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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THERMOREGULATION OF THE BOX TURTLES *Terrapene carolina* AND *Terrapene ornata*

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

José Pedro Sousa do Amaral

Norman, Oklahoma

2001

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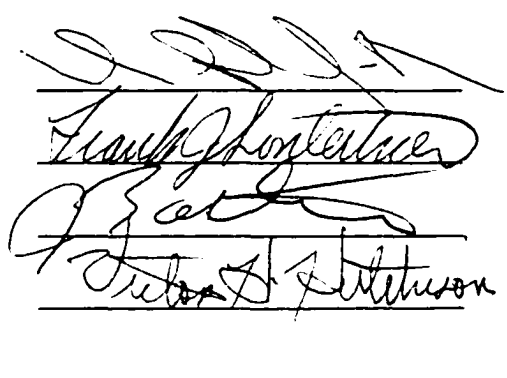
THERMOREGULATION OF THE BOX TURTLES
Terrapene carolina AND Terrapene ornata

A Dissertation APPROVED FOR THE

DEPARTMENT OF ZOOLOGY

BY



ACKNOWLEDGMENTS

I thank Joseph Bastian, Victor Hutchison, Michael Kaspari, Neil Shafer-Ray, and Frank Sonleitner for their suggestions and for their diverse roles as my committee members. I particularly thank Victor Hutchison for his continuous role as a mentor and a supporting friend. I thank Richard Bowker for being a true friend. I apologize to my parents for my being so out of touch. I thank Cari Deen, Dick Durtsche, Shawn Sartorius, and Deborah Lutterschmidt for their help and friendship. And I thank the Junta Nacional de Investigação Científica e Tecnológica for their most excellent support.

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Thermoregulation in two species of closely related box turtles, Terrapene carolina and T. ornata

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To be submitted to the Canadian Journal of Zoology (French abstract and UK English spelling)

Abstract:

Terrapene ornata and T. carolina are closely related box turtles that live in different habitats: grasslands and desert edges, and forested areas, respectively. Considering these species' habitat selection, I predicted that T. ornata would select for higher body temperatures (T_b) and would be a more precise thermoregulator than T. carolina. I recorded time series of cloacal T_b's in thigmothermal linear gradients from acclimatised (LD 12:12; 10, 20° C) box turtles. I used three analytical methods to evaluate and characterise turtles' activity: a ratio-dependent index that measured activity as an indirect function of T_b changes, a comparison of hourly mean variance of T_b (ratio-independent), and autocorrelation. I tested the thermoregulatory differences of active T. carolina and T. ornata with a factorial ANOVA, and characterised the turtles' thermoregulatory cycles with correlograms. Overall, T. ornata had significantly higher mean T_b's than T. carolina. Both species had similar diel thermoregulatory cycles with a period of approximately 24-hr. No clear differences in absolute thermoregulatory precision of T_b's were detected. These species' thermal behaviours were consistent with those reported from field studies, suggesting that there are intrinsically determined differences in thermal preferences that may help explain the different habitat choices.

Résumé:

Terrapene ornata et T. carolina sont des espèces proches des Tortues de cadre qui vivent en différents habitats: zones de prairies et des bords des déserts, et zones de forêt, respectivement. Considérant la sélection d'habitat de ces espèces, je avais présumé que T. ornata choisirait des températures corporelles (T_b) plus élevées et qu'elle serait une thermorégulatrice plus précise que T. carolina. Je avais enregistré, dans des gradients linéaires thigmothermiques, des séries temporelles de T_b cloacales de Tortues de cadre

acclimatées (LD 12:12; 10, 20° C). Je avais employé trois méthodes analytiques pour évaluer et caractériser l'activité de ces tortues: un indice taux-dépendant qui mesurait l'activité comme une fonction indirecte des changements de T_b, une comparaison de variance horaire moyenne de T_b (taux-indépendant), et une autocorrélation. Je avais testé par ANOVA (test factoriel) les différences thermorégulatrices de T_b de T. carolina et de T. ornata actives, et je avais caractérisé les cycles thermorégulateurs des tortues avec des correlogrammes. De façon générale, T. ornata a eu des T_b moyennes sensiblement plus élevées que T. carolina. Les deux espèces ont eu les même cycles quotidiens de thermorégulation avec une période proche de 24-hr. Je n'avais pas détectée aucune différence claire en la précision thermorégulatrice absolue des T_b's. Le comportement thermique de ces espèces était conforme à ceux enregistré dans la nature pour les même espèces, suggérant qu'il y a des différences intrinsèques qui déterminent les préférences thermiques et qui pourraient aider à expliquer les différents choix d'habitat de ces tortues.

Introduction

The differences in morphology and behaviour among closely related species have long intrigued biologists for to understand the manner and extent of such differences is to understand the natural control of biological diversity (Schoener 1974). Closely related species may select different habitats. Habitat selection is extremely important for the survival of species (Reagan 1974). Habitat selection is partially innate and may be reinforced during ontogeny (Klopfer 1962, 1963; Klopfer and Hailman 1967; Wecker 1964). For ectotherms, different habitat selections often imply different thermoregulatory preferences. Reptiles, as many other ectotherms, regulate their body temperature (T_b) within a relatively narrow range by using physiological and behavioural regulation such as shuttling among different thermal microclimates (Cowles and Bogert 1944; Huey 1982; Hutchison 1979).

Terrapene carolina and T. ornata are two species of box turtles that are very closely related phylogenetically (Legler 1960), but occupy different habitats. T. carolina inhabits mesic forested areas, whereas T. ornata occurs in grasslands and desert edges, often in sandy areas, areas that are more xeric than those inhabited by T. carolina (Conant and Collins 1991; Legler 1960). For box turtles, temperature, cover, and moisture are fundamental aspects of the environment (Reagan 1974). This difference in habitat preference may expose the two turtles to different regimens of environmental temperatures. Regarding these regimens, I assumed a fundamental difference in the thermal characteristics of the two species' habitat types at the scale of ectothermic organisms with the size of box turtles. I assumed that the more xeric habitat is overall warmer than the more mesic habitat. In my assumption, that difference in temperature results from differences in the amount and distribution of overhead cover between the two types of habitat.

Box turtles are relatively small, mainly terrestrial, emydid turtles. Terrapene carolina ranges from southern Maine, southern Michigan, and southern Wisconsin southward to Florida and the Gulf of Mexico and westward to south-eastern Kansas, eastern Oklahoma, and eastern Texas. T. ornata ranges from western and southern Illinois, Missouri, Oklahoma, and all but the extreme eastern part of Texas, westward to south-eastern Wyoming, eastern Colorado, eastern and southern New Mexico, and southern Arizona, and from southern South Dakota and southern Wisconsin, southward to northern Mexico (Reagan 1974).

In the central USA, these species' distribution ranges overlap. Turtles from these fringe areas are good subjects to test whether the two species still show different thermal behaviours, even when both wooded areas and grasslands are available. After acclimation, each species' differences in preferred body temperatures (T_b) measured in a laboratory gradient would show an active choice of thermal environment, and thus suggest that the differences in habitat selection in these two species may result primarily from different thermal preferences. Therefore, differences in field T_b's between these species could result mainly from different thermal selections in addition to other selections of habitat features.

Mean body temperature ($\overline{T}_b$) is used frequently as a descriptive measure of the thermal status of a species. However, a single absolute temperature might not be as important to understand thermoregulation as is the temporal sequence of the component individual T_b's. On the other hand, time series are sets of observations recorded sequentially in time (Box et al. 1994). Analyses of time series of thermal data allow for a more complete understanding of the thermoregulation of a given organism, particularly when the organism follows a cyclical thermoregulatory model.

However, the study of combined time series from different organisms can lead to mean time series that are too noisy and thus meaningless. When studying combined time series, noise sources can be as subtle as slight differences in the organisms' cycle synchronicity

that causes individual cycles within the mean time series to be off-phased, or as blunt as having altogether two statistical populations regarding cyclical behaviour. One example of this latter noise source can be when organisms in a gradient either move or do not move, regardless of whether they thermoregulate.

In this study, I used three time series-based analytical methods to characterise turtle activity in the thermal gradients: (1) a ratio-dependent activity index (Hutchison and Spriesterbach 1986) based on relative temperature differentials, (2) a ratio-independent use of variance to measure activity and absolute thermoregulatory precision (sensu Bowker 1984) (although I used variance instead of standard deviation, as a measure of precision), and (3) autocorrelation. Body temperature data for each species were compared considering the following time-related parameters: animals' level of activity as expressed by T_b change, acclimatisation temperature, gender, species, day, and photoperiod. I followed Folk (1974), and Hutchison and Dupré (1992) for the definition of acclimation and acclimatisation (response to a single environmental factor, and to two or more factors, respectively).

I assumed that turtles that live in environments where it is easier to have T_b's above the critical thermal maximum (Lutterschmidt and Hutchison 1997) for the species, would be more precise thermoregulators and more thermophilic: more precise because of the higher probability of thermal death, and more thermophilic because of the higher availability of high temperatures. Therefore, I predicted that Terrapene ornata would select for higher T_b's and would be a more precise (Bowker 1984; Bowker and Johnson 1980; Hutchison and Dupré 1992) thermoregulator than T. carolina.

Materials and methods

I obtained young and adult three-toed and ornate box turtles (Terrapene carolina and T. ornata) in October 1996 from the Oklahoma Department of Wildlife Conservation. These turtles were confiscated from individuals who had collected them from several counties in south-eastern Oklahoma for illegal sale in the pet trade. I also used juvenile box turtles that were hatched and reared in the laboratory (St. Clair 1995). I kept the turtles in three pens (186 cm long, 61 cm wide, and 61 cm high) with sandy soil, a UV light source (General Electric, 20 watts, Black Light), and a heat lamp at one of the ends. I fed the turtles with assorted fruits and vegetables dusted with Reptivite® vitamin mixture and provided water ad libitum. Turtles were code-marked by notching their marginal scutes (Cagle 1939). In this article, I report means plus and minus their 95% confidence intervals.

Turtles were acclimatised on an LD 12:12 photoperiod at either 20.0±1.0 or 10.0±1.0° C for a minimum of 14 days before the experiments. To avoid the postprandial increase of box turtle T_b (Gatten 1974), I did not feed the turtles for 7 days before each experiment, but provided water ad libitum during that period. Experiments were conducted from 6 February to 9 May 1996 and 8 December to 10 May 1997. To measure temperature selection of turtles, I placed animals singly in four linear thigmothermal gradients (209.5 cm long, 16.5 cm wide, and 22.5 cm high) with wood sides and an aluminium plate floor (0.3 cm thick) (Sievert and Hutchison 1988) maintained at temperatures ranging from approximately 6.0±1.5 to 44.0±1.5° C. To produce this range of temperatures, I housed the gradients in an environmental room at 5.0±1.5° C and used a series of heating pads (250 watts) spaced apart to maintain the warm end of each gradient. I controlled the temperature of the heating pads with rheostats to provide a more uniform temperature gradient. Broad-spectrum fluorescent lights (General Electric, 34 watts) were suspended 26 cm above the entire length of each gradient. These lights were on an LD 12:12 photoperiod and provided the

only source of light in the environmental room. Each gradient was covered by clear acrylic plastic that prevented measurable heat from the lights from entering the gradient. In the thermal gradients cover and moisture were kept constant: cover was absent and moisture was kept at about 69% relative humidity.

To measure T_b, I used 32-gauge copper-constantan thermocouples. To prepare the thermally sensitive ends, I entwined the leads at an end of the thermocouple, fused the entwined leads together at the tip, and encapsulated the end with epoxy resin. Approximately 16 hours before recording body temperatures, a previously calibrated thermocouple was inserted about 3 cm into the turtle's cloaca. I used duct tape to secure the exiting thermocouple wire to the turtle's carapace. Cloacal temperatures of Terrapene carolina correlate well with core body temperatures in both shielded and unshielded solar environments (Russo 1972), and thus are good estimations of turtle T_b; I made the same assumption for ornate box turtles. I put each turtle in the middle of a thermal gradient for habituation to the test conditions; each habituation period lasted about 12 hours. Each recorded part of the experiment started at the beginning of the first photophase and lasted 48 hours. I recorded discrete time series of body temperatures at 10-minute intervals with a Model 50 Data Logger (Electronic Controls Design Inc., Milwaukie, Oregon, USA). In addition, I recorded the body mass of each turtle before and immediately after each trial to monitor dehydration.

Species, gender, acclimation temperature, day, and activity were the parameters (or treatments) that defined my statistical treatment blocks of measured turtle T_b's. I was concerned both with the general thermoregulatory behaviour of turtles within each treatment block, and with the time series aspects of that behaviour. I characterised the absolute thermoregulatory precision (turtles with lower s^2 were considered to be more precise thermoregulators than turtles with higher s^2), the cyclical characteristics, and temperature ranges for each species. I determined each animal's activity, as measured by a relative

activity index that measures activity indirectly as a function of changes in body temperature (Hutchison and Spriesterbach 1986):

$$[1] \qquad \% \text{ Relative Activity} = \frac{\sum_{t=1}^{t} \left| T_{b,t} - T_{b,t} \right|}{\sum_{i=1}^{n} \left[\sum_{t=1}^{t} \left| T_{b,t} - T_{b,t} \right| \right]_i} \cdot 100,$$

where T_b is the body temperature read at a given time x, t is the total number of sampled temperatures per animal, and n is the total number of animals per treatment. This index (1) works by comparing each successive absolute temperature differential between sampling events (the numerator) to the sum of all the absolute temperature differentials for that treatment (the denominator). I excluded all turtles that had activity indices lower than the mean activity index for the treatment block to which they belonged (i.e., turtles that were relatively inactive). The sum of the activity indices of all turtles in a given treatment block is always 100%. Therefore, one can assume that if all turtles were to show the same level of activity, then each turtle's activity index could be calculated by dividing the total activity (which is always 100%) by the sample size of that treatment block. This method was deemed necessary because many animals just sat at one place in the thermal gradient, and showed little or no thermal changes during the experiments. I was interested in comparing the active thermoregulation of the two species of turtles and the inclusion of T_b's from such inactive animals would be inappropriate. Although this method reduced in half the useful sample size of each treatment block, it allowed for the unbiased reduction of error caused by inactive animals.

To avoid pseudo-replication, I calculated individual $\bar{T}_b$'s for each turtle, to which each turtle from each treatment contributed once or twice to the treatment mean (twice when day was also considered as a treatment parameter). This approach avoided the problems of pseudo-replicated sample sizes that would be a consequence of averaging time-series

directly. Moreover, I calculated the $\bar{T}_b$ and the variance ($\underline{s}^2$) at each hour during the 48-hr. experiment runs. To calculate these means, I started by synchronising the time series of T_b's of all turtles that were not excluded based on their activity indices. Then, I calculated hourly $\bar{T}_b$ and associated $\underline{s}^2$ from the six source thermal data points recorded for each turtle in the course of one hour. Both individual $\bar{T}_b$ and associated $\underline{s}^2$ were further averaged to produce hourly treatment block $\bar{T}_b$ and mean variance ($\bar{\underline{s}}^2$). I carried over the $\underline{s}^2$ from the temperature sub-sampling, because I wanted an unbiased (and ratio-independent) measure of activity, for I assumed that turtles would have larger $\bar{\underline{s}}^2$ during periods of more movement, and smaller $\bar{\underline{s}}^2$ during periods of less movement. With respect to precision, carrying over the $\underline{s}^2$ from the temperature sub-sampling prevents the inflation of $\underline{s}^2$ that could have occurred as a consequence of different turtles thermoregulating at different temperatures (Hutchison and Dupré 1992). To calculate the 95% confidence intervals for each treatment block hourly $\bar{T}_b$, I used the $\underline{s}^2$ calculated from the individual turtle hourly $\bar{T}_b$. One-way analysis of variance (ANOVA) was used to compare the T_b's partitioned by species, gender, day, and acclimation temperature. Factorial ANOVA was used to compare $\bar{T}_b$'s partitioned by all effects (same as the previous ANOVA with the addition of activity and interactions among the effects). Moreover, the absolute thermoregulatory precision of both species was compared with Wilcoxon Rank Sum tests testing the $\bar{\underline{s}}^2$'s of active and inactive turtles, separated by sex, at both acclimation temperatures. I used this non-parametric test because the distribution of $\underline{s}^2$ was clearly non-normal.

To test the presence and nature of the thermoregulatory cycles, I estimated the autocorrelation function for the time series. In this case, autocorrelation was the correlation of T_b data points with their own lagged values. I changed the lag period ($\underline{k}$) in one-hour intervals between each consecutive series. The most satisfactory estimate of the $\underline{k}^{th}$ lag autocorrelation function is

[2] $$r_k = \frac{c_k}{c_0}$$

where

[3] $$c_k = \frac{1}{N}\sum_{t=1}^{N-k}(z_t - \bar{z})(z_{t+k} - \bar{z}) \qquad \underline{k} = 0, 1, 2, \ldots, \underline{K}$$

is the estimate of the autocovariance, N is the sample size of the time series, and $\bar{z}$ is the sample mean of the time series (Box et al. 1994). For highly random data that have little correlation, the autocorrelation function will drop abruptly to zero, which implies small correlation time. On the other hand, highly correlated data will have a correlation function that varies with $\underline{k}$ but whose amplitude only slowly moves toward zero. Correlograms (plots of the autocorrelation function [1]) were used to measure autocorrelation across 48 non-wrapped around lagged series for each one of the treatment blocks' T_b's.

I used the JMP® statistical package for Macintosh computers (Sall et al. 2000) to analyse my data. I set my α at 0.05. The turtles used in this experiment were housed and tested in accordance with the principles and guidelines of the University of Oklahoma Animal Care and Use Committee (assurance number 73-R-100) and of the Canadian Council on Animal Care.

Results

Overall, Terrapene ornata selected higher T_b than T. carolina across all treatments (Table 1). When the data were not partitioned according to the activity parameter, the treatment $\bar{T}_b$'s were not statistically different. Similarly, daily $\bar{T}_b$ for each gender of each species were not statistically different among treatments (Fig. 1), but the ranges of those grand mean T_b's fell within those described in the literature (Legler 1960). Although non-significant, the means for the second day were consistently lower than those for the first day, with the exception of the $\bar{T}_b$ of male Terrapene ornata acclimatised at 10° C (Fig. 1).

The ratio-independent index of activity showed that the turtles' period of most activity (higher $\bar{s}$) coincided with lower body temperatures, whereas during the period of lowest activity (lower $\bar{s}$) the body temperatures were higher (Fig. 2). Therefore, I studied the body temperatures further partitioned by activity. Moreover, that index also showed that the activity period may be interrupted by transient periods of inactivity that occur around noon and early afternoon. These interruptions showed up more or less clearly on all activity traces for both species at both acclimation temperatures (Fig. 2).

Once the averaged body temperatures were partitioned by activity, I found significant differences among treatments. The factorial ANOVA model explained 60.7% of the variance of $\bar{T}_b$ (Table 2). Day had a highly significant effect on turtle $\bar{T}_b$ (Table 2), where the second-day $\bar{T}_b$ were lower than those during the first day, probably a consequence of the turtles acclimatisation to the gradient itself. Activity had a highly significant effect on $\bar{T}_b$ (Table 2). Inactive animals had higher $\bar{T}_b$ than active animals, which suggested that the turtles while at rest chose places on the gradient with higher temperatures. The interaction between day and activity had no significant effects, which suggested that the turtles did not change the amount of movement from one day to the next. Temperature acclimation had a highly significant effect on turtle $\bar{T}_b$ (Table 2), and animals that were acclimated at lower temperatures chose higher $\bar{T}_b$ on the gradient. There was a significant effect of the interaction between day and temperature acclimation on turtle $\bar{T}_b$ (Table 2), where the cold-acclimatised animals select higher $\bar{T}_b$ in the second day. Moreover, there was a significant effect of the interaction between activity and acclimatisation (Table 2), where the cold-acclimated animals selected higher $\bar{T}_b$ while inactive.

The two species had highly significant differences in $\bar{T}_b$ (Table 2); Terrapene carolina had lower $\bar{T}_b$ than T. ornata. Thermally, the two species did not behave differently from one day to the next; both had higher $\bar{T}_b$ while inactive and lower while active. However, there was a highly significant effect of the interaction between activity and species (Table

2), for while active and inactive T. ornata selected higher $\bar{T}_b$ than T. carolina, the latter had a larger difference between its active and inactive $\bar{T}_b$ than the former. There was a highly significant effect of the interaction between acclimation temperature and species on $\bar{T}_b$ (Table 2), as T. carolina did not seem to be affected by temperature acclimation—the $\bar{T}_b$ were similar for both acclimation temperatures, whereas T. ornata chose higher $\bar{T}_b$ after acclimation at lower temperature.

There was a highly significant effect of gender on $\bar{T}_b$ (Table 2), with females choosing higher $\bar{T}_b$. Furthermore, there was a highly significant effect of the interaction between day and gender (Table 2). Females had higher $\bar{T}_b$ than males during the first day, but about the same $\bar{T}_b$ during the second day. There were no differences in activity or acclimation effects according to gender. Similarly, the interaction between species and gender had no effect on $\bar{T}_b$.

The absolute thermoregulatory precision of Terrapene ornata, as expressed by differences in $\bar{s}$, was significantly different than that of T. carolina only in two instances. T. ornata had highly significantly lower $\bar{s}$ than T. carolina for female turtles active at 10° C (normal approximation Z=-1.94856; p=0.0513), whereas T. carolina had highly significantly lower $\bar{s}$ than T. ornata for female turtles inactive at 20° C (normal approximation Z=-2.36095; p=0.0182).

The autocorrelation results showed cycling for all treatment blocks considering the parameters species, gender, and acclimation temperature (Table 3). Most treatment blocks showed simple dampened sinusoidal correlograms with periods of approximately 24-hr. Male Terrapene carolina acclimated at 10° C and the female T. ornata acclimated at 20° C showed more complex but still sine-based correlograms, with a period of approximately 24-hr. Overall, T. carolina had longer periods (range 24–27 hr.) than T. ornata (range 15–27 hr.) for both acclimation temperatures. The overall aspect of the correlograms (e.g.,

Fig. 3) suggested a diel cycle of activity for all turtles. The dampening (Fig. 3) occurred because I did not wrap around the time series data used to generate the correlograms.

The mean body mass loss in the gradients was 4.88±0.56% of the initial body mass (n=128), part of which due to evacuation of faeces and urine. Evacuation and salivation are important behaviours involved in thermoregulation (Morgareidge and Hammel 1975), but in this study their direct contributions to the turtles' T_b's were not controlled.

