient fluctuating temperatures. Internal body temperatures (T_b) were measured every 15 min for two days, and then the alligators were injected with bacterial lipopolysaccharide (LPS), pyrogen-free saline, or left untreated. Alligators injected intraperitoneally with LPS exhibited maximum T_bs 2.6±1.1 °C and 3.5±1.2 °C higher than untreated control animals on days one and two after treatment, respectively. T_bs for these animals fell to within control ranges by day three postinjection. Similarly, mean preferred body temperatures (MPBTs) were significantly higher for LPS-injected alligators on days one (4.2±1.8 °C) and two (3.5±1.6 °C) after treatment. Intraperitoneal injection of heat-killed *Aeromonas hydrophila*, a gram-negative bacterium known to infect crocodilians, resulted in a fever while injection of *Staphylococcus aureus* (gram positive) did not elicit a febrile response. Injection of LPS in alligators maintained indoors in a constant temperature environment resulted in no increase in internal T_b. These results indicate that alligators did not exhibit a febrile response in the absence of a thermal gradient, and suggest that febrile responses observed are probably behavioral in nature.
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Keywords: Fever; Crocodilian; Lipopolysaccharide

1. Introduction

Alligators are ectothermic vertebrates that regulate body temperatures by shuttling between aquatic and terrestrial environments or moving into/out of shaded or sunny areas (Seebacher and Franklin, 2005). Since internal body temperatures of alligators are linked to the temperatures of their environments, the general physiological and biochemical processes of these organisms are dependant on the temperature of their surroundings.

Several studies have revealed that lizards (Bernheim and Kluger, 1976; Firth et al., 1980, Muchlinski et al., 1989) and turtles (Amoral et al., 2002; Monagas and Gatten, 1978) exhibit elevated internal body temperatures in response to pyrogenic challenge. The fevers demonstrated by these ectotherms are thought to be behavioral in nature (Kluger, 1980). However, not all members of the class Reptilia can produce a fever response to infection and inflammation (Laburn et al., 1981; Zurovsky et al., 1987a, 1987b). Lang (1987) showed that the American alligator (*Alligator mississippiensis*) is capable of febrile responses. This study was conducted to further define the febrile response of American alligators to infectious stimuli.

2. Materials and methods

2.1. Chemical and biochemicals

Bacterial lipopolysaccharide derived from *E. coli* 055:B5 and pyrogen-free saline were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). *Aeromonas hydrophila* (7965) and *Staphylococcus aureus* (29247) were obtained from American Type Culture Collection (Manassas, VA, USA).

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1095-6433/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.cbpa.2007.09.016

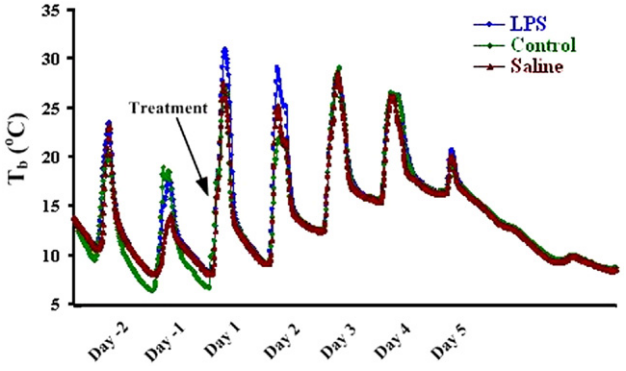

Fig. 1. Thermal regulation curves for alligators treated with bacterial LPS. Thermal probes were placed in captive alligators and, after two days, their internal body temperatures were recorded for two days to measure baseline temperatures. Alligators were injected with LPS, pyrogen-free saline, or left untreated and body temperatures were recorded for another four days. The thermal probes were extracted and the data transferred to a computer for analysis. These data indicate that alligators treated with LPS exhibited higher T_bs on days one and two after injection.

2.2. Treatment of animals

Alligators (*A. mississippiensis*) (128–141 cm in length) were captured at night using a spotlight and a cable noose. The animals were maintained out-of-doors in 3 m × 3 m pens and allowed free access to a pool of 1.25 m^2 of water area (0.75 m depth). The studies were conducted during the months of February (2006 $\bar{x}$ low temp = 7.4 °C, $\bar{x}$ high temp = 20.3 °C, $\bar{x}$ overall temp = 13.8 °C) and March (2006 $\bar{x}$ low temp = 6.6 °C, $\bar{x}$ high temp = 18.0 °C, overall $\bar{x}$ temp = 12.3 °C) (http://www.weather.gov/climate/index.php?wfo=lch). Each experiment was typically scheduled to be conducted after the passage of a low pressure system such that the weather permitted maximal differences between the temperatures of the water and terrestrial environment during the daytime hours. All of the procedures used in this study were approved by the McNeese State University Animal Care and Use Committee.