Discussion

Only one of my predictions was fully supported by the results of my experiment: Terrapene ornata selected higher temperatures whereas differences in thermoregulatory precision were unclear. It was important to partition the T_b by activity, for both species of box turtles showed an alternation of active and inactive periods during day and night, respectively. During the day, both T. ornata and T. carolina moved about in the gradient, whereas at night they chose a place on the gradient and rested there; this behaviour suggested a predominantly diurnal activity. This type of resting choice was affected by acclimation, as only the animals acclimated at 10° C had rest T_b's higher than their activity T_b's. Perhaps the acclimation at lower temperatures prevents certain physiological needs from being fulfilled and once the turtles are exposed to a range of adequate temperatures, they will thermoregulate to satisfy those needs. For example, this would also explain why there is a statistical effect of day on turtles' T_b, as the physiological needs delayed by acclimation are satisfied in the gradient there is an acclimation to the gradient conditions and an overall decrease of T_b during the second day. However, this overall decrease of T_b during the second day might be associated with water loss minimisation. Turtles use water evaporation to thermoregulate (Morgareidge and Hammel 1975), and the absence of potable

water in the gradient may force the turtles to seek lower temperatures during the second day to conserve water.

Individuals of Terrapene carolina when released in unfamiliar surroundings tend to move unidirectionally (Lemkau 1970), and that could have affected the thermoregulation of these animals in a linear thigmothermal gradient. This characteristic may have affected the thermoregulatory behaviour of animals that were excluded by the ratio-dependent activity index (i.e., animals that moved either to the cold or to the warm end, and stayed there). However, the overall thermoregulatory behaviour of the selected animals was similar enough to that described from turtles observed under natural conditions.

Terrapene carolina prefer wooded areas to open grassland (Smith 1956; Webb 1970). However, T. carolina show a seasonal shift in habitat use from grasslands in late spring and early fall to forested areas in early spring, summer, and late autumn (the activity in grasslands coincided with moderate temperatures and peak moisture conditions) (Reagan 1974). This seasonal shift by T. carolina may have confounding effects in thermoregulation experiments, if the turtles are not acclimatised to known light and temperature conditions. In the summer, T. carolina maintained by thermoregulation a T_b of 26–28° C, emerging early in the day and then shuttling between dense vegetation and appropriate microhabitats when choice existed (a daily temperature cycle resulted from such behaviour) (Russo 1972). The early spring ($\bar{T}_b$=8.97° C) and fall ($\bar{T}_b$=17.11° C) were periods of greatest activity and maximal exposure to solar radiation (Russo 1972). The winter $\bar{T}_b$ was just above freezing (1.47° C) (Russo 1972). In this experiment, the $\bar{T}_b$'s for both the active and inactive portion of the daily cycle of T. carolina were consistent with these field-measured temperatures. The highest gradient-measured inactive and active $\bar{T}_b$ were 25.88 and 24.99° C, respectively. The lowest $\bar{T}_b$ was 19.95° C for active female eastern box turtles acclimated at 10° C (the lowest T_b was 13.73° C for active male eastern box turtles acclimated at 20°C).

In T. ornata, there are two daily activity periods: the first around 11:00 and the second beginning in late afternoon (Legler 1960), perhaps to avoid high temperatures around noon. Because this reduction of activity around noon also occurred in the gradient, it is probably the result of time-based cycling more than a thermally based choice. The laboratorial occurrence of this reduction of activity around noon also suggests that these turtles' thermoregulation is truly circadian—the result of an endogenous timing mechanism. In the field, ornate box turtles prefer higher environmental temperatures, and are not active at T_b below 15° C and seldom active at temperatures below 24° C (Legler 1960). A preferred T_b between 28 and 30° C has been suggested for T. ornata (Fitch 1956; Legler 1960). These observations closely match what I measured for T. ornata in the gradients, with a few differences. The inactive $\bar{T}_b$ for female T. ornata acclimated at 10° C is within the suggested range, but not that of the males nor any of the $\bar{T}_b$'s for turtles acclimated at 20° C. The active $\bar{T}_b$ for both males and females was lower (ranging from 24.46 to 26.80° C), but the lowest T_b for T. ornata was 22.08° C (quite above 15° C).

The absolute thermoregulatory precision of T. ornata, the most thermophilic of the two species, was significantly higher than that of T. carolina in only one instance. Higher absolute thermoregulatory precision has been associated with ectotherms that are comparatively more active and have specialised diets (Bowker 1984), or with ectotherms that live in environments where the critical thermal maximum (Lutterschmidt and Hutchison 1997) is more easily attained. However, in this study no clear differences were measured.

Visual recognition plays an important role in habitat selection (Emlin 1956; James 1971; Miller 1942). The absence of visual clues in the thermal gradients further reinforces the seemingly intrinsical choice of temperatures. In the gradient, physical features of the habitat such as cover did not influence the choice of temperatures by the turtles as they could under natural settings. The similarity between laboratory and field thermal information and behaviour suggested that thermal choice might be the most important feature determining

habitat selection in these two species of box turtles. Moreover, the interspecific differences present even in the acclimated turtles further reinforces thermal choice as an important cause for habitat selection.

Measures of T_b over time often result in an inequality of T_b variances. However, in most cases ANOVA is a sufficiently robust test to overcome the assumption of equal variances implicit in the method (Winer 1971). A common confounding effect in the determination of the preferred T_b of organisms is the cyclical changes in that preference over time (Hutchison and Dupré 1992). Therefore, the partitioning of T_b according to activity resulted in more meaningful comparisons between the temperatures of species with similar cycles of activity. Overall, the major disadvantage of the methods used to divide the turtles according to their activity, is the consequent reduction in sample size per treatments.

Linear thigmothermal gradients, although advantageous for studies where movement is inferred from T_b changes, have a disadvantage in that they tie the vector of animal's movements with the changes of T_b. In short, a change in the animal's position always corresponds either to an increase or to a decrease in T_b (assuming short thermal latency). It would be interesting to compare linear thigmothermal results from those obtained in a circular gradient (thigmothermal, heliothermal, and mixed) where the animal's movement is video recorded, for circular gradients untie the vector of movement from the animal's thermal changes. Therefore, one could perhaps separate the behaviours that result from a thermoregulatory drive from those that result from an ambulatory drive.

The few reported cases of hybridisation between Terrapene carolina and T. ornata (Blaney 1968; Ward 1968) suggest a good opportunity to test the thermoregulation of the hybrids, and perhaps show whether the habitat selection and thermal preferences are genetically based. Moreover, the comparison of the thermoregulation of turtle populations from the northern and southern extremes of the species' ranges might further show the effects of acclimatisation and genotype. This has been done for Terrapene ornata, and

turtles from locations close to the extreme north had an $\bar{T}_b$ 6.3° C lower than that of ornate box turtles from locations closer to the extreme south of this species range (Ellner and Karasov 1993). However, these turtles were not thoroughly acclimatised before being tested (Ellner and Karasov 1993) and thus it is unclear whether the lower temperatures selected by the northern turtles are a result of phenotypic rather than genotypic aptations (sensu Gould and Vrba 1982). The vast literature on physiological acclimatisation and acclimation suggests that inter-population differences determined in the absence of proper "common garden" (a tabula rasa approach to acclimatisation) controls may not be genetically based (Garland and Adolph 1991). Furthermore, inter-population differences, regardless of their genetic or environmental origins, cannot be deemed as adaptations without proper test for the adaptive value of the considered aptations (Garland and Adolph 1991).

Acknowledgements

Part of this work is from a dissertation submitted by the author in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Zoology at the University of Oklahoma, USA. I thank Joseph Bastian, Victor Hutchison, Michael Kaspari, Neil Shafer-Ray, and Frank Sonleitner for their suggestions and review of the manuscript. I thank the Oklahoma Department of Wildlife Conservation and Bob St. Clair for supplying the turtles used in this study. I thank Robin Whitekiller, who helped me care for the turtles. This article was written while the first author received a fellowship from Junta Nacional de Investigação Científica e Tecnológica PRAXIS XXI BD11292/97, Portugal.

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Table 1. Summary mean active body temperature ($\bar{T}_b$) and variance ($\bar{s}$) for the several treatment blocks of the box turtles, Terrapene carolina and T. ornata, tested over a 48-hr. period. Variance is used as a measure of absolute thermoregulatory precision.

Acclimatization	Day	Species	Gender	$\bar{T}_b$(° C)	$\bar{s}$	n
10° C	1	carolina	♀	24.99	1.28	18
			♂	23.96	1.47	9
	1	ornata	♀	26.23	0.62	9
			♂	24.93	1.14	9
	2	carolina	♀	19.95	1.28	‡
			♂	22.81	1.00	‡
	2	ornata	♀	26.66	0.80	‡
			♂	26.80	0.44	‡
20° C	1	carolina	♀	24.11	1.44	19
			♂	22.33	0.59	12
	1	ornata	♀	25.49	0.93	8
			♂	25.67	1.13	9
	2	carolina	♀	23.30	0.94	‡
			♂	21.78	1.32	‡
	2	ornata	♀	24.46	1.58	‡
			♂	24.92	0.27	‡

‡—Same sample size as the corresponding value for the first day

Table 2. Comparison between mean body temperatures ($\bar{T}_b$'s) of box turtles (Terrapene carolina) and ornate box turtles (Terrapene ornata). Factorial ANOVA results for $\bar{T}_b$'s with treatment blocks defined by day (first and second), activity (active and inactive), acclimation (10 and 20° C), species, and gender. DF: degrees of freedom; SS=sum of squares; F Ratio=Fisher's value; p=associated probability.

Source of Variation	DF	SS	F Ratio	p
Day	1	108.64	49.16	<0.001
Activity	1	76.71	35.28	<0.001
Day·Activity	1	4.58	2.11	0.148
Acclimation	1	72.69	33.43	<0.001
Day·Acclimation	1	12.15	5.59	0.019
Activity·Acclimation	1	12.17	5.60	0.019
Day·Activity·Acclimation	1	18.82	8.65	0.004
Species	1	349.16	160.57	<0.001
Day·Species	1	7.26	3.34	0.069
Activity·Species	1	50.51	23.23	<0.001
Day·Activity·Species	1	46.05	21.18	<0.001
Acclimation·Species	1	68.61	31.55	<0.001
Day·Acclimation·Species	1	99.02	45.54	<0.001
Activity·Acclimation ·Species	1	13.58	6.25	0.013
Day·Activity·Acclimation ·Species	1	0.28	0.13	0.719
Gender	1	18.32	8.43	0.004
Day·Gender	1	20.11	9.25	0.003
Activity·Gender	1	3.71	1.71	0.192
Day·Activity·Gender	1	5.77	2.66	0.104
Acclimation·Gender	1	3.31	1.52	0.218
Day·Acclimation·Gender	1	18.16	8.35	0.004
Activity·Acclimation·Gender	1	32.98	15.17	<0.001
Day·Activity·Acclimation·Gender	1	1.89	0.87	0.352
Species·Gender	1	3.22	1.48	0.224
Day·Species·Gender	1	0.73	0.34	0.563
Activity·Species·Gender	1	8.52	3.92	0.049
Day·Activity·Species·Gender	1	4.02	1.85	0.175
Acclimation·Species·Gender	1	46.95	21.59	<0.001
Day·Acclimation·Species·Gender	1	23.92	11.00	0.001
Activity·Acclimation·Species·Gender	1	1.73	0.80	0.373
Day·Activity·Acclimation·Species·Gender	1	3.88	1.78	0.183

Table 3. Duration and profile of the thermoregulatory periods of each treatment block of box turtles, Terrapene carolina and T. ornata, as determined by correlograms of time series of body temperature. Profile is a qualitative description of the correlogram.

Treatment	Period (hr.)	Profile
♀carolina 10°C	27	single sine
♀carolina 20°C	24	single sine
♂carolina 10°C	26	double in-phase sine
♂carolina 20°C	26	single sine
♀ornata 10°C	27	single sine
♀ornata 20°C	15	complex sine
♂ornata 10°C	18.5	single sine
♂ornata 20°C	21	single sine

Legends

Figure 1—Time series of average body temperature ($\bar{T}_b$) and variance ($\bar{s}^2$) for female (♀) and male (♂) Terrapene ornata and T. carolina acclimated at two temperatures (10 and 20° C). The $\bar{T}_b$'s are shown by the solid circles, whereas $\bar{s}^2$'s are shown by the open circles. Black bars indicate the scotophase of the photoperiod of LD 12:12. These turtles were deemed as active thermoregulators (see text for details).

Figure 2—Comparison of mean body temperature ($\bar{T}_b$) of male and female Terrapene ornata and T. carolina acclimated at two temperatures (10 and 20° C). The $\bar{T}_b$'s are shown partitioned by day. Horizontal mid-lines show sample means, black rectangles show the 95% confidence interval of the means, white rectangles show the standard deviations, and vertical lines with terminal ticks indicate the ranges. These turtles were deemed as active thermoregulators (see text for details).

Figure 3—Sample plot of the autocorrelation function (r_k) of the T_b's from males of Terrapene carolina acclimated at 10° C. The dampening is the result of the series not being wrapped around when the autocorrelation functions were calculated.

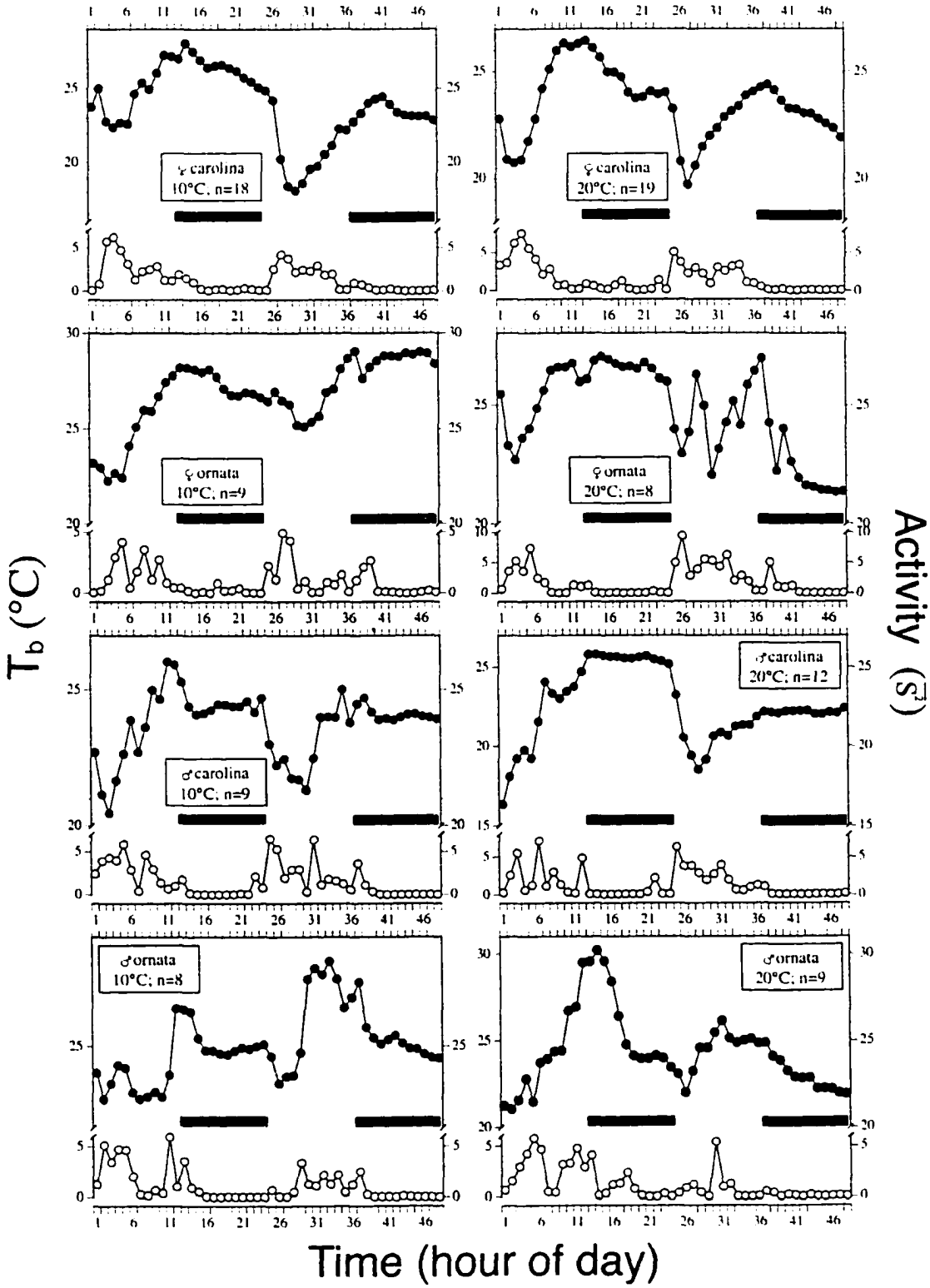
♀ carolina
10°C; n=18
♀ carolina
20°C; n=19
♀ ornata
10°C; n=9
♀ ornata
20°C; n=8
♂ carolina
10°C; n=9
♂ carolina
20°C; n=12
♂ ornata
10°C; n=8
♂ ornata
20°C; n=9
T_b (°C)
Activity (s⁻¹)
Time (hour of day)

Figure 1

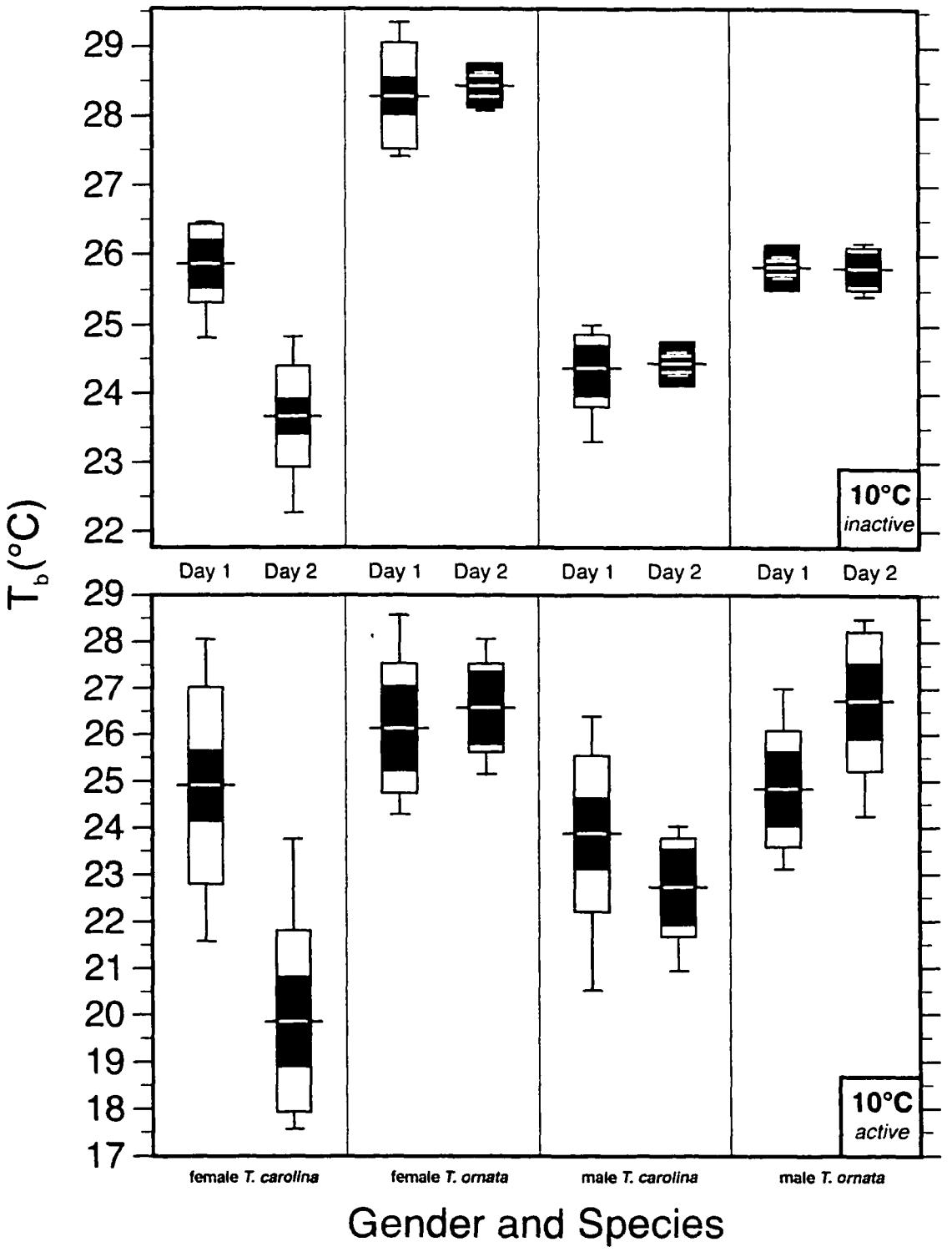
T_b (°C)
29
28
27
26
25
24
23
22
Day 1
Day 2
Day 1
Day 2
Day 1
Day 2
Day 1
Day 2
10°C
inactive
29
28
27
26
25
24
23
22
21
20
19
18
17
10°C
active
female T. carolina
female T. ornata
male T. carolina
male T. ornata
Gender and Species

Figure 2

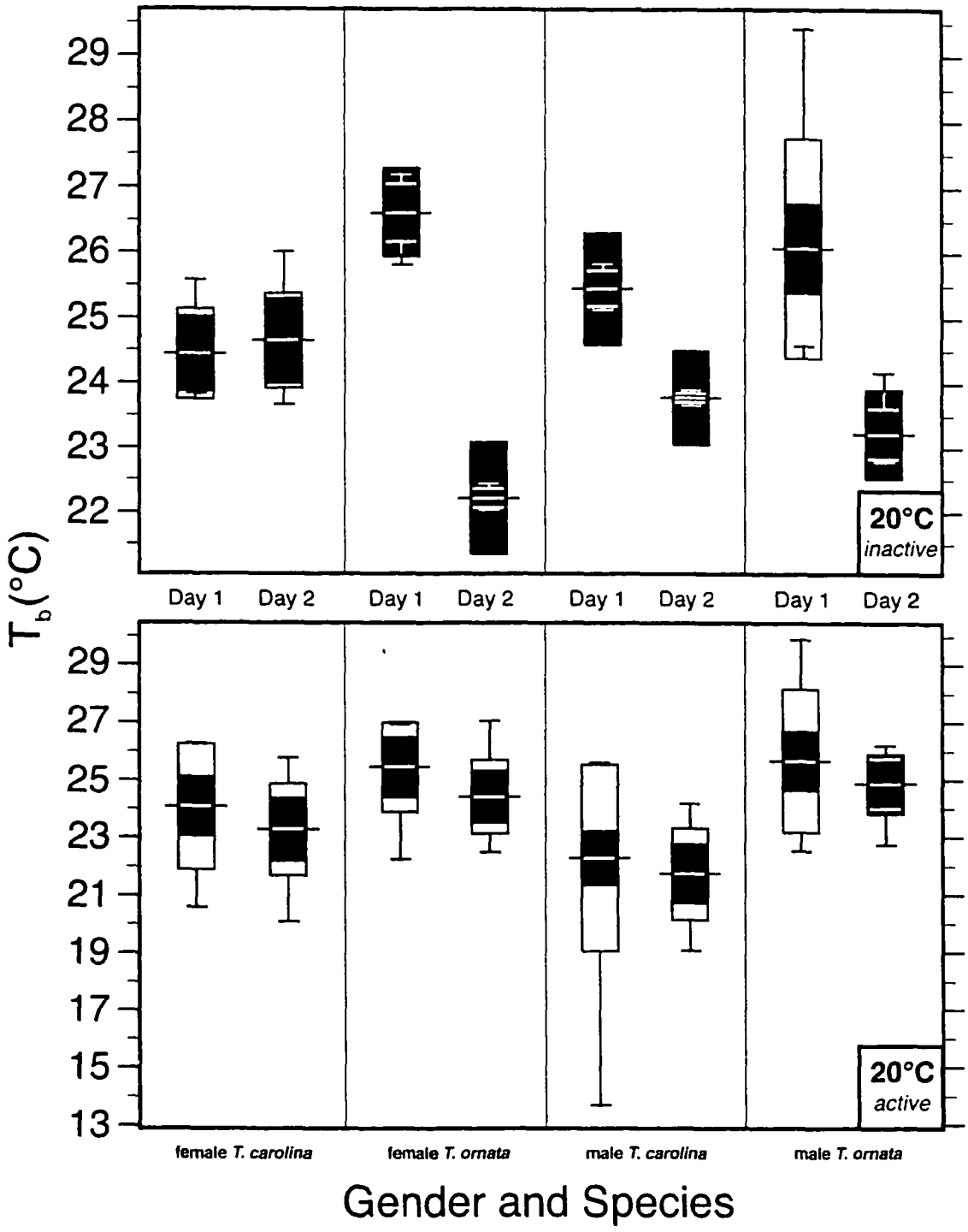

Figure 2 (continued)

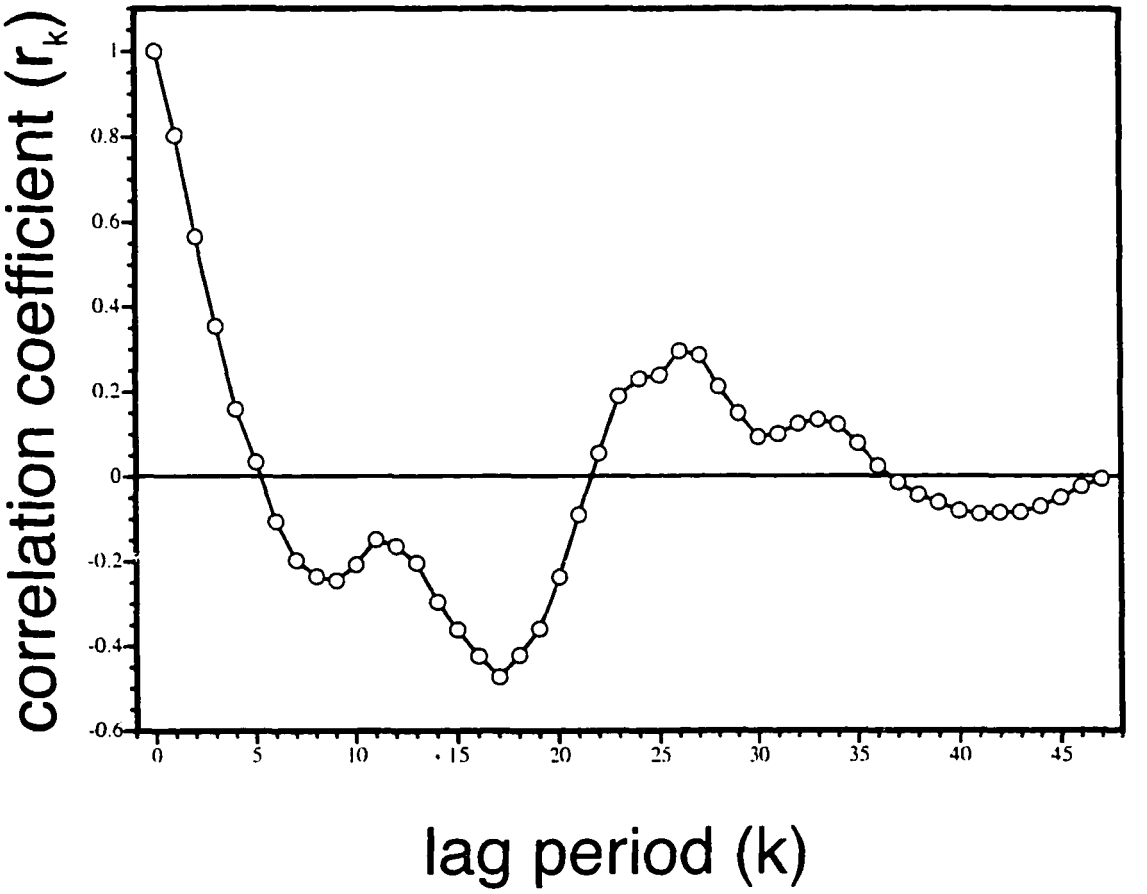
correlation coefficient (r_k)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
0
5
10
15
20
25
30
35
40
45
lag period (k)

Figure 3

The Influence of Bacterial Lipopolysaccharide on the Thermoregulation of the Box Turtle Terrapene carolina

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Running page head: LPS effects on box turtle thermoregulation

To be submitted to Physiological and Biochemical Zoology

Abstract

Ectotherms can adjust their thermoregulatory set points in response to bacterial infection; the result may be similar to endothermic fever. I examined the influence of dose on the set point of body temperature (T_b) in Terrapene carolina. After acclimation to 20° C, I injected postprandial turtles with two doses of bacterial endotoxin (LPS, lipopolysaccharide from Escherichia coli), 0.0025 or 0.025 mg of LPS per gram of non-shell body mass, or with reptilian saline (control group). I placed the animals singly in linear thigmothermal gradients and recorded their T_b's for 48 hours. The turtles showed dose-influenced thermal selection. Turtles injected with the high dose had T_b's significantly higher than control turtles, whereas low dose turtles had T_b's significantly lower than control turtles. Also, there was a daily effect on the T_b of the turtles injected with the high dose. High dose turtles had significantly higher T_b's than the control turtles during the first day, but not during the second. My results support the prediction of Romanovsky and Székely that an infectious agent may elicit opposite thermoregulatory responses depending upon quality and quantity of the agent, and the host health status.

Introduction

Ectotherms can adjust their thermoregulatory set points in response to bacterial infection. This ability to develop fever improves the ectotherms' survival to bacterial infections (Bernheim and Kluger 1976; Kluger 1977; Kluger et al. 1975; Vaughn et al. 1974). By definition, ectotherms do not have autonomous thermoregulation. Therefore to be feverish, infected ectotherms must produce and maintain their fever behaviorally. However different the means to ectothermic fever may be, the results are similar to endothermic fever. Fever does not reflect an inability to regulate core body temperature (T_b), but it is the regulation of T_b at a higher level (Liebermeister 1887; International Union of Physiological Sciences 1987). Therefore, fever is not strictly a hyperthermic state. Fever is also part of a complex physiological defense strategy by the host against invading microorganisms, or against non-microbial agents recognized as foreign by mobile immune cells of the body (Zeisberger 1999).

Fever can be induced by inflammatory mediators (endogenous pyrogens such as prostaglandins and cytokines) released by immune cells activated by contact with foreign molecules (exogenous pyrogens) (Zeisberger 1999). Many multicellular organisms have as part of that complex physiological defense strategy an early response to infection by microorganisms called "acute phase reaction." This reaction comprises changes in the plasma concentrations of trace metals (e.g., iron) (Hacker, et al. 1981) and certain glycoproteins, and the appearance of various peptides in the blood plasma, several of which have been identified as mediators that alter the function of the leukocytic, lymphatic and other systems, and may act as endogenous pyrogens (International Union of Physiological Sciences 1987). Fever is concomitant with the acute phase response. The central nervous system may have the ability to recognize the nature of the infectious challenge through non-thermal neural signals in addition to humoral and thermal-signal

feedback (Székely and Romanovsky 1998), and accordingly activate a defense strategy (Zeisberger 1999). A proposed mechanism for central nervous system recognition of fever is the activation of the subdiaphragmatic vagal afferent nerves by products of liver macrophages, followed by the transmission of such input through noradrenergic pathways to fever-producing sites in the brain, with perhaps prostaglandin E_2 as the ultimate pyrogenic mediator (Blatteis and Sehic 1997).

The thermal responses of animals to injections of a variety of gram-negative bacteria (dead or alive) or extracted pyrogenic bacterial lipopolysaccharide (LPS) have been studied in reptiles, and the results showed both the presence and the absence of febrile responses (Don, et al. 1994). The ubiquity and utility of fever response among reptiles has been questioned, because of the number of species that were afebrile in response to pyrogen injection (Laburn, et al. 1981; Zurovsky, et al. 1987; Hallman, et al. 1990; Muchlinski, et al. 1995).

Even though a few of the reptilian species or groups previously deemed as afebrile now have been shown as capable of developing fever (Monagas and Gatten 1983; Don, et al. 1994; Burns, et al. 1996; Muchlinski, et al. 1999), it still is important to trace the evolution of fever to test its putative adaptive value under the assumption that if fever were an old and adaptive physiological feature then it would be conserved phylogenetically. Turtles were considered the most primitive group among amniotes, and thus the best models for primitive amniote organization and physiology (Rieppel 1999). Despite recent changes (Hedges and Poling 1999; Rieppel 1999) in the relative phylogenetic position of chelonians among reptilian clades, chelonians are interesting models to help trace the reptilian phylogeny of fever because turtles diverged early from squamates (the earliest known fossil turtle is from the Upper Triassic of Germany). Moreover, most studies on reptilian fever were done with squamates as models, thus studies of other groups of reptiles are particularly important.

Terrapene carolina (Linné) (box turtles) are relatively small, mainly terrestrial, emydid turtles that range from southern Maine, southern Michigan, and southern Wisconsin southward to Florida and the Gulf of Mexico and westward to southeastern Kansas, eastern Oklahoma, and eastern Texas (Reagan 1974). This species can develop behavioral fever (Monagas and Gatten 1983).

In comparison to mammals, emydid turtles have little thermal insulation. At 37° C their resting level of heat production is only 6–8% of that of mammals of comparable size (Gatten 1974b). Therefore, if Terrapene carolina is to develop a fever in response to an injection of LPS, then it has to do so behaviorally by moving about in the gradient and selecting appropriate temperatures. In the linear thigmothermal gradients, animal movements are tied to the changes of T_b. Therefore, the thermoregulatory drive and the locomotory drive may conflict if a turtle needs to thermoregulate more precisely, as potentially is the case of the animals injected with pyrogen. For example, if a turtle moves in that type of gradient then its T_b will change, and the drive to move will conflict with the drive to thermoregulate precisely. However, linear thigmothermal gradients by the same reason also allow the extrapolation of animal activity based on T_b changes.

Mean body temperature ($\bar{T}_b$) is used frequently as a descriptive measure of the thermal status of a species. However, a single absolute temperature might not be as important to understand specific thermoregulation as is the temporal sequence of the component individual T_b's. Time series are sets of observations recorded sequentially (Box, et al. 1994). Analyses of time series of thermal data allow for a more complete understanding of the thermoregulation of an organism, particularly when the organism follows a cyclical thermoregulatory model.

To test what kind of response the turtles would show to an increasing dose of pyrogen, I examined the influence of three dosages of LPS (0.000, 0.0025, and 0.025 mg of LPS per gram of non-shell body mass dissolved in sterile reptilian saline) on the set point of T_b

and on the thermoregulatory cycle of Terrapene carolina. I followed Folk (1974), and Hutchison and Dupré (1992) for the definition of acclimation and acclimatization (response to a single environmental factor, and to two or more factors, respectively); and followed the glossary of terms for thermal physiology (International Union of Physiological Sciences 1987). I used two time series-based analytical methods to characterize turtle activity in the thermal gradients: (1) variance as a measurement of activity and absolute thermoregulatory precision (Bowker 1984) and (2) autocorrelation as a measurement of cycling.

Material and Methods

I obtained young and adult three-toed box turtles (Terrapene carolina triunguis) in October 1996 from the Oklahoma Department of Wildlife Conservation. These turtles were confiscated from individuals who had collected them from several counties in southeastern Oklahoma for illegal sale in the pet trade. I also used juvenile box turtles that were hatched and reared in the laboratory (St. Clair 1995). I kept the turtles in three pens (186 cm long, 61 cm wide, and 61 cm high) with sandy soil, a UV light source (General Electric, 20 watts, Black Light), and a heat lamp at one of the ends. I fed the turtles with assorted fruits and vegetables dusted with Reptivite® (Zoo Med, San Luis Obispo, California, USA) vitamin mixture and provided water ad libitum. Turtles were code-marked by notching their marginal scutes (Cagle 1939). In this article, I report means plus and minus their 95% confidence intervals.

Turtles were acclimatized on an LD 12:12 photoperiod at 20.0±1.0° C for a minimum of 14 days before the experiments. To avoid the postprandial increase of box turtle T_b (Gatten 1974a), I did not feed the turtles for 7 days before each experiment, but provided water ad libitum during that period. Experiments were conducted from 28 May to 12 August 1997.

To measure temperature selection of turtles, I placed animals singly in four linear thigmothermal gradients (209.5 cm long, 16.5 cm wide, and 22.5 cm high) with wood sides and an aluminum plate floor (0.3 cm thick) maintained at temperatures ranging from approximately 6.0±1.5 to 44.0±1.5° C. To produce this range of temperatures, I housed the gradients in an environmental room at 5.0±1.5° C and used a series of heating pads (250 watts) spaced to maintain the warm end of each gradient. I controlled the temperature of the heating pads with rheostats to provide a more uniform thermal gradient. Broad-spectrum fluorescent lights (General Electric, 34 watts) were suspended 26 cm above the entire length of each gradient, thus keeping light intensity constant throughout the thermal gradients. These lights were on a synchronized LD 12:12 photoperiod and provided the only source of light in the environmental room. Each gradient was covered by clear acrylic plastic that prevented measurable heat from the lights from entering the gradient. Cover and moisture also were kept constant in the thermal gradients: cover was absent and moisture was kept at about 69% relative humidity.

To measure T_b, I used 32-gauge copper-constantan thermocouples. To prepare the thermally sensitive ends, I stripped off about 2 cm of the insulation plastic, entwined the leads at an end of the thermocouple, fused the entwined leads together at the tip, and encapsulated the end with epoxy resin. Approximately 15 hours before recording body temperatures, a previously calibrated thermocouple was inserted about 3 cm into the turtle's cloaca. I used duct tape to secure the exiting thermocouple wire to the turtle's carapace. Cloacal temperatures of Terrapene carolina correlate well with core body temperatures in both shielded and unshielded solar environments (Russo 1972), and thus are good estimations of turtle T_b. Then, I put each turtle in the middle of a thermal gradient for habituation to the test conditions; each habituation period lasted about 12 hours.

At the beginning of the first photophase after habituation to test conditions, I gave each turtle an intraperitoneal injection of either sterile reptile saline (control treatment) or

endotoxin (LPS from Escherichia coli; Sigma, St. Louis, Missouri, USA) dissolved in reptile saline (experimental treatment). Escherichia sp. have been isolated from reptilian abscesses (Frye 1973) and thus are a known reptilian pathogen. Moreover, the use of purified LPS instead of bacteria (dead or alive) allows for the preparation of more precise dosages, thus allowing for better comparisons of results from different experiments.

Individuals in the first experimental group (n=17) received 0.0025 mg of endotoxin per gram of non-shell body mass (delivered in 0.375-mg LPS per ml of solution). Because the dry shell mass of this species is approximately 25% of the total body mass (Marvin and Lutterschmidt 1997), I used a value of 75% of body mass to determine the amount of solution to inject in each individual. Based upon its non-shell body mass, I injected each turtle in the control group (n=17) with a volume of reptile saline equivalent to the volume of fluid injected into experimental turtles. Sixteen individuals from the control group then were re-tested (at least 7 days later) as an experimental group with a higher concentration of injected endotoxin. Individuals in this second experimental group (n=16) received 0.025 mg of endotoxin per gram of non-shell body mass (delivered in 3.75-mg LPS per ml of solution). I used a repeated-measures design for the animals treated with the high dose of LPS because of time limitations imposed by this species' annual activity cycle. Terrapene carolina shifts seasonally its habitat use from open areas, such as grasslands, in late spring and early fall to forested areas in early spring, summer, and late autumn (the activity in grasslands coincides with moderate temperatures and peak moisture conditions) (Reagan 1974).

Day (first and second), activity (active and inactive), time of day (1–24), treatment (low dose, high dose, and control), and, in the repeated-measures design, subjects (the 15 turtles tested under both control and high dose treatments), were the parameters (independent variables) that defined the statistical treatment blocks of measured turtle T_b's. Considering the diel patterns of box turtle thermoregulation seen elsewhere (do Amaral,

unpublished data), I defined the treatment block activity as coinciding with photoperiod. Therefore, turtles during photophase were considered to be active, and during scotophase inactive. I characterized the absolute thermoregulatory precision (turtles with relatively smaller s^2 were considered to be precise thermoregulators) (Hutchison and Dupré 1992), the cyclical characteristics, and temperature ranges for each treatment. I was interested both in the general thermoregulatory behavior of turtles within each treatment block, and in the time series aspects of that behavior.

Following injection, I recorded discrete time series of body temperatures at 10-minute intervals with a Model 50 Data Logger (Electronic Controls Design Inc., Milwaukie, Oregon, USA). Each recorded part of the experiment started at the beginning of the first photophase and lasted 48 hours. The turtles were not disturbed during the experimental runs. In addition, I recorded the body mass of each turtle before and immediately after each trial to monitor any excessive dehydration.

In a previous study of active thermoregulation of box turtles (do Amaral, unpublished data), I determined each animal's overall activity with an activity index that indirectly measures activity as a function of changes in body temperature (Hutchison and Spriestersbach 1986). The exclusion was deemed necessary because some animals just sat at one place in the thermal gradient, and showed little or no thermal changes during the experiments. I was interested in comparing the active thermoregulation of two species of box turtles and the inclusion of T_b's from such inactive animals would have been inappropriate. In the present study, the situation was some somewhat different. Because I injected into the turtles an agent that modifies the thermoregulation of ectotherms, exclusive examination of the thermoregulation of active turtles could have been a biased approach. Therefore, I decided to analyze turtle T_b's without excluding any animals. The major assumption behind this decision was ignorance of the allocated time to thermoregulation by

the turtles during disease; if a turtle were to allocate most of its time to thermoregulation during disease, then immobility could be a thermoregulatory behavior.

The individual turtle thermal data were processed in steps, from each turtle's run time series to the final treatment blocks' means and associated statistics. To avoid pseudo-replication, I calculated individual mean body temperatures ($\bar{T}_b$'s) for each turtle, to which each turtle from each treatment contributed once or twice to the treatment mean (twice when day was also considered as a treatment parameter). Moreover, I calculated the $\bar{T}_b$ and the variance (s^2) at each hour during the 48-hr. experiment runs. To calculate these means, I started by calculating hourly $\bar{T}_b$ and associated s^2 from the six source thermal data points recorded for each turtle in the course of one hour (Fig. 1). Both individual $\bar{T}_b$ and associated s^2 were further averaged to produce hourly treatment block $\bar{T}_b$ and mean variance ($\bar{s}^2$) (Fig. 1). I carried over the s^2 from the temperature sub-sampling, because I wanted an unbiased measure of activity, for I assumed that turtles would have larger $\bar{s}^2$ during periods of more movement, and smaller $\bar{s}^2$ during periods of less movement (Fig. 1). With respect to precision, carrying over the s^2 from the temperature sub-sampling prevents the inflation of s^2 that could have occurred as a consequence of different turtles thermoregulating at different temperatures (Hutchison and Dupré 1992). Two factorial ANOVA were used to compare $\bar{T}_b$'s partitioned by all effects (with the addition of interactions among the effects). A factorial ANOVA to compare the low dose turtles to the controls, and a repeated measures factorial ANOVA to compare the high dose turtles and the control. The latter factorial ANOVA accounted for repeated measures by the use of the random term in the model (in this case subject turtles) as the error term instead of the residual error (Sall and Lehman 1996). Moreover, the absolute thermoregulatory precision for the three treatments was compared with two Kruskal-Wallis tests: one testing the $\bar{s}^2$'s of animals during their activity period and the other those of animals during their inactivity period.

To test the presence and nature of the thermoregulatory cycles, I estimated the autocorrelation function for the time series. In this case, autocorrelation was the correlation of T_b data points with their own lagged values. I changed the lag period ($\underline{k}$) in one-hour intervals between each consecutive series. The most satisfactory estimate of the $\underline{k}^{th}$ lag autocorrelation function is

[1] $$r_k = \frac{c_k}{c_0}$$

where

[2] $$c_k = \frac{1}{N}\sum_{t=1}^{N-k}(z_t - \bar{z})(z_{t+k} - \bar{z}) \qquad \underline{k} = 0, 1, 2, \ldots, \underline{K}$$

is the estimate of the autocovariance, N is the sample size of the time series, and $\bar{z}$ is the sample mean of the time series (Box, et al. 1994). For highly random data that have little correlation, the autocorrelation function will drop abruptly to zero, which implies small correlation time. On the other hand, highly correlated data will have a correlation function that varies with $\underline{k}$ but whose amplitude varies smoothly. Correlograms were used to measure autocorrelation across 47 lagged series. I used the JMP statistical package for Macintosh computers (SAS Institute Inc 1997) to analyze my data. I set the experimental α at 0.05. The turtles used in this experiment were housed and tested in accordance with the principles and guidelines of the University of Oklahoma Animal Care and Use Committee (assurance number 73-R-100).

Results

The two dose experiments caused opposite changes in the turtles' T_b (Table 1). When compared with the 17 control turtles, the 17 treatment turtles injected with the low dose

(0.0025 mg of endotoxin) selected lower T_b in the gradient (behavioral anapyrexia). However, when 16 of the 17 control turtles from the previous experiment were injected with the high dose (0.025 mg of endotoxin) they selected higher T_b (behavioral fever) (Table 1). In experiment one, the treatment group injected with the low dose of LPS showed behavioral anapyrexia and decreased its $\bar{T}_b$ by about 2° C during both days (Table 1). Terrapene carolina showing behavioral fever increased their $\bar{T}_b$ by about 1 °C (Table 1). The factorial ANOVA model used to compare the time series of low dose and control turtles explained 86.44% of the variance of $\bar{T}_b$, and the repeated measures factorial ANOVA model used to compare high dose and control animals explained 61.67%.

Day had a highly significant effect on turtle $\bar{T}_b$ (Table 2, Table 3). During the second day of the experiment turtles from each treatment block had lower $\bar{T}_b$ than during the first day (Fig. 2). During the second day, the $\bar{T}_b$'s of turtles injected with the high dose were similar to the $\bar{T}_b$'s of the control treatment turtles (Fig. 2), whereas low dose turtles kept lower $\bar{T}_b$'s.

Activity had no statistical effect on $\bar{T}_b$ (Table 2, Table 3). Therefore, turtles maintained T_b similarly through the photoperiod. Moreover, there was no significant effect of the interaction between day and activity on turtle $\bar{T}_b$ (Table 2, Table 3), which suggested that in each dose treatment turtles moved similarly on both days. Consequently, the overall decrease of T_b from the first to the second day, is the result of a change in thermal selection more than a change in activity.