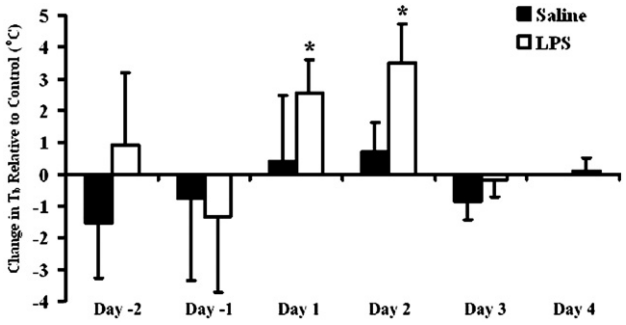

Fig. 2. Effects of LPS on maximum T_b of alligators. Alligators injected intraperitoneally with bacterial LPS exhibit increases in T_b on days one and two after injection. The body temperatures of alligators treated with pyrogen-free saline did not vary significantly from untreated control animals. Results represent the means ± standard deviations of four different alligators per treatment group. *= significantly higher than untreated controls ($p < 0.05$).

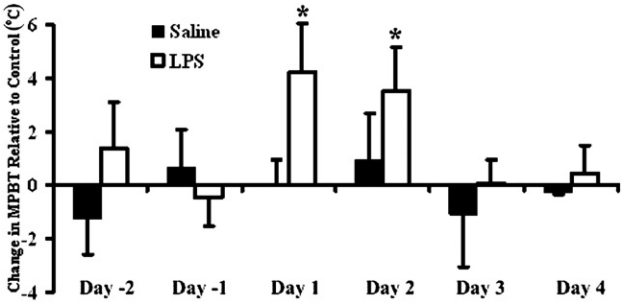

Fig. 3. Effects of LPS on MPBT of alligators. Alligators injected intraperitoneally with bacterial LPS exhibit increases of MPBT on days one and two after injection. The body temperatures of alligators treated with pyrogen-free saline did not vary significantly from untreated control animals. Results represent the means ± standard deviations of four different alligators per treatment group. *= significantly higher than untreated controls ($p < 0.05$).

2.3. Collection of data

Tidbit™ temperature data loggers (Onset Computer Corp., Bourne, MA, USA) were placed nonsurgically into the stomachs of juvenile alligators by gentle insertion through the oral cavity, down the esophagus, and into the stomach. This method of data collection was chosen to eliminate the possibility of infection due to surgical implantation. The loggers were programmed to start collecting data every 15 min, two days after insertion. After two days of baseline thermal data collection, the animals were injected intraperitoneally with a pyrogen, pyrogen-free saline, or left untreated. The potential pyrogens were bacterial LPS (10 mg/kb body mass), *A. hydrophila* (10^7 CFU, heat-killed), or *S. aureus* (10^7 CFU, heat-killed). The bacteria were grown in nutrient broth at 37 °C using standard microbiological methods. Temperature data were typically collected for five days after the treatment. The probes were removed from the animals, cleaned, and the thermal data were transferred to a computer using a Tidbit™ computer coupler.

2.4. Statistics and controls

Each experiment was performed using four animals in each treatment group. The data presented in the thermal regulation plots (Figs. 1 and 6) represent the mean temperatures at each time point for each treatment group. The maximum T_bs (Figs. 2 and 4) and mean preferred body temperature (MPBTs, Figs. 3 and 5) reflect the means ± standard deviations for each treatment group. The results for the T_bs and MPBTs were subjected to analysis of variance using Sheffe's post-hoc comparisons (Tamhane and Dunlop, 2005) to determine the level of significance between the different treatment groups. A value of $p < 0.05$ was considered to be significant.