The two experiments showed different effects on their populations. The dose treatment had a highly significant effect: low dose had lower $\bar{T}_b$ (Table 2) and high dose turtles had higher $\bar{T}_b$ (Table 3). There was no statistical effect of the interaction between day and low dose (Table 2), whereas the same interaction was highly significant for the high dose animals (Table 3). These results suggest that the LPS effects on turtle thermoregulation last

about one day for the high dose and more than one day for the low dose. During the second day, high dose and control turtles had similar thermoregulatory patterns, whereas low dose turtles maintained relatively lower $\bar{T}_b$ than the other two treatments. The interaction between activity and dose had a significant effect on low dose turtle $\bar{T}_b$ (Table 2), but not on high dose (Table 3). Therefore, turtles injected with LPS had altered movement patterns (Fig. 2). The low dose-injected animals were active only during the first part of the photophase, and even then less active than the other two groups (Fig. 2). The controls remained active at similar levels throughout the photophase of both days (Fig. 2). There was a highly significant effect of the interaction among day, activity, and dose on turtle $\bar{T}_b$ (Table 2, Table 3).

Hour of day had a significant effect in the low dose comparison (Table 2), but not in the high dose (Table 3). This again reflects the merging of the thermoregulatory behavior of high dose-injected animals with that of controls during the second day, and the absence of such convergence among the low dose-injected animals (Fig. 2). Reflecting turtle variability, subjects had a highly significant effect on turtle $\bar{T}_b$.

Both low and high dose-injected Terrapene carolina had higher absolute thermoregulatory precision (as expressed by smaller $\bar{s}$). When all turtles were considered, both treatment blocks of turtles injected with LPS had highly significant lower $\bar{s}$ than the saline-injected turtles for both active (Kruskal-Wallis $X^2=16.21$; $p=0.0003$) and inactive (Kruskal-Wallis $X^2=7.02$; $p=0.0299$) periods.

The autocorrelation results showed no diel cycling for any of the three treatment blocks (Fig. 3). Even the treatment block injected with saline showed disruption of its diel cycling. In a different study, similarly acclimatized turtles showed simple dampened sinusoidal correlograms with periods of approximately 24-hr (Fig. 3) (do Amaral, unpublished data).

The mean body mass loss in the gradients was 3.10±0.37% (n=59), part of which was due to evacuation of feces and urine. Evacuation and salivation are important behaviors involved in thermoregulation (Morgareidge and Hammel 1975), but in this study their direct contributions to the turtles' T_b's were not controlled.

Discussion

The injection of LPS changed the thermoregulatory behavior of acclimatized Terrapene carolina. The low dosed turtles selected lower T_b's than the controls, and the high dosed turtles selected higher T_b's than controls. In addition, the controls turtles (injected with sterile saline) showed a thermoregulatory pattern different than that seen elsewhere (do Amaral, unpublished data) in the same species of turtles similarly acclimatized (Fig. 3).

Injection of saline, regardless of whether carrying LPS, caused a change in the thermoregulation of Terrapene carolina. Although stress alone may fail to induce T_b changes under some circumstances (Cabanac and Laberge 1998), it frequently causes emotional T_b changes (usually fever) in ectotherms (Casterlin and Reynolds 1980; Cabanac and Gosselin 1993) and in endotherms (Briese, et al. 1991; Urison, et al. 1993; Wachulec, et al. 1997). Therefore, it is not surprising that the injection of an inert liquid would cause a thermoregulatory response in T. carolina. In contrast, the disruption of the diel cycling of T_b was unexpected. The decrease in $\bar{T}_b$ in all treatments from the first to the second day, can be explained as the turtles' acclimatization to the conditions of the gradient. Perhaps the absence of a diel cycle is a consequence of seasonal effects on the thermoregulation of T. carolina, for this study took place in the summer, whereas the study where the diel cycles were seen took place in the winter and spring (do Amaral, unpublished data). Terrapene carolina has a seasonal shift in habitat use (Reagan 1974), and that is why I interrupted the study at the end of the summer.

The high dose-injected turtles recovered to normothermy at least one day sooner that the low dose-injected animals. High dose turtles also maintained a behavioral fever during the first day, whereas low dose animals maintained a behavioral anapyrexia during both days. Therefore, the high dose turtles recover faster from the injection of toxin than the low dose animals, perhaps the warmer bodies of the high dose turtles were able to detoxify the LPS faster. The control animals were less precise than either of the LPS treated turtles. Perhaps this increase in absolute thermoregulatory precision reflects an increase of the thermoregulatory drive in the turtles injected with LPS, in detriment of their ambulatory drive. Therefore, turtles treated with LPS showed an increase thermoregulatory focus regardless of their direction of departure from normothermy.

Age-dependent toxicity of LPS was suggested as a cause for anapyrexia (Habicht 1981). However, in Terrapene carolina that did not seem to be the case, for my treatment blocks had turtles of different age groups. There are also many findings that moderate doses of LPS are followed by anapyrexia instead of fever (Székely and Romanovsky 1998).

After bacterial infection (live Aeromonas hydrophila at $3x10^9$ bacterium per ml), Terrapene carolina increased their $\bar{T}_b$ by 4.6° C (Monagas and Gatten 1983), thus maintaining a behavioral fever at higher levels than those recorded in the present study (where the highest difference was 1.1° C during the first day for turtles injected with high dose). Other differences notwithstanding, perhaps the main reason for the differences between the two studies were the type of pyrogen used: in the study with live bacteria, the pyrogen levels will increase after injection as long as bacteria keep growing and dividing; whereas in the LPS injection, exogenous pyrogen levels can only decrease after injection.

If instead of using LPS I had used live Escherichia coli, then bacterial growth would have been drastically different in each of the two groups of treated turtles. In the turtles that were maintaining a fever, E. coli would have been near its optimum growth rate, whereas

in the turtles maintaining an anapyrexia E. coli would have had a very slow growth rate (Fig. 4). At first look, it is paradoxical that the turtles injected with the most "bacteria" would enhance bacterial growth even further, whereas the turtles with the least would depress their growth.

Apparently, the reduction of the concentrations of certain plasma trace metals such as iron, compounded with an increase in temperature, is what suppresses bacterial growth in a host (Grieger and Kluger 1978; Kluger and Rothenburg 1979; Hacker, et al. 1981). The reduction of plasma iron is independent of the increase in T_b, but depends on the protein levels of the host, where protein-deprived hosts maintain relatively high levels of blood iron during infection (Hoffman-Goetz and Kluger 1979). Iron (and perhaps other trace metals) is a limiting factor for bacteria. During fever, an increase in temperature promotes bacterial growth thus increasing their demand for more iron. However, the concomitant reduction in plasma iron further reduces the iron supply available to bacteria. Eventually, this situation prevents continued bacterial growth.

Ideally, an invasion by pathogens should elicit a scalable response from the organism's defense system. Considering that an organism's time and resources are limited, defense scaling would allow for a more tailored response to the quantity and quality of the pathogenic agents. Consequently, there might be times when it would be beneficial to spend more time or resources subduing the invading agent, whereas at other times it would be prejudicial for the host to allocate time or resources to that same response (e.g., a weakened animal may not have enough energy reserves to fight an infection).

When the quantity or quality of the pathogens are below a certain threshold, an ectotherm may lower its T_b set point (behavioral anapyrexia). This lowering of the set point and implied decrease of its $\bar{T}_b$ will allow it to decrease the bacterial growth rate, while keeping its immune system operative. This strategy may be used when the infection is incipient, and although it takes more time to subdue the infection, it is energetically less

costly due to the decrease in $\bar{T}_b$. Moreover, this strategy may be used when the ectotherm was already debilitated at the time of the infection, regardless of the quality and quantity of the pathogen. However, when the quantity or quality of the pathogens are above a certain threshold, the ectotherm may increase its T_b set point (behavioral fever). This increased set point and implied increase of $\bar{T}_b$ will allow the host to increase the bacterial growth rate, thus amplifying the limiting effects caused by the active reduction of the plasma iron. This strategy may be used when the infection is large, and although it takes less time to subdue the infection, it is energetically very costly due to the increase in energy expenditure caused by the increase in $\bar{T}_b$, and also by the potential increase of time allocated to thermoregulation to the detriment of feeding activity (Baracos, et al. 1987). My results support the Romanovsky and Székely (1998) preliminary hypothesis of two antagonistic thermoregulatory strategies against pathogens, for in Terrapene carolina the same agent caused either a fever or an anapyrexia depending upon dose.

The diversity of methods used to prepare pyrogens and doses thereof, and other incongruities among studies on fever suggest that better controls should be used. Additional treatment blocks dosed with antibiotics and antipyretics would be useful extensions to this study. The treatment with antibiotics would allow for a better baseline against which to compare the pyretic or anapyretic responses. The treatment with antipyretics would contrast not only the effects of the pyrogen, but also the effects of antibiotics. The latter would be particularly useful if one were to test the adaptive value of fever as expressed by increased survivorship of turtles able to reset their thermoregulatory set points in response to a bacterial infection (Bernheim and Kluger 1975; Bernheim and Kluger 1976). In addition, the use of other acclimation temperatures could enhance the influence of the average environmental temperature on the degree of departure from normothermy.

Acknowledgements

Part of this work is from a dissertation submitted by the author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology at the University of Oklahoma, USA. I thank Joseph Bastian, Victor Hutchison, Michael Kaspari, Neil Shafer-Ray, and Frank Sonleitner for their suggestions and review of the manuscript. I thank the Oklahoma Department of Wildlife Conservation and Bob St. Clair for supplying the turtles used in this study. I thank Robin Whitekiller, who helped with animal care. Part of this work was funded by a grant from the Graduate Student Association of the University of Oklahoma. This paper was written while the author held a fellowship from Junta Nacional de Investigação Científica e Tecnológica PRAXIS XXI BD11292/97, Portugal.

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Tables

Table 1: Summary of mean body temperatures ($\bar{T}_b$'s) for all treatment blocks of box turtles (Terrapene carolina) tested over a 48-hr. period. Thermal data were partitioned by day. n=sample size; CI=confidence interval of the mean.

Experiment	Treatment Dose	n	$\bar{T}_b$±95% CI
	Day 1		
One	Saline control.....	17	24.03±4.10
	0.0025 mg LPS	17	22.08±4.52
Two	0.025 mg LPS.....	16	25.17±4.13
	Day 2		
One	Saline control.....	17	22.03±5.63
	0.0025 mg LPS	17	19.13±4.74
Two	0.025 mg LPS.....	16	22.15±4.18

Table 2: Comparison between mean body temperatures ($\bar{T}_b$'s) of low dose-treated and control box turtles (Terrapene carolina). Factorial ANOVA results for $\bar{T}_b$'s with treatment blocks defined by day (first and second), photoperiod-based activity (active and inactive), treatment dose (low dose and control), and hour of day (1–24). Treatment consisted of two doses of LPS (0.000 and 0.0025 mg per gram of non-shell body mass, all dissolved in sterile reptilian saline). DF: degrees of freedom; SS=sum of squares; F Ratio=Fisher's value; p=associated probability.

Source of Variation	DF	SS	F Ratio	p
Day	1	1000.95	462.96	<0.0001
Activity	1	2.95	1.36	0.2463
Day x Activity	1	4.08	1.89	0.1729
Treatment Dose	1	86.24	39.89	<0.0001
Day x Treatment Dose	1	15.84	7.33	0.0082
Activity x Treatment Dose	1	12.98	6.00	0.0163
Day x Activity x Treatment Dose	1	13.74	6.36	0.0135
Hour of Day	1	13.14	6.08	0.0156

Table 3: Comparison between mean body temperatures ($\bar{T}_b$'s) of high dose-treated and control box turtles (Terrapene carolina). Repeated measures factorial ANOVA results for $\bar{T}_b$'s with treatment blocks defined by day (first and second), photoperiod-based activity (active and inactive), treatment (low dose, high dose, and control), hour of day (1–24), and subjects (the 15 turtles tested under both treatments; the repeated-measures effect). Treatment consisted of two doses of LPS (0.000 and 0.025 mg per gram of non-shell body mass, all dissolved in sterile reptilian saline). DF=degrees of freedom; SS=sum of squares; F Ratio=Fisher's value; p=associated probability.

Source of Variation	DF	SS	F Ratio	p
Day	1	2818.20	92.44	<0.0001
Activity	1	2.94	0.10	0.7564
Day x Activity	1	1.87	0.06	0.8044
Treatment Dose	1	454.30	14.90	0.0001
Day x Treatment Dose	1	74.31	2.44	0.1187
Activity x Treatment Dose	1	1.57	0.05	0.8203
Day x Activity x Treatment Dose	1	135.57	4.45	0.0351
Subjects	14	65809.10	154.19	<0.0001
Hour of day	1	57.74	1.89	0.1690

Figure Legends

Figure 1. Hypothetical time series of average body temperature (T_b) for two Terrapene carolina to illustrate how treatment-block mean body temperatures ($\bar{T}_b$'s) and mean carry-over variances ($\bar{s}^2$) were calculated. One turtle's T_b's are shown by the solid circles, whereas the other turtle's T_b's are shown by the open circles.

Figure 2. Time series of average body temperature ($\bar{T}_b$) and variance ($\bar{s}^2$) for different treatment blocks of turtles (Terrapene carolina acclimated at 20° C) injected with three dosages of LPS (0.000 (saline), 0.0025, and 0.025 mg per gram of non-shell body mass, all dissolved in sterile reptilian saline). The $\bar{T}_b$'s are shown by the solid circles, whereas $\bar{s}^2$'s are shown by the open circles. Black bars indicate the scotophase of the photoperiod of LD 12:12.

Figure 3. Plot of the autocorrelation function (r_k) of the T_b's from box turtles (Terrapene carolina) acclimated at 20° C over the autocorrelation lag period (k). For comparison, also plotted are the autocorrelation functions of non-injected female (♀) and male (♂) Terrapene carolina acclimatized at similar conditions (do Amaral, unpublished data). The vertical thin line marks the 24-hour lag. The dampening is the result of the series not being wrapped around.

Figure 4. Representation of the overlap between Terrapene carolina (box turtles) body temperatures (T_b) and the temperature-dependent growth model for Escherichia coli, illustrating how by changing their T_b's box turtles can alter greatly bacterial development. The single-hatched areas represent the intersection between the turtles' T_b's and the E. coli growth curve, the crosshatched area represents the intersection between the turtle's mean body temperatures ($\bar{T}_b$) and the E. coli growth curve, and the solid area represent the upper thermal limit for T. carolina (Hutchison, et al. 1966). The bacterial growth model was

redrawn from Barber (1908). LRR=loss of righting response; CTMax=critical thermal maximum.

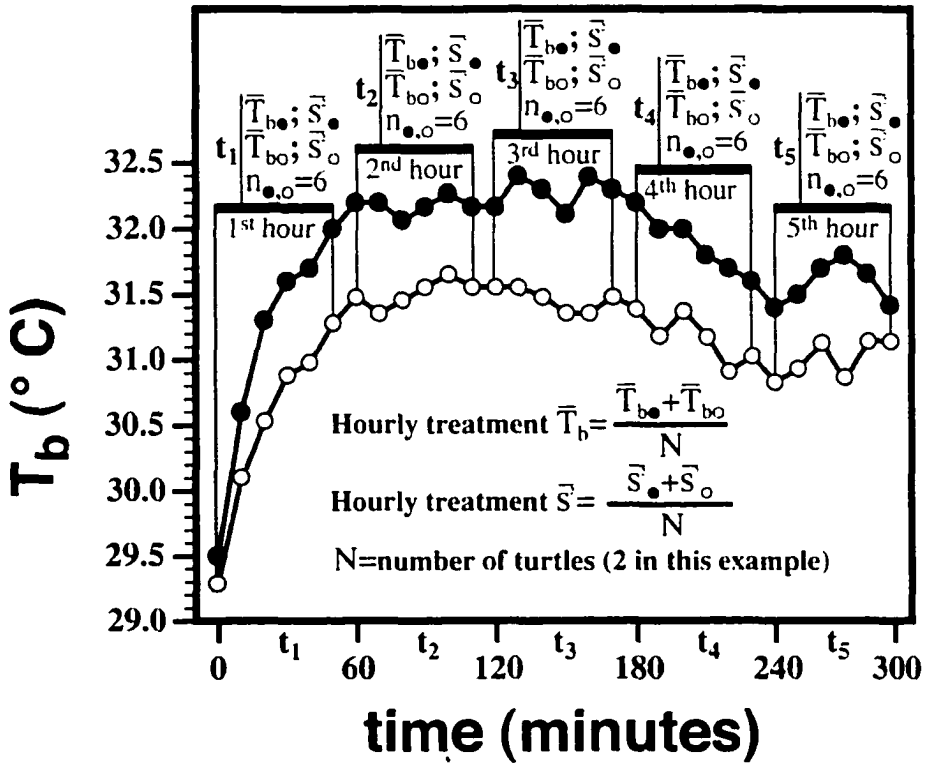
T_b (° C)
32.5
32.0
31.5
31.0
30.5
30.0
29.5
29.0
t_1
t_2
t_3
t_4
t_5
1st hour
2nd hour
3rd hour
4th hour
5th hour
$n_{\bullet,o}=6$
Hourly treatment $\bar{T}_b=\frac{\bar{T}_{b\bullet}+\bar{T}_{bo}}{N}$
Hourly treatment $\bar{S}=\frac{\bar{S}_{\bullet}+\bar{S}_{o}}{N}$
N=number of turtles (2 in this example)
0
60
120
180
240
300
time (minutes)

Figure 1

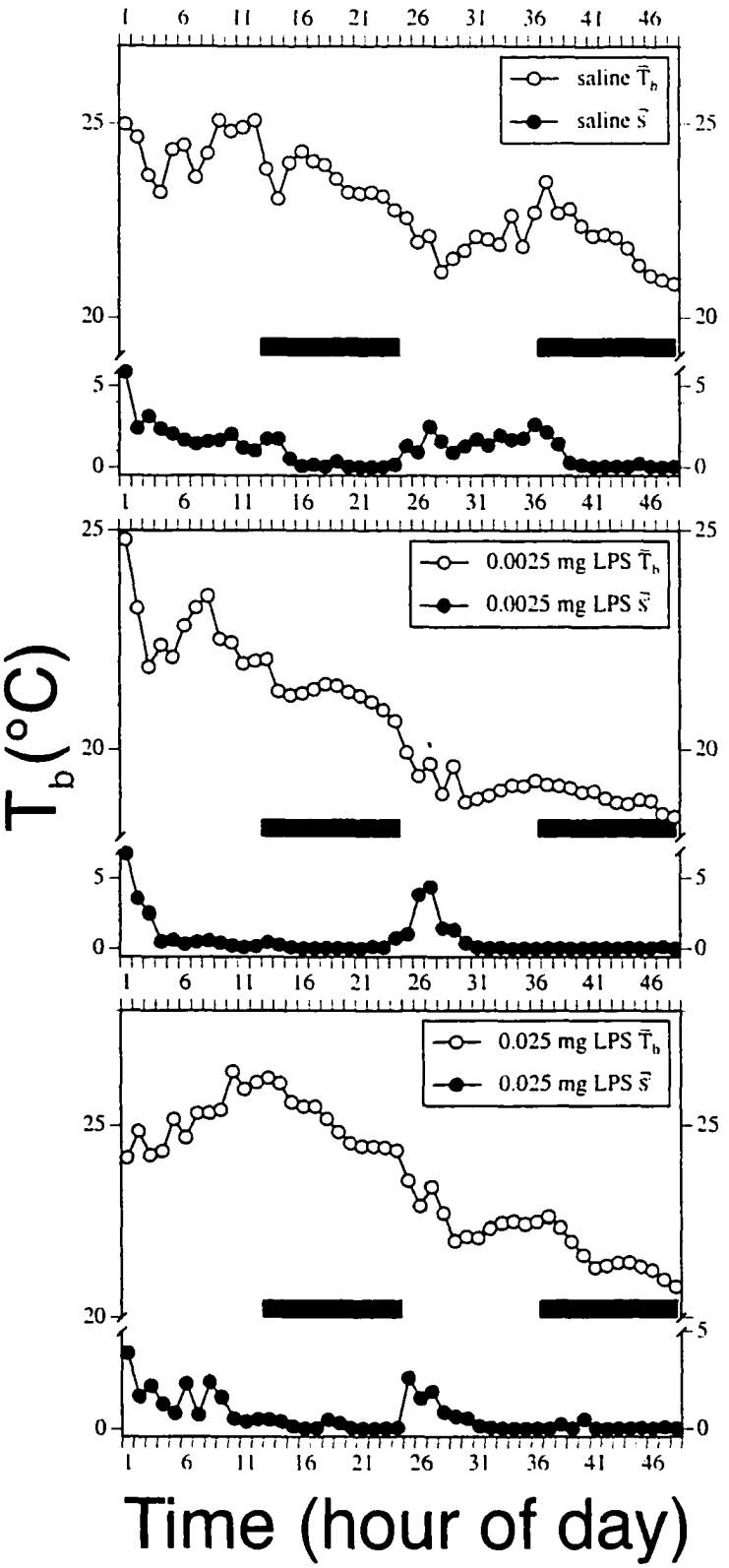
saline $\bar{T}_b$
saline $\bar{s}$
0.0025 mg LPS $\bar{T}_b$
0.0025 mg LPS $\bar{s}$
0.025 mg LPS $\bar{T}_b$
0.025 mg LPS $\bar{s}$
T_b (°C)
Time (hour of day)

Figure 2

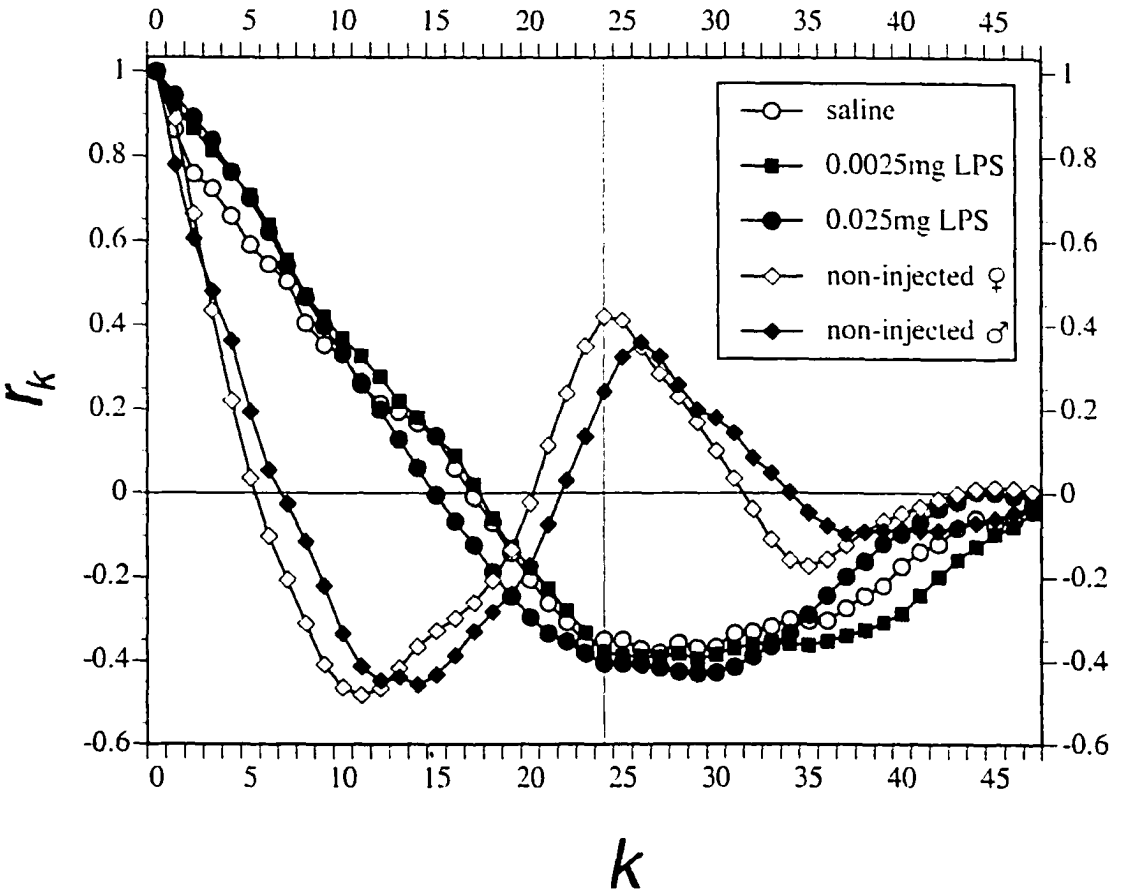
saline
0.0025mg LPS
0.025mg LPS
non-injected ♀
non-injected ♂
r_k
k

Figure 3

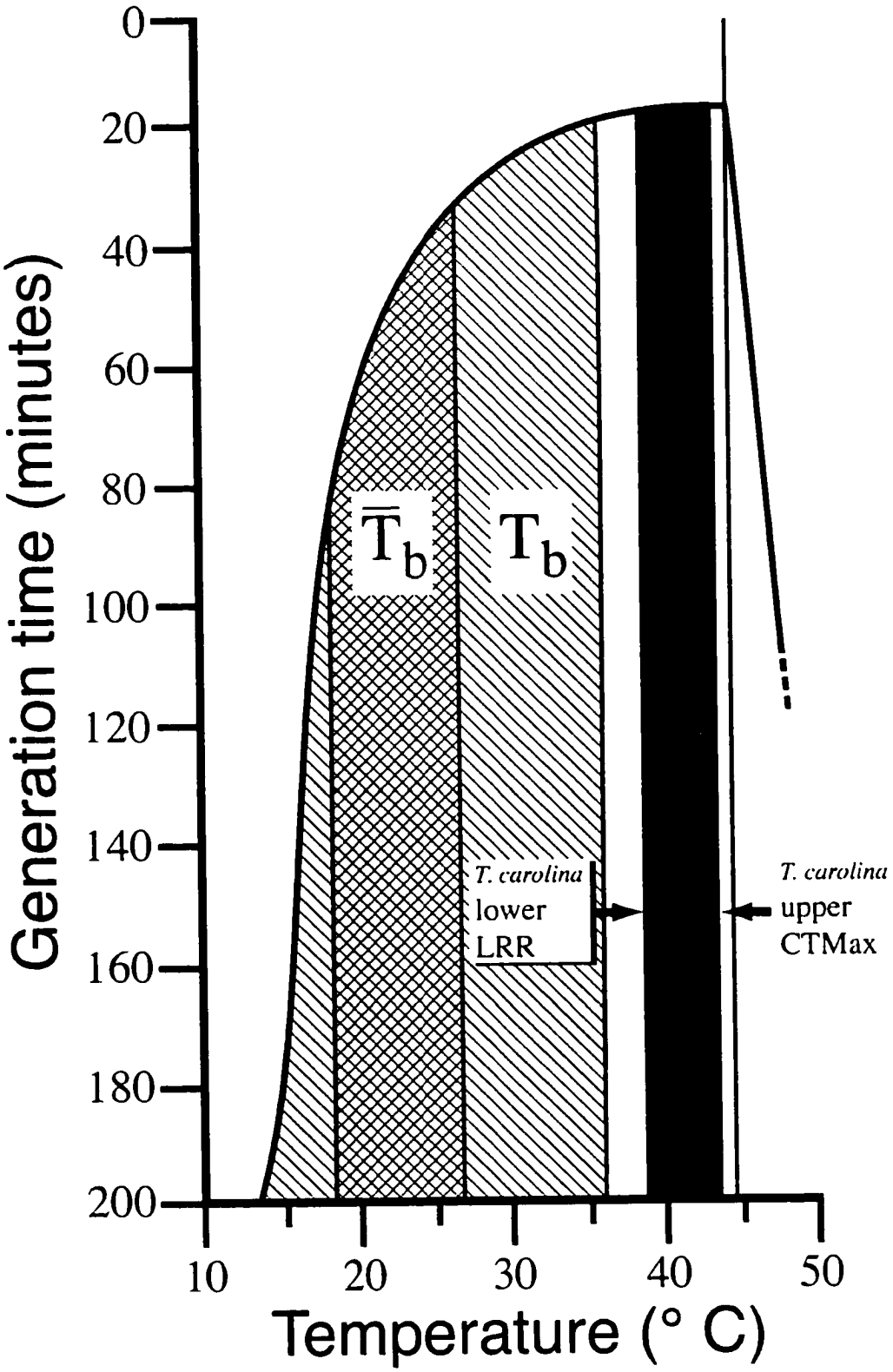
0
20
40
60
80
100
120
140
160
180
200
Generation time (minutes)
$\overline{T}_b$
T_b
T. carolina
lower
LRR
T. carolina
upper
CTMax
10
20
30
40
50
Temperature (° C)

Figure 4

The presence of chaos in the thermoregulatory cycles of the box turtles Terrapene carolina and T. ornata

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Keywords: chaos, thermoregulation, box turtles, time series

Running head: chaotic thermoregulation in box turtles

To be submitted to The American Naturalist

Abstract:

Chaos theory offers deterministic models to explain some complex cyclical phenomena. Time series of body temperatures (T_b) of reptiles often show complex and difficult to characterize oscillatory cycles within longer diel cycles. Furthermore, diseases and other disturbing agents may force normal regulatory mechanisms to change. I tested for chaos in the thermoregulatory cycles of Terrapene ornata and T. carolina. Then, I examined the influence of two doses 2.5 and 25 μg of Escherichia coli lipopolysaccharide (LPS) pyrogen on the thermoregulatory chaosticity of T. carolina, and compared specifically the thermoregulation of T. ornata and T. carolina regarding chaos. I recorded time series of cloacal T_b in thigmothermal linear gradients from acclimatized (LD 12:12; 10, 20° C) box turtles. Quantitative and qualitative methods were used to evaluate and characterize the presence of chaos: the presence of strange attractors in state space plots, positive Lyapunov exponents, and smaller-than-five correlation dimensions. Both species of turtles showed chaotic thermoregulation. Terrapene carolina had a higher percentage of chaotic thermoregulatory behaviors than T. ornata. The injection of LPS reduced the number of chaotic thermoregulatory behaviors in T. carolina, and increased the number of noisy thermoregulatory behaviors. Deterministic chaos was confirmed as a good thermoregulatory model for turtles.

Introduction

The spirit of Plato dies hard. We have been unable to escape the philosophical tradition that what we can see and measure in the world is merely the superficial and imperfect representation of an underlying reality.

—S.J. Gould, "The Mismeasure of Man"

Mean body temperature ($\bar{T}_b$) is used frequently as a descriptive measure of the thermal status of an animal. However, a single absolute temperature might not be as important to understand specific thermoregulation as the temporal sequence (time series) of the component individual body temperatures (T_b). Time series are sets of observations recorded sequentially in time (Box et al. 1994). Analyses of time series of thermal data allow for a more complete understanding of the thermoregulation of a given organism, particularly when the organism follows a cyclical thermoregulatory model. However, it is common for time series of T_b to look noisy and to show complex high-frequency oscillatory patterns. Frequently, small oscillations in time series of T_b are ignored or treated as noise, and thus part of the information contained in the series may be lost.

Time series of T_b of reptiles often show complex and traditionally difficult to characterize oscillatory cycles within longer diel cycles. Most natural systems are nonlinear and changes in the systems' behaviors are not a simple function of the changes in the systems' conditions (Ditto and Pecora 1993). Chaos theory offers deterministic models to explain some complex cyclical phenomena. The larger framework underlying chaos is the theory of dynamical systems. A dynamical system has two parts: a state or initial condition (the essential information about the system), and a dynamic or rule (which specifies how a

system evolves) (Crutchfield et al. 1986; Tuffilaro et al. 1991). The use of chaos has ranged from theologians (Davies 1997) to astrophysicists (Sussman and Wisdom 1992), and increasingly has been considered as a deterministic model for complex systems.

Chaos characterizes many biological processes spanning biochemistry (Thomas 1995), biological control systems (Cavalieri and Koçak 1994; Glass and Malta 1990; Endresen 1997; Leloup and Goldbeter 1999; Weiss et al. 1994), cardiology (Bassingthwaighte 1994; Goldberger 1991; Hokkanen 2000; but see: Le Pape 1997, and Weiss et al. 1999), cytology (Keough et al. 1991; Li et al. 1992; Shen and Larter 1995), ecology (Constantino et al. 1997; Huppert and Stone 1998; Perry et al. 1997; Ruxton and Rohani 1998; Suárez 1999), neurology (González et al. 1999), general physiology (Rossler and Rossler 1994), and thermoregulation (Hahn et al. 1992). Many of the characteristics of chaos make it a very good working model for thermoregulation because of its sensitivity to the system's initial conditions, its ability to return to a stable trajectory after being disturbed, and its underlying determinism. Furthermore, the behavior of chaotic systems is a collection of many orderly behaviors, none of which prevails under ordinary circumstances, and among which the chaotic systems may rapidly switch (Ditto and Pecora 1993). Although chaos is unpredictable, it is deterministic; thus two identical systems driven by the same initial conditions will produce the same output (Ditto and Pecora 1993).

To look for chaosticity in thermoregulation, I chose turtles for two reasons: the results would complement and refine my own use of more traditional methods to study the same T_b time series for the same data sets (do Amaral 2001a; do Amaral 2001b), and would complement and contrast Bowker et al. (Bowker et al. 2001) work with lizards. More

traditional approaches are greatly sensitive to the noise present in the time series (do Amaral 2001a; do Amaral 2001b). Moreover, turtles used to be considered the most primitive group among amniotes, and thus considered the best models for primitive amniote organization and physiology (Rieppel 1999). Despite recent changes in their phylogenetic position among reptilian clades (Hedges and Poling 1999; Rieppel 1999), chelonians are interesting models to help trace the reptilian phylogeny of thermoregulation because of their ancient divergence from squamates (the earliest known fossil turtle is from the Upper Triassic of Germany) and of their different lifestyles (Hutchison 1979).

Box turtles are relatively small, mainly terrestrial, emydid turtles. In the USA, Terrapene carolina ranges from southern Maine, southern Michigan, and southern Wisconsin southward to Florida and the Gulf of Mexico and westward to southeastern Kansas, eastern Oklahoma, and eastern Texas. Terrapene ornata ranges from western and southern Illinois, Missouri, Oklahoma, and all but the extreme eastern part of Texas, westward to southeastern Wyoming, eastern Colorado, eastern and southern New Mexico, and southern Arizona, and from southern South Dakota and southern Wisconsin, southward to northern Mexico (Reagan 1974). Both species can thermoregulate (do Amaral 2001b; Sturbaum 1982). Terrapene carolina and T. ornata are species that are very closely related (Legler 1960), but have different habitat preferences. Terrapene carolina inhabits mesic forested areas, whereas T. ornata occurs in grasslands and deserts, often in sandy areas that are more xeric than the places inhabited by T. carolina (Legler 1960). For box turtles, temperature, cover, and moisture are critical aspects of the environment (Reagan 1974). This difference in habitat preference may have an influence on the chaosticity of the

thermoregulatory mechanisms for these species. Assuming that an ectothermic organism with chaotic thermoregulation preempts changes in environmental temperatures by having a regulatory system that is both highly responsive to change and intrinsically variable, then the degree of chaosticity in the organism's thermoregulation may be proportional to the environment's thermal heterogeneity. I assumed a fundamental difference in the thermal heterogeneity (scaled to ectothermic organisms with box turtle size) of the two species habitat types. I assumed that the warmer habitat is less thermally diverse than the cooler habitat. In my assumption, those differences in thermal heterogeneity result from differences in the amount and distribution of overhead cover between the two types of habitat.

In addition, diseases and other disturbing agents may force normally chaotic regulatory mechanisms to change, and thus switch the systems' behaviors from chaotic to linear (Goldberger 1991). I assumed that a diseased organism has a narrower range of physiological activities than a healthier conspecific. Therefore, a possible test for the intrinsic nature of chaotic regulation could be the administration of disturbing agents to an organism that shows chaotic regulation, and the consequent comparison of the behaviors before and after the disturbance. Injections of a low and a high dose of Escherichia coli lipopolysaccharide (LPS) caused behavioral anapyrexia and behavioral fever, respectively, in T. carolina (do Amaral 2001a). However, a further test of whether LPS modulates the underlying thermoregulatory model is particularly important, because it would corroborate a hypothetical physiological nature of the chaos generating mechanism in T. carolina.

The main goal of my study was to test whether chaos was present in the thermoregulation of these two turtle species. Additionally, if chaos were present then treatment differences due to turtle species or injection with pyrogen could correspond to varying degrees of chaotic thermoregulation. Considering the thermal properties of these species' habitats and my assumption regarding environmental thermal heterogeneity, I predicted that T. carolina would show a higher degree of thermoregulatory chaosticity than T. ornata. Moreover, considering the disturbing effects of bacterial lipopolysaccharide (LPS) on turtle thermoregulation and my assumption regarding increased physiological focus of diseased organisms, I predicted that turtles treated with both doses of LPS would show a lesser degree of thermoregulatory chaosticity than control turtles.

Materials and methods

I obtained young and adult eastern and ornate box turtles (T. carolina and T. ornata) in October 1996 from the Oklahoma Department of Wildlife Conservation. These turtles were confiscated from individuals who had collected them from several counties in southeastern Oklahoma for illegal sale in the pet trade. I also used juvenile box turtles that were hatched and reared in the laboratory (St. Clair 1995). I kept the turtles in three pens (186 cm long, 61 cm wide, and 61 cm high) with sandy soil, a UV light source (General Electric, 20 watts, Black Light), and a heat lamp at one of the ends. I fed the turtles with assorted fruits and vegetables dusted with Reptivite® vitamin mixture and provided water ad libitum. Turtles were code-marked by notching their marginal scutes (Cagle 1939).

Turtles were acclimatized on an LD 12:12 photoperiod at 20.0±1.0° C for a minimum of 14 days before the experiments. To avoid postprandial effects, I did not feed the turtles for 7 days before each experiment, but provided water ad libitum during that period. Experiments were conducted from February 6 to May 9 of 1996 and from December 8 of 1996 to August 12 of 1997. I followed Folk (1974), and Hutchison and Dupré (1992) for the definition of acclimation and acclimatization (response to a single environmental factor, and to two or more factors, respectively).

To measure temperature selection of turtles, I placed animals singly in four linear thigmothermal gradients (209.5 cm long, 16.5 cm wide, and 22.5 cm high) with wood sides and an aluminum plate floor (0.3 cm thick) maintained at temperatures ranging from approximately 6.0±1.5 to 44.0±1.5° C. To produce this range of temperatures, I housed the gradients in an environmental room at 5.0±1.5° C and used a series of heating pads (250 watts) spaced apart to maintain the warm end of each gradient. I controlled the temperature of the heating pads with rheostats to provide a more uniform temperature gradient. Broad-spectrum fluorescent lights (General Electric, 34 watts) were suspended 26 cm above the entire length of each gradient, thus keeping light intensity constant throughout the thermal gradients. These lights were on a synchronized LD 12:12 photoperiod and provided the only source of light in the environmental room. Each gradient was covered by clear acrylic plastic that prevented measurable heat from the lights from entering the gradient. Cover and moisture also were kept constant in the thermal gradients: cover was absent and moisture was about 69% relative humidity.

To measure T_b, I used 32-gauge copper-constantan thermocouples. To prepare the thermally sensitive ends, I stripped about 2 cm of the insulation plastic, entwined the leads at an end of the thermocouple, flame fused the entwined leads together at the tip, and encapsulated the end with epoxy resin. Approximately 15 hours before recording body temperatures, a previously calibrated thermocouple was inserted about 3 cm into the turtle's cloaca. I used duct tape to secure the exiting thermocouple wire to the turtle's carapace. Cloacal temperatures of T. carolina correlate well with core body temperatures in both shielded and unshielded solar environments (Russo 1972). Then, I put each turtle in the middle of a thermal gradient for the 12-hour habituation period to the test conditions.

Turtles that were tested for the effects of bacterial LPS were injected at the beginning of the first photophase after the habituation period. In these tests, I gave each turtle an intraperitoneal injection of either sterile reptile saline (control treatment) or endotoxin (LPS from Escherichia coli; Sigma Chemical Co., St. Louis, Missouri, USA, item L-2630) dissolved in reptile saline (experimental treatment). Escherichia sp. have been isolated from reptilian abscesses (Frye 1973) and are a known reptilian pathogen (Marcus 1981). Moreover, the use of a purified LPS pyrogen instead of bacteria (dead or alive) allows for the preparation of more precise doses, thus allowing for better comparisons of results from different experiments.

Individuals in the first experimental group (n = 17) received 2.5 μg of endotoxin per gram of non-shell body mass (delivered in 0.375 mg endotoxin per ml solution). Because the dry shell mass of this species is approximately 25% of the total body mass (Marvin and Lutterschmidt 1997), I used a value of 75% of body mass to determine the amount of

solution to inject in each individual. Based upon its non-shell body mass, I injected each turtle in the control group (n = 17) with a volume of reptile saline equivalent to the volume of fluid injected into experimental turtles. Sixteen individuals from the control group then were re-tested at least 7 days later as an experimental group with a higher concentration of injected endotoxin. Individuals in this second experimental group received 25 μg of endotoxin per gram of non-shell body mass (delivered in 3.75 mg endotoxin per ml solution).

Following injection, I recorded discrete time series of body temperatures at 10-minute intervals with a Model 50 Data Logger (Electronic Controls Design Inc., Milwaukie, Oregon, USA). Each recorded part of the experiment started at the beginning of the first photophase and lasted 48 hours. The turtles were not disturbed during the experimental runs. In addition, I recorded the body mass of each turtle before and immediately after each trial to monitor any excessive dehydration.

Qualitative and quantitative methods were used to evaluate and characterize the presence of chaos. Qualitatively, I looked at the attractors of time series of turtle T_b, created by embedding the series in state space. Quantitatively, I measured the largest Lyapunov exponent (λ_1) and the correlation dimension (D_2) for each time series of turtle T_b, and these series were considered as chaotic when the Lyapunov exponents were positive and the correlation dimensions were smaller than five. For general statistical data analysis and manipulation, I used the JMP statistical package for Macintosh computers (Sall et al. 2000), and for chaos analyses I used the peer-reviewed program collection CDA Pro

(Sprott and Rowlands 1998) for IBM compatible computers. In this article, I report means plus and minus their 95% confidence intervals, and set α at 0.05.

State space is a useful concept for the visualization of a dynamical system's behavior. It is an abstract space whose coordinates are the degrees of freedom of the system. For example, the motion of a pendulum is completely determined by its initial position and velocity (Crutchfield et al. 1986). Therefore, its state is a point in a plane whose coordinates are position and velocity. While swinging back and forth, an ideal frictionless pendulum follows a circular orbit through the 2-dimensional state space. However, with friction, the pendulum's orbit is a spiral. Attractors are geometric forms that characterize long-term system behavior in state space (Crutchfield et al. 1986), and strange attractors are attractors with non-integer dimensions (Tuffilaro et al. 1991).

The Lyapunov exponents of a system are a set of invariant geometric measures that describe intuitively the dynamical content of the system (Banbrook et al. 1996). Lyapunov exponents are measures of the rate at which nearby trajectories diverge in state space (Sprott and Rowlands 1995). I calculated the largest Lyapunov exponent (λ_1) for each turtle T_b time series. The units of the calculated Lyapunov were bits per data sample (e.g., in this study, a Lyapunov exponent of +1.00 meant that the separation of nearby orbits doubled on the average every ten minutes). I measured the separation in units of diameter of the smallest D-dimensional hypersphere that enclosed the attractor at three successive time steps. The Lyapunov exponent error (λ_e) was calculated as 2.5 times the standard deviation of the slopes of the three successive time steps divided by the square root of the

number of trajectories followed. Chaotic orbits have at least one positive Lyapunov exponent.

Given the time series $x(t)$, an m-dimensional phase portrait is reconstructed with delay coordinates, i.e., a point in the attractor is given by $\{x(t), x(t+\tau), \ldots, x(t+[m-1]\tau)\}$ where τ is the almost arbitrarily chosen delay time. Then, the nearest Euclidean neighbor to the initial point was located $\{x(t_0), x(t_0+\tau), \ldots, x(t_0+[m-1]\tau)\}$ and the distance between the two points $L(t_0)$ denoted. At a later time t_1, the initial length will have evolved to length $L'(t_1)$. The length element is propagated through the attractor for a time short enough so that only small scale attractor structure is likely to be examined. If the evolution time is too large one may see L' shrink as the two trajectories that define it pass through a folding region of the attractor. This would lead to an underestimation of λ_1. Now, one looks for a new datum that satisfies two criteria reasonably well: its separation, $L(t_1)$, from the evolved fiducial point is small, and the angular separation between the evolved and replacement elements is small. If an adequate replacement point cannot be found, the initially used points are retained. This procedure is repeated until the fiducial trajectory has traversed the entire data file, at which point one estimates

$$\lambda_1 = \frac{1}{t_M - t_0} \cdot \sum_{k=1}^{M} \log_2 \frac{L'(t_k)}{L(t_{k-1})},$$

where M is the total number of replacement steps.