3. Results

Fig. 1 shows the results of a 7-day thermoregulation study. Thermal curves depicting internal T_b of alligators from all three treatment groups (LPS-treated, saline-treated, untreated) show

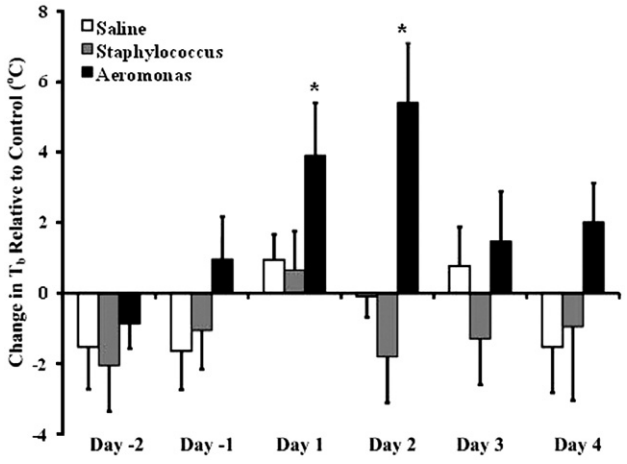

Fig. 4. Effects of *Aeromonas hydrophila* and *Staphylococcus aureus* bacteria on maximum T_b of alligators. Alligators injected with 10^7 CFU of *A. hydrophila* exhibited increases in maximum T_bs on days one and two after injection. However, the maximum T_bs of alligators injected with 10^7 CFU of *S. aureus* or pyrogen-free saline were not statistically different from untreated control animals. Results represent the means±standard deviations of four different alligators per treatment group. *=significantly higher than untreated controls ($p<0.05$).

rapid increases during late morning/early afternoon hours. Temperatures typically began to rise after 11 am and peaked at approximately 2 pm, followed by sharp decreases during late afternoon. The minimal nighttime temperatures ranged from 7–17 °C, while daytime highs typically ranged from 27–33 °C. The daily amplitudes of T_bs ranged from 7–21 °C, which are substantially larger ranges than those observed by Seebacher et al. (2003). Alligators injected with bacterial LPS derived from *E. coli* exhibited a significant increase ($p<0.05$) in T_b relative to controls on day one (2.6 °C) and day two (3.5 °C) after injection (Fig. 2). However, the T_bs of LPS-treated alligators were not

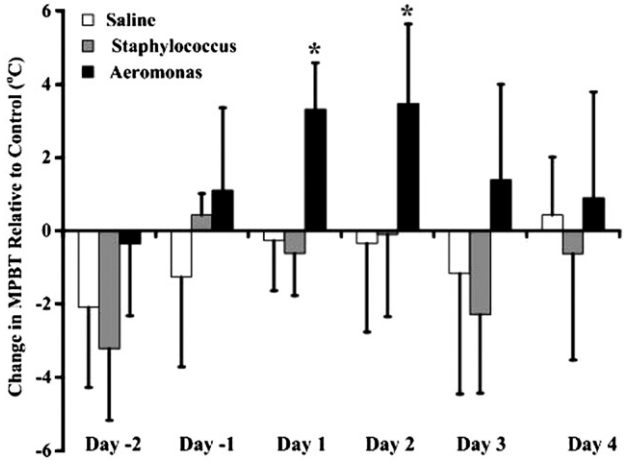

Fig. 5. Effects of *Aeromonas hydrophila* and *Staphylococcus aureus* bacteria on maximum MPBT of alligators. Alligators injected with 10^7 CFU of *A. hydrophila* exhibited increases in MPBT on days one and two after injection. However, the MPBTs of alligators injected with 10^7 CFU of *S. aureus* or pyrogen-free saline were not statistically different from untreated control animals. Results represent the means±standard deviations of four different alligators per treatment group. *=significantly higher than untreated controls ($p<0.05$).

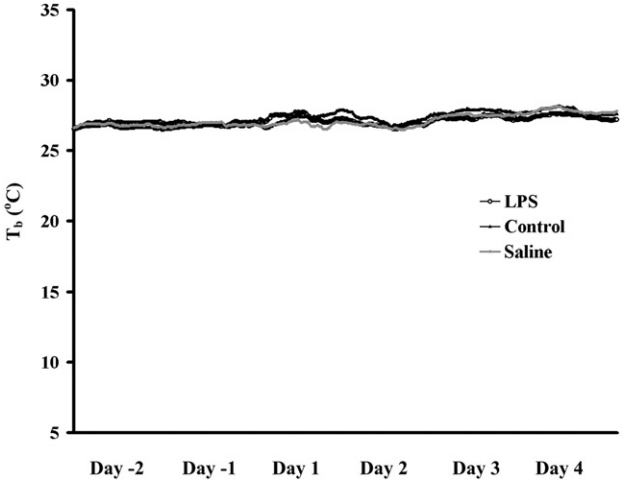

Fig. 6. Effects of LPS on T_b of alligators in a constant temperature environment. Alligators injected with bacterial LPS or pyrogen-free saline and maintained in a constant temperature environment (31 °C) did not exhibit changes in T_b relative to untreated control animals. Results represent the means±standard deviations of four different alligators per treatment group.

statistically different ($p>0.05$) than untreated control animals at any time point before treatment or after day two. Fig. 3 displays the effects of intraperitoneal LPS treatment on the MPBT. LPS induced 4.2 °C and 3.5 °C increases in MPBT on day one and day two postinjection, respectively, relative to untreated controls. The MPBT of LPS-treated animals were not significantly different from control animals before treatment or three-five days after treatment (Fig. 3). In addition, treatment of alligators with pyrogen-free saline did not result in significant ($p>0.05$) changes in T_b or MPBT, relative to untreated control animals, at any time point.