Both chaotic and certain noisy systems have positive Lyapunov exponents, thus to further test the chaosticity of a time series I measured the dimension of each attractor of turtle T_b time series. Systems with positive Lyapunov exponents and smaller-than-five

dimensions are considered to be chaotic (Sprott and Rowlands 1995). If one reduces (or normalizes) by $1/r$ the linear size in each spatial direction of an object residing in the Euclidean dimension D, then its measure N (length, area, or volume) would increase to $N = r^D$ times the original. If one takes the logarithm of both sides of the latter expression and solves for D, one gets a dimension D that needs not be an integer, $D = \log N / \log r$. This generalized treatment of D is called Hausdorff dimension. By and large, the concept of certain dimensions quantifiers is that the weight $p(\varepsilon)$ of a typical ε-sphere covering part of the invariant set scales with its diameter like $p(\varepsilon) \approx \varepsilon^D$, where the value for D depends also on the precise way one defines the weight. Using the square of the probability p_i to find a point of the set inside the ball, the dimension is called the correlation dimension D_2, which is computed most efficiently by the correlation sum (Grassberger and Procaccia 1983b):

$$C(m,\varepsilon) = \frac{1}{N_{pairs}} \cdot \sum_{j=m}^{N} \sum_{k<j-w} \Theta\left(\varepsilon - \left|\mathbf{s}_j - \mathbf{s}_k\right|\right)$$

where $\mathbf{s}_i$ are m-dimensional delay vectors, $N_{pairs} = (N - m + 1)(N - m - w + 1)/2$ is the number of pairs of points covered by the sums, Θ is the Heaviside step function, and w is the Theiler window. On sufficiently small length scales and when the embedding dimension m exceeds the box-dimension of the attractor (Sauer and Yorke 1993),

$$C(m,\varepsilon) \propto \varepsilon^{D_2},$$

Since one does not know the box-dimension a priori, one checks for convergence of the estimated values of D_2 in m.

Capacity dimension (similar to the Hausdorff dimension) is calculated by dividing the phase space into equal hypercubes with embedding dimension D, and then plotting the log

of the fraction of hypercubes that contain data points versus the normalized linear dimension of the hypercubes. The capacity dimension is taken as the average slope of the latter curve. Correlation dimension (D_2) is a lower bound on the capacity dimension, which in most cases approaches the capacity dimension (Sprott and Rowlands 1995), and it is the most common method of characterizing strange attractors (Hilborn and Tuffilaro 1997). Moreover, D_2 is a more accurate measurement of attractor dimensions when the data sets are small (Grassberger and Procaccia 1983a; Grassberger and Procaccia 1984). Therefore, I measured the D_2 for those series of T_b's. To calculate the D_2, a new datum was taken with each pass through the series, and a hyperdimensional sphere of embedding dimension D (in many cases, $D = 8$) and radius r was centered on the chosen datum (Sprott and Rowlands 1995). The fraction of subsequent data points in the recorded series within that sphere was calculated for several values of r, and a plot was made of the log of that number versus the log of the radius (Sprott and Rowlands 1995). The correlation dimension (D_2) was determined as the average slope of the cumulative curve over the middle one-quarter of the vertical scale, and the error ($D_{2\varepsilon}$) was determined as half the difference of the maximum and minimum slope over the same range (Sprott and Rowlands 1995). A correlation dimension greater than about five implies essentially random data (Sprott and Rowlands 1995).

To test further the appropriateness of using nonlinear techniques to study turtle T_b time series, I statistically compared turtle time series against surrogate series (or null hypotheses) generated from the same data. I haphazardly chose ten turtle time series from among all experimental runs that had had chaotic thermoregulation. Then, I generated two

types of surrogate time series (or null hypotheses) from those ten series that were studied for their largest Lyapunov exponents (λ_1) and correlation dimensions (D_2). One type of surrogate series was generated by randomly shuffling the data. This method preserves the probability distribution but generally produces series with a different power spectrum and correlation function (Sprott and Rowlands 1995). The other type of surrogate series was generated by multiplying the Fourier transform of the data by random phases with equal probability ($0 \leq \phi < 2\pi$), and then inverse Fourier transforming the series back to the time domain. This method preserves the power spectrum and correlation function but generally produces a different probability distribution (Sprott and Rowlands 1995). The normal series λ_1 and D_2 were compared with the λ_1 and D_2 of the surrogated series through a repeated measures analysis of variance (ANOVA) model. This ANOVA accounted for repeated measures by the use of the random term in the model (in this case the pairs of turtle T_b series and their surrogate series) as the error term instead of the residual error (Sall and Lehman 1996).

In the environmental room, the small oscillations in the working levels of the heating and the cooling systems could have introduced extraneous dynamics in the turtle thermoregulation . To test the influence of those potential extrinsic sources of variability on turtle chaosticity, I measured the λ_1 and D_2 of the time series of temperatures of the environmental room that were concomitant with each one of the series of the ten turtles haphazardly selected for the surrogate series experiments. The turtle T_b series λ_1 and D_2 were compared with the λ_1 and D_2 of the environmental room series through a repeated measures analysis of variance (ANOVA) model. This ANOVA accounted for repeated

measures by the use of the random term in the model (in this case the pairs of turtle and environmental room temperature time series) as the error term instead of the residual error (Sall and Lehman 1996).Based on the values of λ_1 and λ_{1e}, and D_2 and D_{2e}, time series were classified into one of four types: Type I ($\lambda_1 - \lambda_{1e} > 0$; $D_2 + D_{2e} < 5$), corresponded to low-dimensional deterministic chaos; Type II ($\lambda_1 - \lambda_{1e} > 0$; $D_2 + D_{2e} \geq 5$), corresponded to high-dimensional noise; Type III ($\lambda_1 - \lambda_{1e} \leq 0$; $D_2 + D_{2e} < 5$), corresponded to linear determinism; and Type IV ($\lambda_1 - \lambda_{1e} \leq 0$; $D_2 + D_{2e} \geq 5$), corresponded to highly correlated noise (colored Gaussian noise). These manipulations of λ_1 and D_2 made conservative the classification of any given thermoregulatory behavior as chaotic by sorting the turtles' behavior based on the ranges of the 95% confidence intervals conceivably farther from the chaotic type (Type I). Furthermore, to test the degree of association between λ_1 and D_2 within each treatment, I used analysis of covariance. This test assumed that if the turtles had no preferred type of thermoregulation, then λ_1 and D_2 would not covary.

Results

Turtles from both species showed chaotic thermoregulation, thus indicating that a deterministic model may explain their thermoregulatory behavior. Moreover, the chaosticity of T. carolina injected with doses of bacterial lipopolysaccharide (LPS) was altered. In many cases, turtles treatment blocks had time series of T_b with periods of about 24 hours, which reflected the photoperiod during the experiment (do Amaral 2001b) (Fig. 1a).

However, all injected turtle treatment blocks showed disruption of diel cycling (do Amaral 2001a).

Many of the attractors looked like a varying ellipsoid coil with clustering nuclei of thermal data points (Fig. 1b), and their complex shape was a promising indication of deterministic chaos. However, not all turtles showed complex attractors in state space that suggested chaotic thermoregulation. After testing different embedding periods (τ), I settled on a general purpose embedding τ of 3 (30 min.) because it was small enough to express correlation between the temperatures and their delayed pairs and it generated attractors that did not collapse on the xy-axes median.

The λ_1 were normally distributed (normal distribution goodness-of-fit Shapiro-Wilk W test, $W = 0.99$, $p = 0.88$). However, the D_2 were not ($W = 0.97$, $p < 0.01$) and had a left skewed bell-shaped frequency distribution. After the D_2 data were square-transformed (Zar 1996), they became normally distributed ($W = 0.99$, $p = 0.75$). All parametric tests done with λ_1 and D_2 used the square-transformed D_2 data. Overall, the Lyapunov exponents (λ_1) and the correlation dimensions (D_2) were highly significantly negatively correlated in all experiments (Figs. 5a, 5b, and 5c). The negative correlation between λ_1 and D_2 is perhaps a concrete manifestation of the Kaplan-Yorke conjecture that there is a connection between Lyapunov exponents and the dimensions that quantify the geometric character of the system (Kaplan and Yorke 1979), but its significance for these data sets is unclear other than that λ_1 and D_2 do not occur randomly in the tested turtles. At 10° C acclimation the overall model showed a highly significant negative correlation between λ_1 and D_2 ($r = -0.35$, $p < 0.01$, $n = 94$) (Fig. 5a). For all treatments, the Lyapunov exponents were mostly positive

(Figs. 2a, 3a, and 4a). However in many instances, the correlation dimensions were above five (Figs. 2b, 3b, and 4b), and at this level of analysis the effects of endotoxin dose and species on the chaosticity of the thermoregulation of these species of box turtles were unclear. Nevertheless, the turtles acclimated at 20° C and the control turtles in the LPS experiment showed a trend toward a higher degree of chaosticity than the other blocks in the same experiments.

When the two species were analyzed separately, the same trend was apparent but T. carolina showed a slightly higher correlation coefficient (T. carolina: $r = -0.38$, $p < 0.01$, $n = 60$; T. ornata: $r = -0.35$, $p = 0.04$, $n = 34$). At 20° C acclimation the overall model showed a highly significant negative correlation between λ_1 and D_2 ($r = -0.37$, $p < 0.01$, $n = 100$) (Fig. 5b), but the degree of association between the two indices was smaller than in the previous case. When the two species were analyzed separately, a different trend was apparent, this time T. carolina showed a highly significant correlation coefficient ($r = -0.47$, $p < 0.01$, $n = 64$) whereas T. ornata had a non-significant correlation coefficient ($r = -0.11$, $p = 0.49$, $n = 36$). For the LPS experiments, the overall model again showed a highly significant negative correlation between λ_1 and D_2 ($r = -0.54$, $p < 0.01$, $n = 50$) (Fig. 5c). When the three treatment blocks within the LPS experiment were analyzed separately, the saline-injected controls had a significantly high correlation coefficient, whereas the animals injected with the low dose of LPS showed little differences from the controls in the association between the two indices, and the correlation for the high dose animals was non-significant (controls: $r = -0.55$, $p = 0.02$, $n = 17$; low dose: $r = -0.55$, $p = 0.02$, $n = 17$; high dose: $r = -0.42$, $p = 0.11$, $n = 16$).

The shuffled surrogate series had highly significantly higher Lyapunov (λ_1) exponents than the original time series ($\bar{x}_{\lambda 1}$ = 0.12±0.02, $\bar{x}_{\lambda 1 shuf}$ = 0.20±0.05; $F_{\lambda 1,\lambda 1 shuf}$ = 11.97, $p < 0.01$, n = 20), but not the Fourier transformed ($\bar{x}_{\lambda 1FT}$ = 0.11±0.02; $F_{\lambda 1,\lambda 1FT}$ = 0.46, p = 0.51, n = 20). In the correlation dimension comparison (D_2), both (shuffled and Fourier transformed) surrogate series' D_2 were highly significantly higher than the original time series' D_2 ($\bar{x}_{D2}$ = 4.49±0.22, $\bar{x}_{D2shuf}$ = 5.46±0.41; $F_{D2,D2shuf}$ = 25.28, $p < 0.01$, n = 20; $\bar{x}_{D2FT}$ = 5.84±0.39, $F_{D2,D2FT}$ = 55.36, $p < 0.01$, n = 20). The environmental room temperature series had highly significantly higher λ_1 than the original time series ($\bar{x}_{\lambda 1}$ = 0.12±0.02, $\bar{x}_{\lambda 1room}$ = 0.18±0.02; $F_{\lambda 1,\lambda 1room}$ = 25.05, $p < 0.01$, n = 20), and highly significantly higher D_2 than the original time series' D_2 ($\bar{x}_{D2}$ = 4.49±0.22, $\bar{x}_{D2room}$ = 5.48±0.32; $F_{D2,D2room}$ = 32.76, $p < 0.01$, n = 20). Robust statistics rather than parametric methods have been suggested as more appropriate methods of comparing series to surrogates (Schreiber and Schmitz 2000). However, the normal distribution of λ_1 and D_2 validated the use of parametric statistics.

Finally, when the percentages of thermoregulatory behaviors classified into one of the four types were compared across treatments, several important differences could be seen (Table 1). Overall, the predominant type of thermoregulation was high-dimensional noise (Type II). No turtle showed linear deterministic thermoregulation (Type III). With respect to the presence of chaos, turtles acclimated to the lower temperature (10° C) showed no clear differences between the two species (Table 1). However, at the higher acclimation temperature of 20° C, the two species showed clear differences with respect to their chaotic thermoregulation (Table 1). Terrapene carolina had more turtles showing chaotic

thermoregulatory behaviors (Type I) than T. ornata (Type II) (Table 1). Again, both species did not show linear deterministic thermoregulatory behavior (Type III) and mostly showed high-dimensional noise (Type II) (Table 1). In the LPS experiments, T. carolina showed a drastic reduction in the number of chaotic behaviors after injections of LPS. The control turtles injected with sterile saline had a large number of chaotic thermoregulatory behaviors, whereas turtles injected with doses of bacterial LPS showed mostly noisy (of both Type II and IV) thermoregulation (Table 1). No treatment blocks injected with either saline or doses of LPS showed linear deterministic thermoregulatory behavior (Type III) (Table 1).

Discussion

Deterministic chaos was confirmed as a good thermoregulatory model for turtles. Furthermore, both hypotheses were supported by the results. The degree of chaosticity of T_b time series was higher in T. carolina than it was in T. ornata. Moreover, turtles treated with LPS showed a lesser degree of thermoregulatory chaosticity than control turtles. Although a good analytical tool in thermoregulation studies, chaos analysis should be complemented by a complete set of thermal null hypotheses in the form of surrogate series and operative temperature (T_e) series.

Undoubtedly, the most important finding is the overall presence of a fairly large number of chaotic thermoregulatory behaviors in any given treatment. This suggests that in box turtles, a thermoregulatory model that intrinsically generates variability seems to be

common among individuals. Bowker et al. (2001) have suggested a nonlinear model for both the physiology and the control of lizard thermoregulation, whereby T_b's are attracted to a final state but that also generates events of T_b's exceeding normal limits. Bowker et al. (2001) justify this model considering that at any given time of their lives, lizards might encounter environments or situations that are thermally disadvantageous. Most reptiles are ectothermic and show some degree of thermoregulation, and many thermoregulate by shuttling between warmer and cooler places (Cowles and Bogert 1944). Lizards, due to their small size (small thermal inertia) and fast movements, are likely to use chaos in their thermoregulation, for they are more likely to face a fast-changing thermal environment. However, even slow-moving reptiles such as turtles show chaotic thermoregulation. This suggests that the main purpose of a chaotic model of thermoregulation is the generation of intrinsical unpredictability, and not simply the maintenance of a complex shuttling behavior between sun and shade. Additionally, many tested box turtles showed noisy thermoregulatory behaviors (non-deterministic thermoregulation), but none showed linear deterministic thermoregulation.

In the two-species comparisons, acclimation had an effect in the overall distribution of thermoregulatory types. At 10° C, the overall pattern across species and gender is unclear, except for the absence of occurrences of Type III (linear deterministic thermoregulation). This overall lack of pattern of turtles acclimated at 10° C may be explained by what may happen to the turtles (and potentially to other ectotherms) during acclimation to low temperatures. In ectotherms, physiological activities can have different optimal temperatures and temperature-influenced rates (Aleksiuk 1976; Arnold 1989; Dawson

1975). When the turtles were picked up from their pens, where they had ad libitum food, water, and basking sites, and were placed at 10° C, they may have become unable, while in acclimation, to fulfill individual physiological that required temperatures higher than 10° C. The disparate physiological states of individual turtles that required temperatures higher than 10° C may have lead to disparate thermoregulatory responses of individual turtles once exposed to a wide range of environmental temperatures, as happened while they were tested (do Amaral 2001b). At 20° C, the overall interspecific thermoregulatory pattern was clearer: Terrapene carolina had more occurrences of Type I thermoregulation (chaotic) than T. ornata.

Overall, male box turtles of either species consistently showed fewer chaotic thermoregulatory behaviors than the females. Physiological activities with different thermal requirements can be gender specific (e.g., embryogenesis in females and testicular recrudescence in males), and will impose different thermoregulatory strategies for each gender. For example, if the ability of ectothermic females to maintain T_b within a certain range is fundamental to follicle development, then female ectotherms may have a greater need to thermoregulate more frequently than males. On the other hand, increasing T_b also increases energetic costs, and it may be advantageous to have lower T_b's to conserve energy. Males may choose the latter strategy and be thermoconformers, possibly with episodic thermoregulatory bouts whenever their physiological activities such require. Other ectotherms (snakes) switch from thermoconformers to thermoregulators to digest food (Kollar 1988; Lutterschmidt and Reinert 1990; Regal 1966; Sievert 1989; Slip and Shine

1988), to shed (Reinert 1984; Reinert 1993; Reinert and Zappalorti 1988), or to promote testicular recrudescence (Viitanen 1967).

Postponing the discussion of the significance of noise, the species that lives in more thermally heterogeneous environments was also the one that showed a predominantly chaotic thermoregulation. Again, the intrinsically ever-changing, ever-testing, but deterministic model that chaos is, may give a good lifestyle strategy to box turtles. This allows them to live in environments that thermally do not allow the simultaneous fulfillment of all physiological activities. In a more traditional study of the same T_b time series, the two species showed different thermoregulatory preferences: T. carolina had higher $\overline{T}_b$ and higher thermoregulatory precision than T. carolina (do Amaral 2001b). My working assumption that the warmer habitat is less thermally diverse than the cooler habitat is a necessary oversimplification. Habitat thermal heterogeneity is a very complex phenomenon to measure. A habitat's thermal heterogeneity is affected by many variables such as size scale, activity times, and habitat structure.

In the LPS study, the injection of both doses of pyrogen was associated with a decrease in the thermoregulatory behaviors of Type I. Behaviors of Type II and IV dominated throughout. Type II is a thermoregulatory behavior that is stochastic, whereas Type IV even though it is a noisy behavior it has periods of high correlation. Regardless of the intricacies of the noise types, the injections of pyrogen caused a decrease in the determinism of the thermoregulation of T. carolina. In a more traditional study of the same T_b time series, it was determined that the two different doses caused opposite adjustments

of the thermal set-points: low dose caused behavioral anapyrexia, whereas high dose caused behavioral fever (do Amaral 2001a).

In the surrogate series generated by either method, the D_2's were different from those of the original series. The λ_1's for the shuffled surrogate series were statistically different from those of the original series, but the λ_1's for the Fourier transformed surrogate series were not statistically different from those of the original series. Therefore, the overall statistical differences in the surrogate time series suggested that the determinism, seen in the original time series, was the result of deterministic dynamic components of the time series. However, the statistical similarities between the λ_1 of the temperature series and the Fourier transformed surrogate are difficult to explain. As suggested by the crinkles in the series, the Fourier transformed surrogates may have been contaminated with periodicity artifacts (Schreiber and Schmitz 2000). Several cautions to the use of Fourier transformed surrogates have been suggested (Schreiber and Schmitz 2000), but with the software used such cautions could not be addressed.

Disease and stress can cause behaviors to became more linear deterministic. For example, healthy hearts have a chaotic heart rate, whereas heart condition caused the rate to become linear deterministic (Goldberger 1991), and thermal stress reduced the fractal dimension of the time series of cattle (Bos taurus) T_b (Hahn et al. 1992). In the box turtle experiments, linear determinism was always an absent thermoregulatory behavior. Instead, the type of thermoregulatory behavior that increased after injections of pyrogen was noise. Therefore, the thermoregulatory response of T. carolina to pyrogen does not follow the model observed in the heart rate. However, the vital maintenance of the organism's internal

milieu through homeodynamic processes (sensu Bassingthwaighte (1994)) does not have to follow necessarily a chaotic model, for non-linear control models with stochastic limit cycles can control biological phenomena such as EEG (Hernandéz 1996) or heart rate (Le Pape 1997).

With respect to the noise measured in the time series, chaotic systems are fairly insensitive to added noise and remain in the basin of attraction despite white Gaussian noise (Bassingthwaighte 1994). However, when turtles remained inactive in the gradient, their T_b did not remain stable. Instead, they acquired the oscillatory dynamics of the environmental room itself. This did not seem to affect the chaotic behavior outcomes, for the environmental room temperature series tested were non-chaotic. All of the tested environmental room temperature series were of Type II. Therefore, the strictly noisy thermoregulatory behaviors seen in many turtle T_b time series may have been environmental artifacts and not true thermoregulatory behaviors. But then again, there were turtles that remained active and yet showed noisy thermoregulatory behavior. At high dimensions, the distinction between chaotic and noise-contaminated systems becomes a matter of semantics (Sprott and Rowlands 1995). Noise is likely order that one cannot yet perceive, but as our understanding of chaos is expanding, so is the study of noise. If not an artifact, the presence of colored Gaussian noise thermoregulatory behavior in box turtles is remarkable. Colored Gaussian noise is a Gaussian random event with a finite correlation time or finite bandwidth (Jung and Hänggi 1988).

The presence of noisy time series of turtle T_b may show that during thermoregulation there are periods of instability or non-equilibrium (transient non-homeodynamic

physiological states). Therefore, turtles would show noisy thermoregulatory behaviors during periods of deregulation. However, one should not preclude linear determinism as yet another model of box turtle physiological strategy: a strategy used during periods of increased stress. However, in this study linear determinism may have been completely masked by environmental noise. Thermal stress was characterized by the fractal analysis of the time series of cattle (Bos taurus) T_b, where the animals exposed to increased heat stress showed a progressive reduction of the fractal dimension of their time series (Hahn et al. 1992). This suggests that the thermoregulatory models of endotherms and ectotherms may share some characteristics. The reduction of dimensions was also suggested as a way to recover a physiological limit cycle from a pathological one (Claude 1995).

The underlying controlling mechanism of thermoregulation may be relatively simple and yet generate thermoregulatory patterns of varying complexity. For example, non-linear control models with multiple negative feedback loops can show periodicity, quasiperiodicity, and period-doubling bifurcations leading to chaos (Glass and Malta 1990). The occurrence of many time series of turtle T_b with positive Lyapunov exponents and smaller-than-five correlation dimensions, and the consistent change in turtle chaosticity attributed to species and treatment with bacterial pyrogen, confirmed chaos as a good thermoregulatory model for turtles. Further research is necessary, especially to determine the influence of phylogenetic relationships on chaosticity through the use of the comparative method, to improve surrogate and operative temperature null hypotheses, to determine the influence of the sampling granularity on chaos detection and biological relevance (to reflect a balance between organism specific thermal inertia and maximal

information measured), and to determine whether chaosticity depends on the thermoregulatory rates of the organisms (slow-paced versus fast-paced animals). Moreover, the use of an open source (code available to the user) chaos software package is recommended for a total control of the algorithms used to analyze the time series.

Acknowledgements

I thank Richard G. Bowker for his introduction, suggestions, and guidance on the use of chaos to study thermoregulation. I thank J. Bastian, V. Hutchison, M. Kaspari, N. Shafer-Ray, and F. Sonleitner for reviewing this manuscript and for making helpful suggestions. Part of this work is from a dissertation submitted by the author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology at the University of Oklahoma, USA. I thank the Oklahoma Department of Wildlife Conservation and Bob St. Clair for supplying the turtles used in this study, and Robin Whitekiller, who helped with animal care. Part of this work was funded by two grants from the Graduate College of the University of Oklahoma. This paper was written while the author held a fellowship from Junta Nacional de Investigação Científica e Tecnológica PRAXIS XXI BD11292/97, Portugal.

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Table 1. Summary percentages of turtles (Terrapene sp.) classified into one of four types of thermoregulatory behavior, based on the values of the largest Lyapunov exponent (λ_1) and the correlation dimension (D_2) of their series of body temperatures. Type I ($\lambda_1 - \lambda_e > 0$; $D_2 + D_{2e} < 5$), corresponded to low-dimensional deterministic chaos; Type II ($\lambda_1 - \lambda_e > 0$; $D_2 + D_{2e} > 5$), corresponded to high-dimensional noise; Type III ($\lambda_1 - \lambda_e < 0$; $D_2 + D_{2e} < 5$), corresponded to linear determinism; and Type IV ($\lambda_1 - \lambda_e < 0$; $D_2 + D_{2e} > 5$), corresponded to highly correlated noise (colored Gaussian noise). The percentages marked with an asterisk are the highest percentages of each data row.

Acclimation	Species		Type I	Type II	Type III	Type IV	N
10° C	T. carolina	♀	30.00	52.50*	0.00	17.50	40
		♂	25.00	50.00*	0.00	25.00	20
	T. ornata	♀	22.22	55.56*	0.00	22.22	18
		♂	6.25	75.00*	0.00	18.75	16
20° C	T. carolina	♀	40.00*	40.00*	0.00	20.00	40
		♂	12.5	79.17*	0.00	8.33	24
	T. ornata	♀	10.53	78.95*	0.00	10.53	19
		♂	5.88	94.12*	0.00	0.00	17
20° C	T. carolina	control	35.29	47.06*	0.00	17.65	17
		low	17.65	47.06*	0.00	35.29	17
		high	18.75	68.75*	0.00	12.50	16

Figure Legends

Figure 1—(a) Representative time series of body temperatures of an active female box turtle (Terrapene carolina) acclimated at 20° C. The sampling period was 10 minutes, and the series spans 48 hours. The black bars represent the scotophase of the photoperiod. (b) State space embedding (τ = 30 min.) of the previous time series of body temperatures. The time series has a positive Lyapunov exponent ($\lambda_1 \pm \lambda_e = 0.23 \pm 0.07$) and the correlation dimension of the attractor is less than five ($D_2 \pm D_{2e} = 2.27 \pm 1.23$).

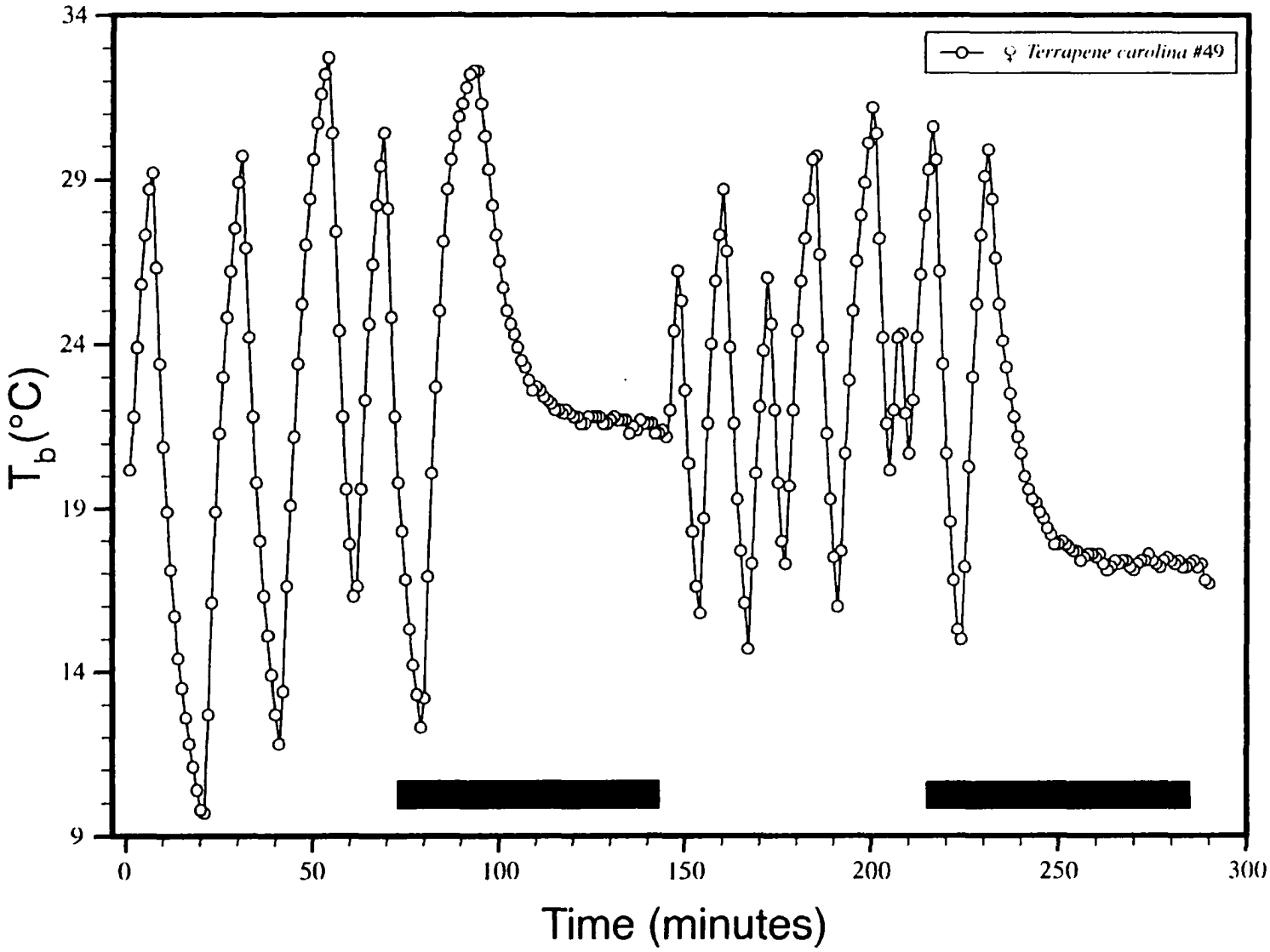
Figure 2—(a) Species comparison Lyapunov exponents (λ_1) for Terrapene carolina and T. ornata acclimated at 10° C. Positive λ_1 may indicate chaotic time series. (b) Species comparison correlation dimensions (D_2) for Terrapene carolina and T. ornata acclimated at 10° C. D_2's larger then 5 basically indicate noise. Deterministic chaos has D_2's lower than five. The vertical lines are 95% confidence intervals (see text for details). The values were sorted in decreasing order for clarity.

Figure 3—(a) Species comparison Lyapunov exponents (λ_1) for Terrapene carolina and T. ornata acclimated at 20° C, positive λ_1 may indicate chaotic time series; and (b) correlation dimensions (D_2) for Terrapene carolina and T. ornata acclimated at 20° C. D_2's larger then 5 basically indicate noise, deterministic chaos has D_2's lower than five. The vertical lines are 95% confidence intervals (see text for details). The values were sorted in decreasing order for clarity.

Figure 4—(a) Lyapunov exponents (λ_1) for Terrapene carolina treated with pyrogen, and acclimated at 20° C. Positive λ_1 may indicate chaotic time series. (b) Correlation dimensions (D_2) for Terrapene carolina treated with pyrogen, and acclimated at 20° C. D_2's larger then 5 basically indicate noise. Deterministic chaos has D_2's lower than five. The vertical lines are 95% confidence intervals (see text for details). The values were sorted in decreasing order for clarity.

Figure 5—(a) Analysis of covariance of Lyapunov exponents and correlation dimensions of the time series of Terrapene carolina and T. ornata acclimated at 10° C, (b) acclimated at 20° C, and (c) analysis of covariance of Lyapunov exponents and correlation dimensions of the time series of Terrapene carolina treated with saline (controls: triangles) or doses of pyrogen (low dose, 2.5 μg pyrogen: open circles; low dose, 25 μg pyrogen: solid circles) and acclimated at 20° C. The density ellipses indicate a probability of 95 (dotted) and 50% (dashed). Positive λ_1 may indicate chaotic time series. D_2's larger then 5 basically indicate noise. Deterministic chaos has D_2's lower than five. Details of the contributions of each species and treatment to each plot in this figure are listed in Table 1.

Figure 1a


♀ Terrapene carolina #49
T_b (°C)
34
29
24
19
14
9
0
50
100
150
200
250
300
Time (minutes)

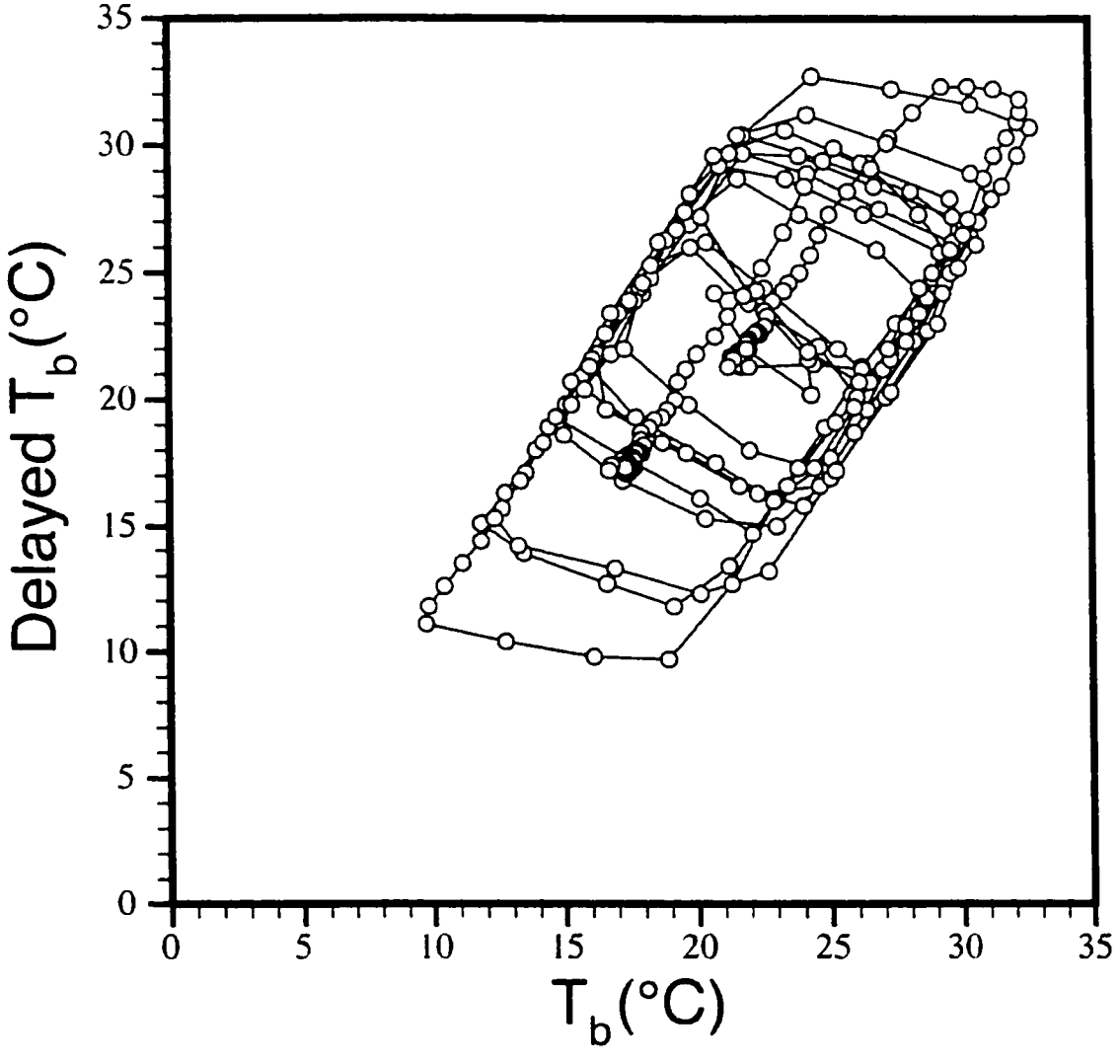


Figure 1b

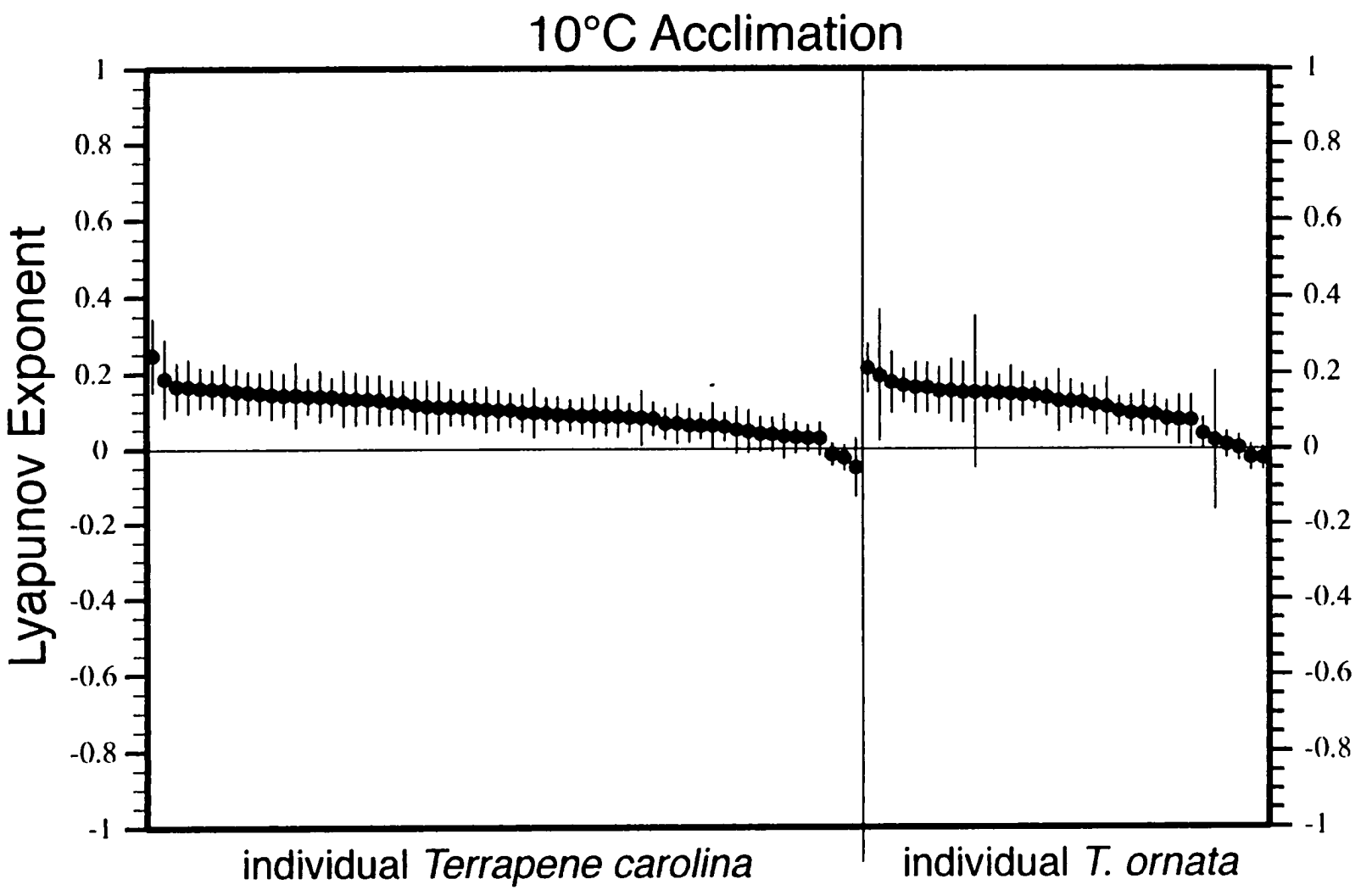

10°C Acclimation
Lyapunov Exponent
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
individual *Terrapene carolina*
individual *T. ornata*

Figure 2a

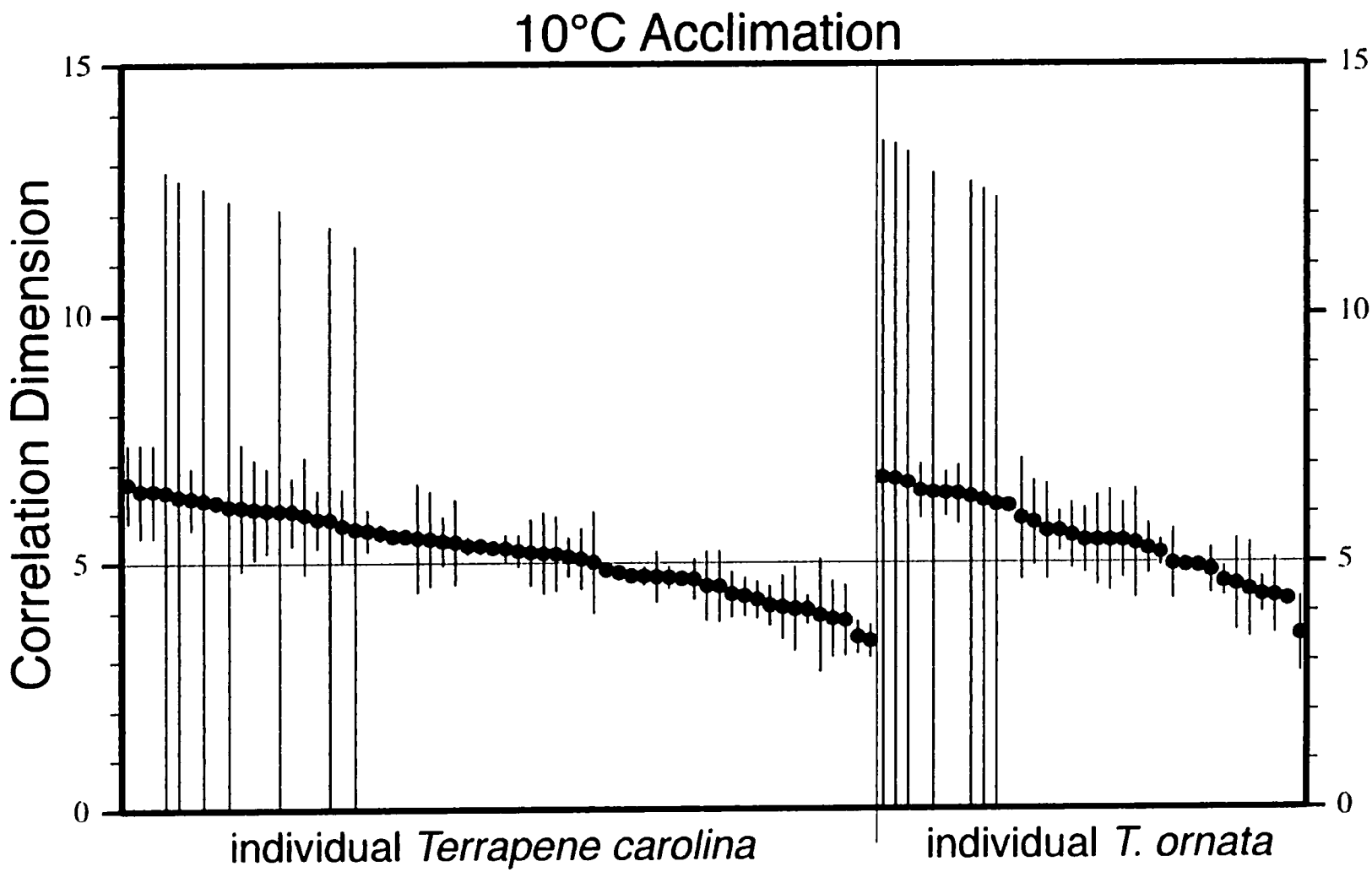
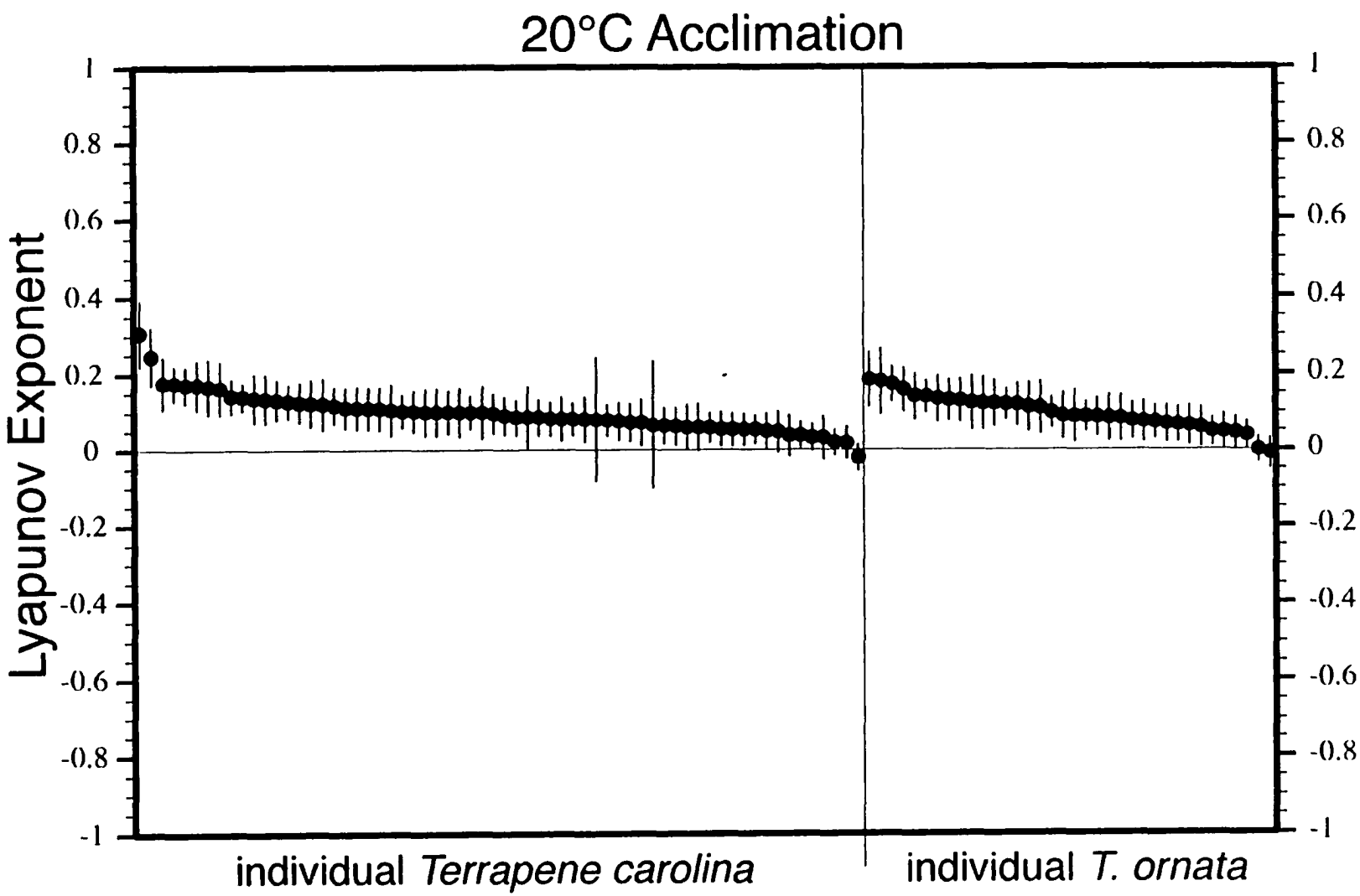