The results of intraperitoneal injection of alligators with heat-killed *A. hydrophila* and *S. aureus* bacteria on alligator internal T_b are displayed in Fig. 4. Immunological challenge with 10^7 CFU of gram-negative *A. hydrophila* (heat-killed) resulted in 3.9 °C and 4.5 °C increases ($p<0.05$) in T_bs on days one and two of treatment, respectively. T_bs of *Aeromonas*-treated animals were not elevated prior to treatment, and returned to control ranges after day two posttreatment. In contrast, treatment with 10^7 CFU of gram positive *S. aureus* (heat-killed) did not significantly change the T_b, relative to untreated control animals. Fig. 5 summarizes the effects of *A. hydrophila* and *S. aureus* bacteria on MPBT. *Aeromonas*-treated animals exhibited MPBTs 3.3 °C and 3.5 °C higher than untreated control animals on days one and two of treatment, respectively. However, treatment with *Staphylococcus* did not significantly alter the MPBT ($p>0.05$) of alligators.

Alligators maintained at constant temperature (30 C) under indoor laboratory conditions did not exhibit a febrile response to bacterial LPS (Fig. 6). Neither maximum T_b nor MPBT were elevated ($p>0.05$) relative to untreated control animals (data not shown).

4. Discussion

Fever can be defined as a regulated elevation of body temperature in response to infection or inflammation. This innate

immune function is thought to be an important adaptive immunological response to infection because of its conservation across a broad diversity of phyla (Kluger et al., 1998). Changes in thermal regulation as a result of immunological challenge have been shown to occur in a wide variety of vertebrates, including teleost fish (Reynolds et al., 1978), amphibians (Bicego-Nahas et al., 2002), reptiles (Ortega et al., 1991), birds (Maloney and Gray, 1998), and mammals (Bernheim and Kluger, 1979). The febrile response has also been shown to occur in invertebrates (Kluger, 1991). Because of its ubiquitous nature across a broad spectrum of phyla, this response is thought to be an ancient innate defense mechanism.

Thermoregulation in reptiles is a complex, and can involve thermoreception of environmental temperatures, changes in physiological and biochemical parameters, and changes in behavior to achieve altered T_bs (Seebacher and Franklin, 2005; Seebacher, 2005). Thermoregulation is important for an effective immune response in reptiles. Increased T_b has been shown to increase the effectiveness of the immune system of snakes to fight parasitic helminth infections (Deakins, 1980). Furthermore, the severity of viral and bacterial infections can be intensified by artificially low T_bs that can hinder the reptilian immune response, or by artificially high temperatures that can increase physiological stress and thus immunosuppress the host (Jacobsen, 1980; Kleese, 1980; Marcus, 1980). T_b is also important for the health and immunity of crocodilians. The results from in vitro studies in our laboratory have shown that innate immunities of *A. mississippiensis* (Merchant et al., 2003), and *Crocodylus johnstoni* and *Crocodylus porosus* (Merchant and Britton, 2006) are temperature-sensitive. Results from in vivo studies have demonstrated that the ability of *A. mississippiensis* to fight infection is optimal near 30 °C (Glassman and Bennett, 1978).

The alligators used were for this study fell within a narrow size window (128–141 cm, 6.3–9.2 kg), which is advantageous for comparisons as it is known that rates of heating and cooling are dependent on sizes of crocodilians (Seebacher et al., 1999). In addition, the rates of heating and cooling are near optimal for crocodilians in this size range (Turner and Tracy, 1986). In addition, the fact that the experiments were conducted during the winter months provided large temperature fluctuations, thereby allowing for ample opportunity to monitor the large daily T_b amplitudes observed in the small alligators (Seebacher et al., 2003). Fig. 1 shows the T_bs of alligators during a seven day febrile response study. The thermal curves show the typical daytime heating and nighttime cooling oscillations observed during the winter months during which this study was conducted. The minimal temperatures typically reflect the temperature of the water during the scotophase, while the scotophase temperature peaks are reflective of the daytime basking temperatures. A notable stepwise change in the minimal temperatures is evident on days 1–3 of the experiment, which is due to the temperature stabilizing after the passage of a low pressure system. These results were typical of the data obtained on the three replicate experiments. The lack of T_b spikes after day five was due to a period of heavy cloud cover, cold temperatures, and rain that persisted for several days and eliminated the opportunity for thermoreguation. Injection of bacterial LPS into alligators caused an elevation of T_b (Figs. 1 and 2) and MPBT (Fig. 3) relative to controls and saline-injected animals. The increases in T_b are consistent with those reported by Lang (1987) for *A. mississippiensis*. LPS is a major component of the outer membrane of gram-negative bacteria. The results from this experiment are consistent with those that showed that LPS challenge induced a febrile response in the box turtle, *Terrapene carolina* (Amoral et al., 2002) and the Cururu toad, *Bufo paracnemis* (Bicego-Nahas et al., 2000).