10°C Acclimation
Correlation Dimension
15
10
5
0
individual *Terrapene carolina*
individual *T. ornata*

Figure 2b

Figure 3a


20°C Acclimation
Lyapunov Exponent
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
individual *Terrapene carolina*
individual *T. ornata*

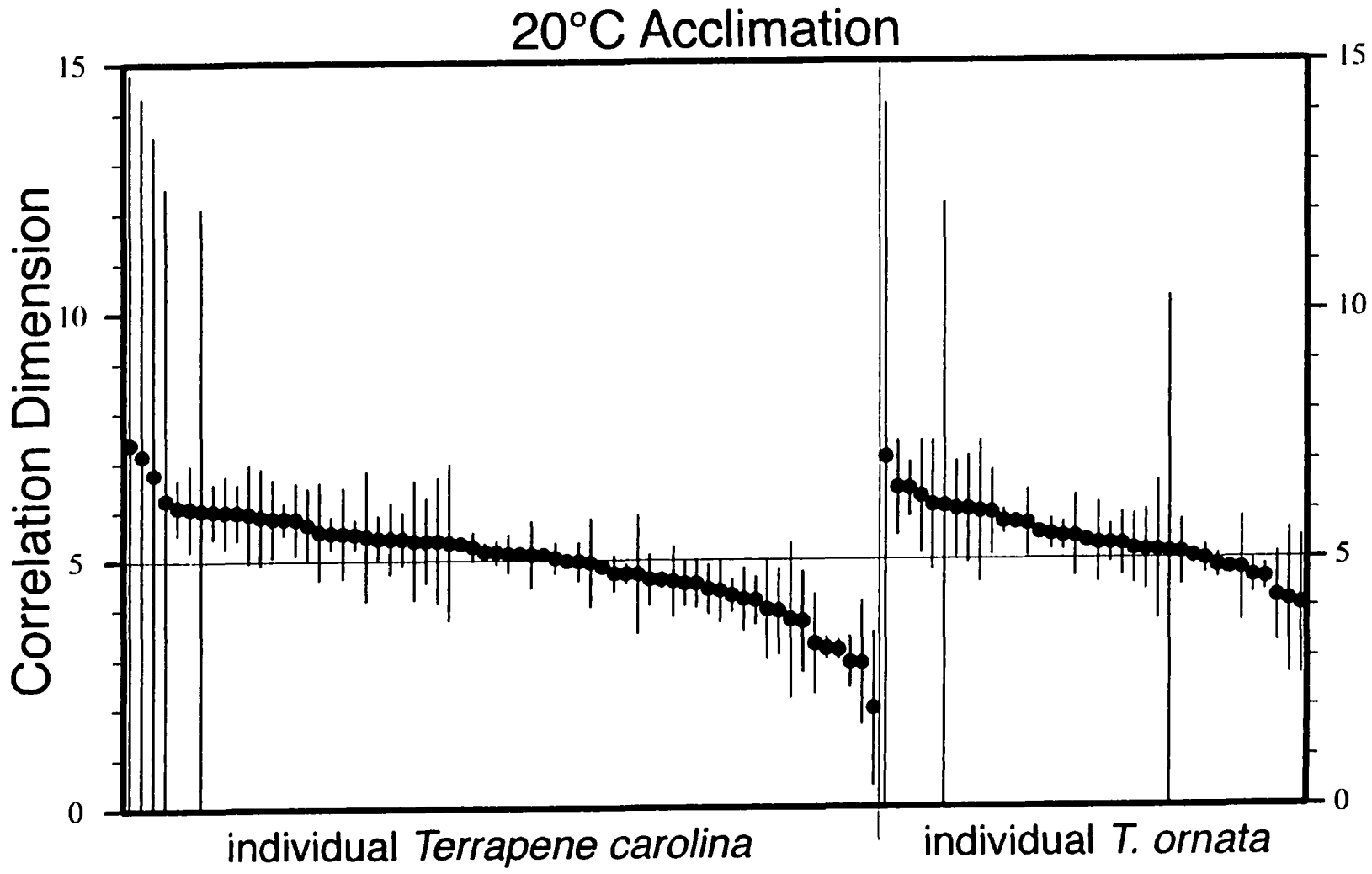

20°C Acclimation
Correlation Dimension
15
10
5
0
individual *Terrapene carolina*
individual *T. ornata*

Figure 3b

Figure 4a

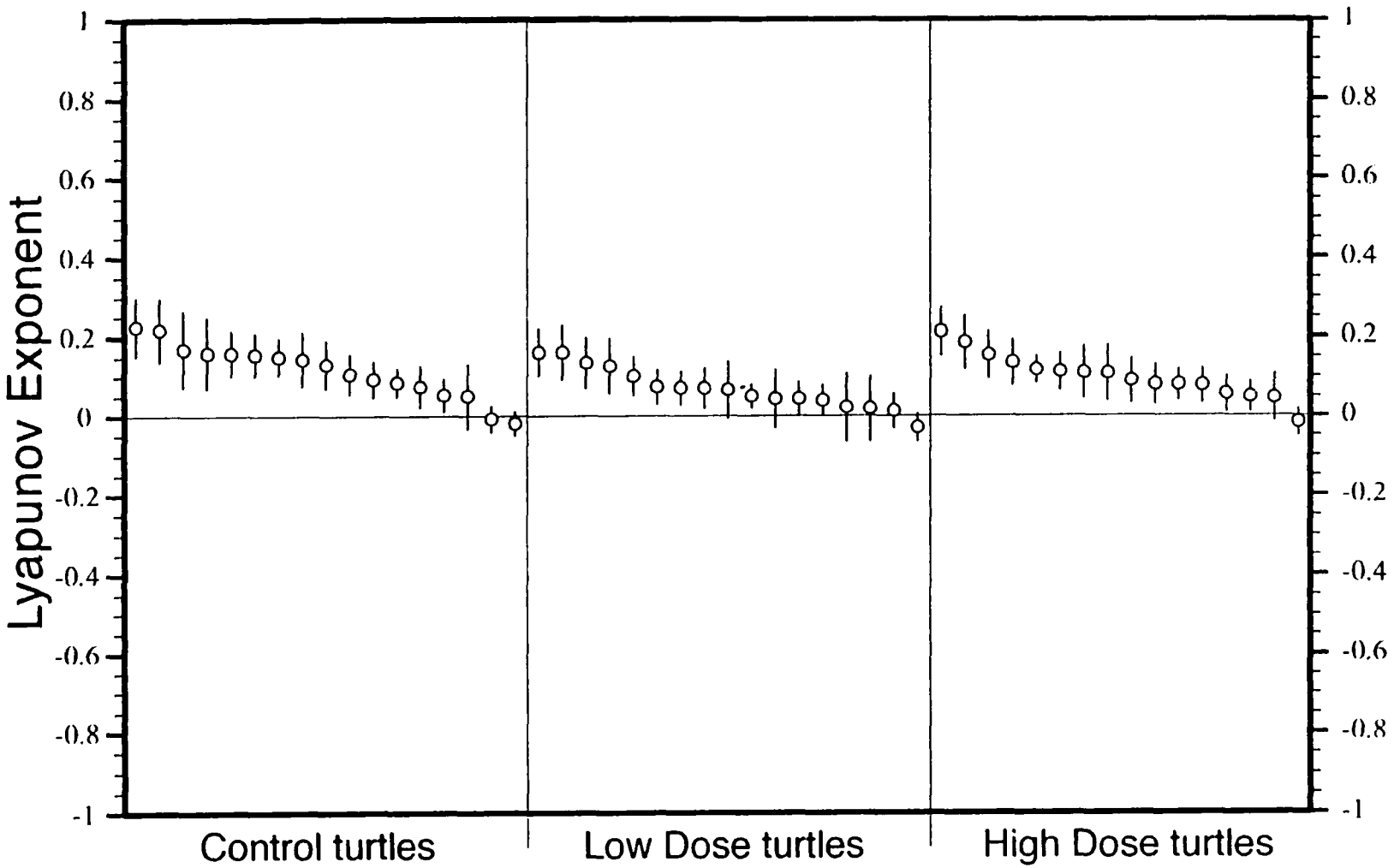
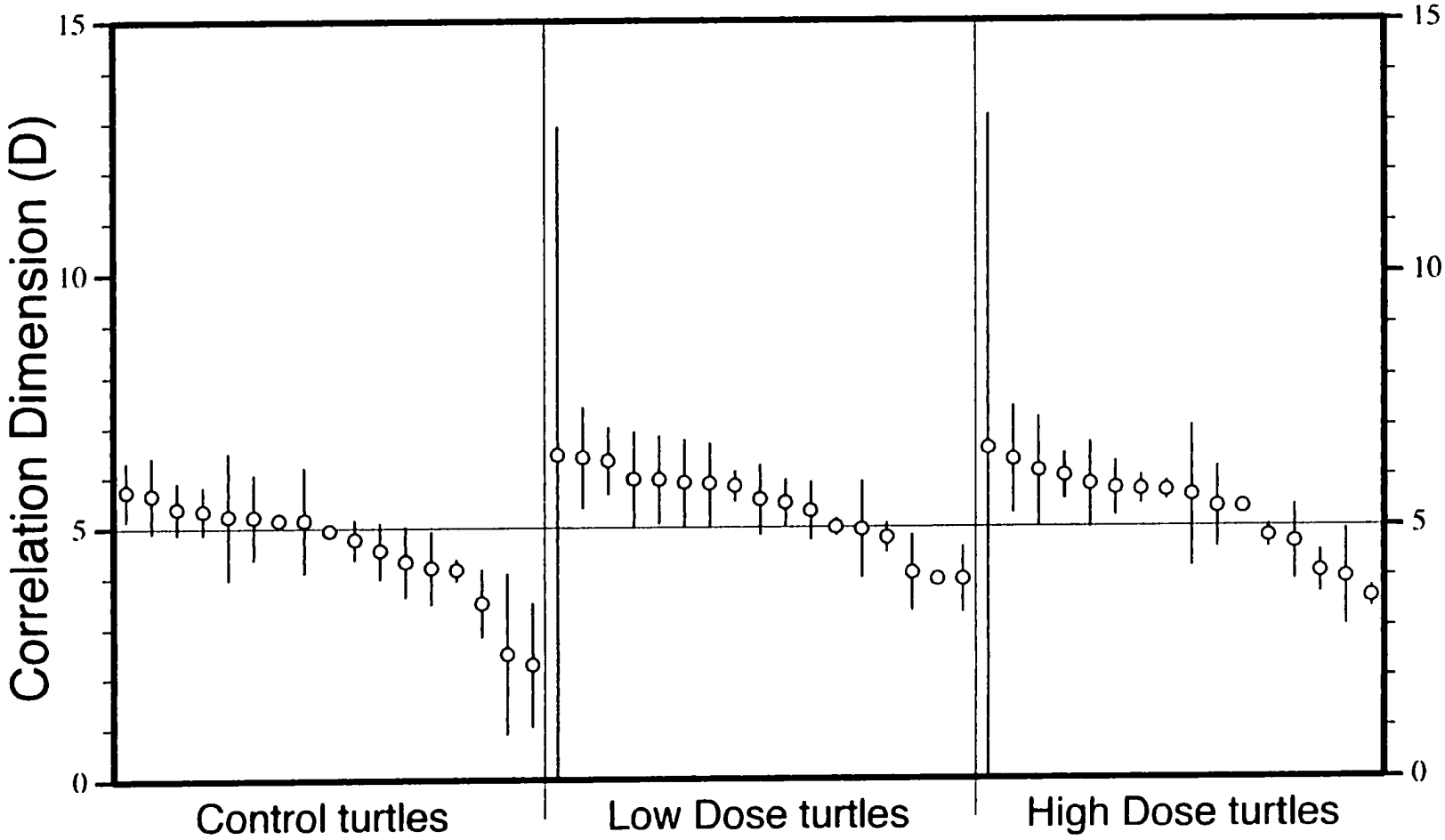

Lyapunov Exponent
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Control turtles
Low Dose turtles
High Dose turtles

Figure 4b


Correlation Dimension (D)
15
10
5
0
Control turtles
Low Dose turtles
High Dose turtles

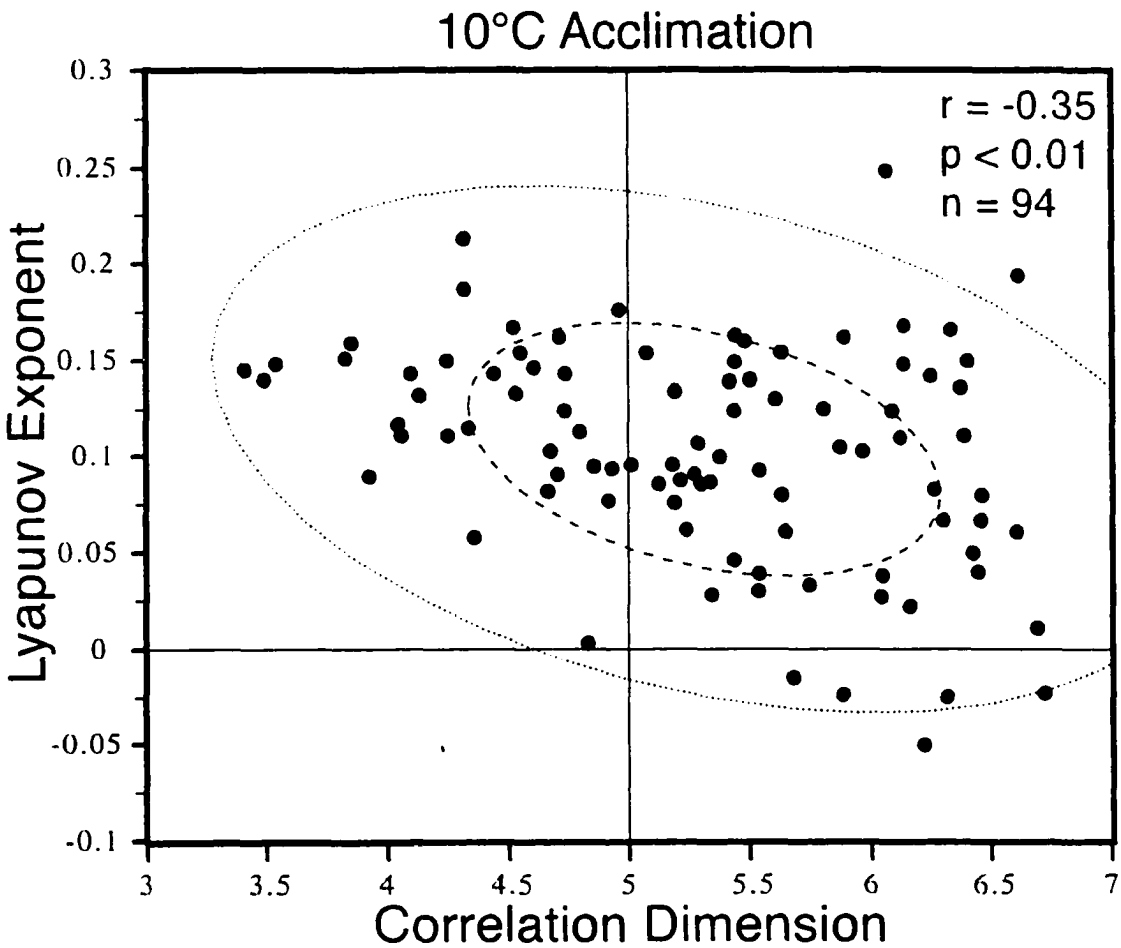

10°C Acclimation
r = -0.35
p < 0.01
n = 94
Lyapunov Exponent
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
3
3.5
4
4.5
5
5.5
6
6.5
7
Correlation Dimension

Figure 5a

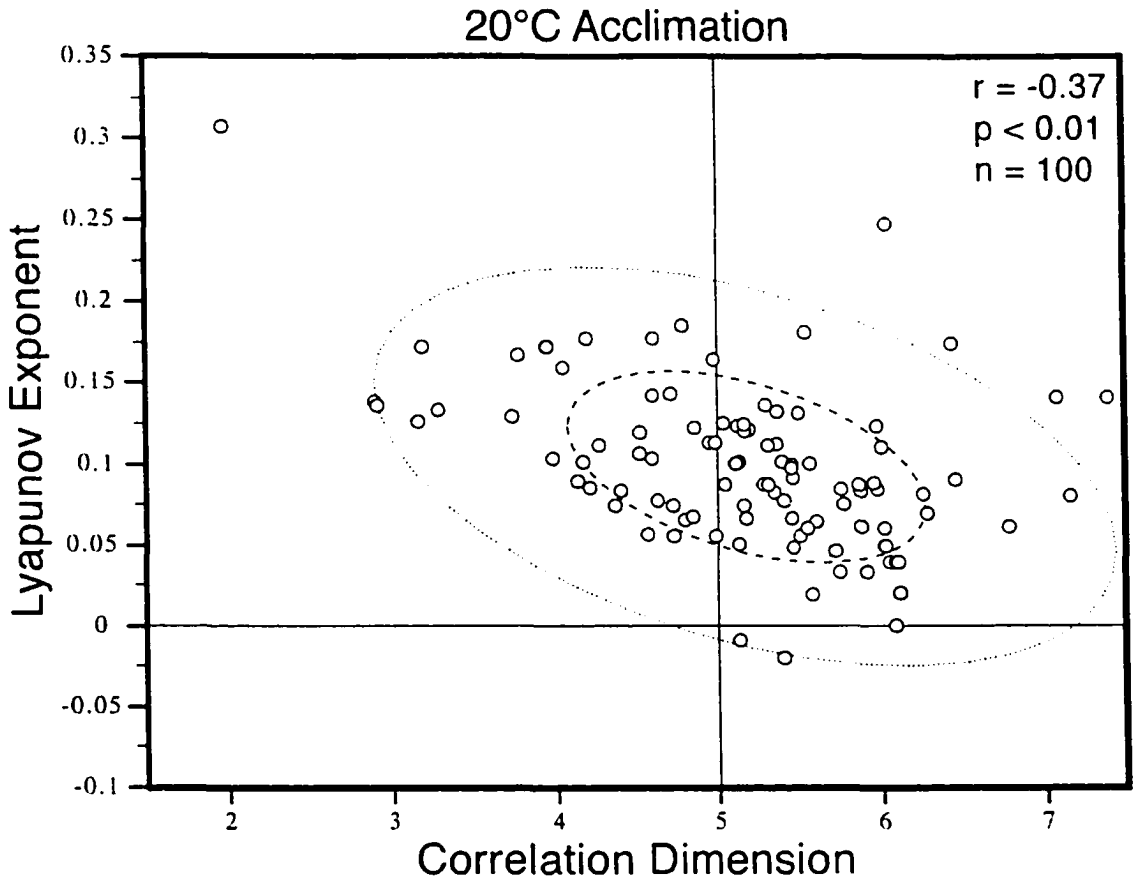

20°C Acclimation
r = -0.37
p < 0.01
n = 100
Lyapunov Exponent
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
2
3
4
5
6
7
Correlation Dimension

Figure 5b

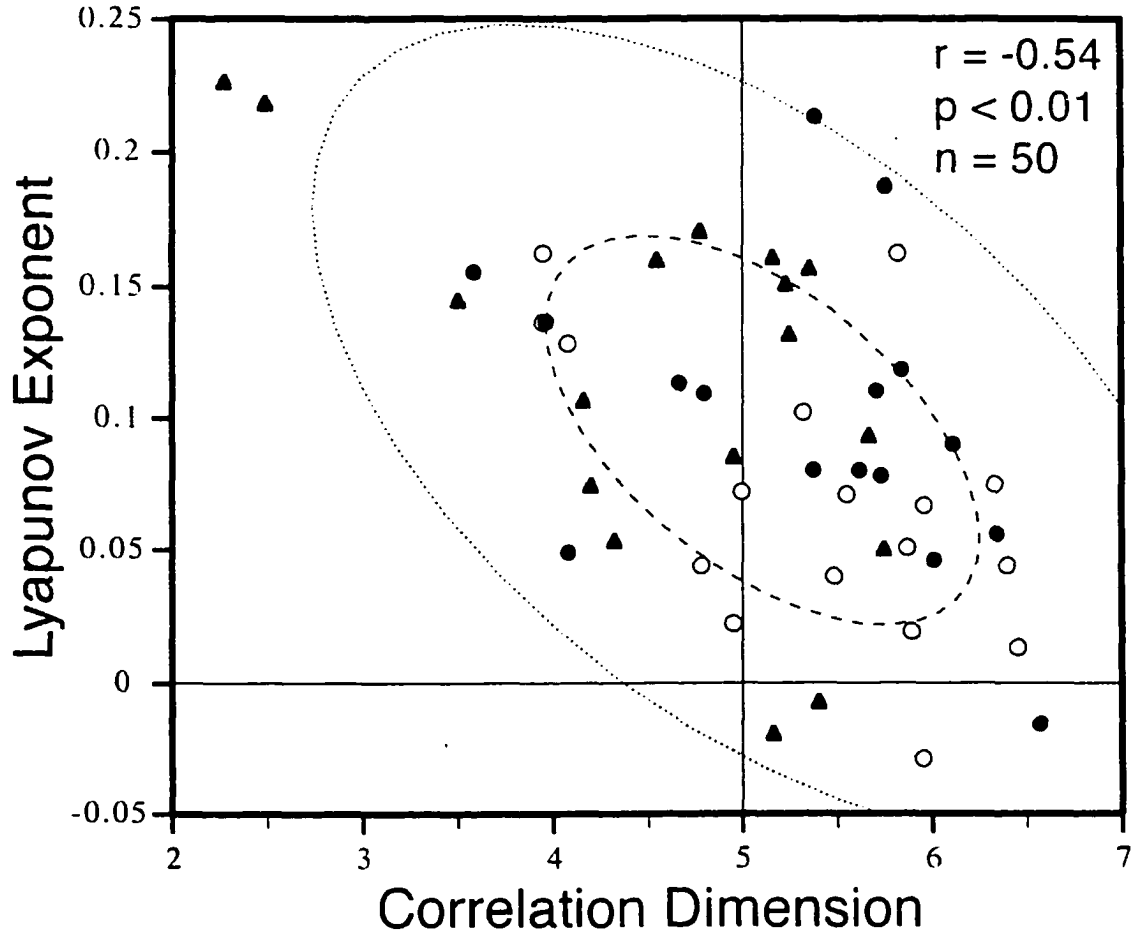

r = -0.54
p < 0.01
n = 50
Lyapunov Exponent
Correlation Dimension
0.25
0.2
0.15
0.1
0.05
0
-0.05
2
3
4
5
6
7

Figure 5c