Injection of LPS is known to elicit an increase in total circulating leukocytes in *A. mississippiensis* (Merchant et al., 2006). These data indicate that alligators are immunologically responsive to bacterial LPS. The results from the present study demonstrate that LPS induces a febrile response in alligators. These studies were conducted in outdoor pens in which the animals were provided free access to water and basking areas, and could therefore thermoregulate. Therefore, these data indicate that alligators show a febrile response to LPS challenge in an environment similar to their natural habitat. It is interesting to note that the rates of heating and cooling, as revealed by the slopes of the increases and decreases of body temperatures (Fig. 1), respectively, were nearly identical for untreated animals, saline-injected, and LPS-injected animals. However, the maximum temperature attained by the LPS-treated animals was significantly higher than that achieved by the two control groups (Fig. 2). Although we did not observe the animals during the studies, we suggest that the LPS-treated animals may have selected basking environments within the study area that maximized their sunlight exposure. Alternatively, the treated animals may have adjusted their orientation to the sun such that increased thermal energy could be absorbed. To eliminate the possibility that social interactions influenced the results of the study, the experiment was repeated with the same animals. In the second experiment, the animals that originally served as untreated controls were injected with LPS, and vice versa. The observation of the same results showed that the elevated T_bs were due to LPS challenge, and not dominance of specific individuals for optimal basking areas.

Injection of alligators with 10^7 CFU of heat-killed *A. hydrophila* resulted in elevated T_bs (Fig. 4) and MPBTs (Fig. 5). Other studies have reported induction of febrile responses in lizards (*Agama agama*, *Scleroporus orcutti*, *Sauromalus obesus*, and *Dipsosaurus dorsalis*) to *Aeromonas* bacteria (Ortega et al., 1991; Muchlinski et al., 1989; Bernheim and Kluger, 1976). The fever response reported in these studies was typically 2–3 °C higher than control animals. The alligators used in the present study exhibited a more intense response (maximum T_bs 4–4.5 °C and MPBTs approximately 3.5 °C higher than controls) than observed in lizards. However, the two-day duration of the response was similar to those measured in lizards. Injection with 10^7 CFU of heat-killed *S. aureus* did not result in increased maximum T_b (Fig. 4) or MPBT (Fig. 5). Although only one gram positive bacterium was used in this study, this result may be an indication that alligators do not respond to gram positive challenges with a febrile response.

Alligators maintained at constant temperature in a controlled laboratory environment did not exhibit increased T_b in response to bacterial LPS (Fig. 6). These data suggest that the febrile response observed in previous experiments may be behavioral in nature. These results are consistent with those reported previously for *S. obesus* (Muchlinski et al., 1989). Although alligators may not have evolved biochemical mechanisms to regulate internal T_b, there may be some mechanism for the immune system to detect exposure to infectious agents and relay messages to the central nervous system, thus allowing for a change in behavior which results in the increased body temperatures observed in this study. It is known that the production of eicosanoids, particularly prostaglandins, can cause vasodilation, and thus increase blood flow thus increasing heat distribution. It is possible that the onset of infection might induce the expression of phospholipases, which could influence subcutaneous eicosanoid levels, thus causing vasodilation and increased blood flow and rates of heating. Alternatively, infection could cause increases in nitric oxide synthesis, which is known to cause vasodilation in reptiles (Axelsson et al., 2001; Broughton and Donald, 2007). Future work in this area will focus on these potential biochemical signaling mechanisms.

Acknowledgements

The authors would like to thank Mr. Dwayne LeJuene for his technical assistance. We thank Mr. W. Parke Moore III of the Louisiana Department of Wildlife and Fisheries for administrative support. The research presented in this study was supported by the McNeese State University Chauvanne–Miller Professorship.

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