EFFECTS OF INCUBATION TEMPERATURE ON THE PHYSIOLOGY, BEHAVIOR, AND MORPHOLOGY OF TURTLES

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EFFECTS OF INCUBATION TEMPERATURE ON

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MORPHOLOGY OF TURTLES

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I. OVERVIEW

This project measured effects of incubation temperature (T_{inc}) on a suite of embryonic and post-embryonic characteristics. Turtles were selected as model organisms for two reasons. First, many taxa exhibit an enigmatic temperature-driven mechanism of gonad differentiation commonly referred to as 'temperature-dependent sex determination' (TSD), although recent research regarding possible interactions between temperature, steroid hormones and/or sex genes suggest that 'temperature-*influenced* sex determination' might more accurately describe the phenomenon. In either case, this research was conducted in part to investigate the evolutionary persistence of TSD, the ancestral sex-determining mechanism in turtles. Second, species comprising the Order Chelonia are among the vertebrate taxa most threatened with extinction. Threats to turtles derive from multiple sources, including ever-increasing habitat alteration and destruction, surges in populations of subsidized predators (species that benefit from encroaching anthropogenic development), demand for turtle meat, especially by an exponentially growing Chinese middle class and, finally, the prospect of unprecedented rates of climate change. This latter issue strongly motivated my research, because although the degree to which climatic conditions are likely to change is significant, many questions remain regarding turtles' resilience to large-scale temperature shifts.

Climate change is a complex phenomenon that is expected to affect aspects of turtle biology at all life stages. Although an upward shift in the global mean temperature is expected, effects on temperature are expected to be variable across spatial and temporal

scales. In addition, climate change is likely to affect a suite of other variables, including rainfall patterns, groundwater tables, primary productivity, and floral assemblages, to name a few. I elected to focus my work on the effects of temperature during embryonic development because, whereas post-embryonic life stages can behaviorally influence the microclimate that they experience, embryos lack this capacity. Therefore, in order to survive, turtle embryos must either be physiologically tolerant of conditions that elicit anhomeostatic conditions, or possess physiological means of modulating the effects of temperature. Additionally, temperature-effected changes during embryogenesis are more likely to be organizational (i.e., nonreversible) than similar exposure later in life, and therefore have lasting consequences for individual fitness.

Originally described in a European lizard (*Agama agama*) and characteristic of approximately two-thirds of turtle species, one of the more astounding organizational effects of T_{inc} is the previously mentioned phenomenon of TSD. This single trait affects individuals' reproductive potential and may have profound effects on population dynamics. Whereas this unusual trait has been studied and described in a broad cross-section of turtles, studies of other more subtle effects of incubation temperature have been performed on a much narrower subset of chelonians. I investigated the effects of T_{inc} on embryo development and metabolism, as well as the occurrence and persistence of temperature effects on post-embryonic size, growth, and metabolic rate in a cross-section of chelonian taxa.

The species I elected to work with represent four families, exhibit two distinct modes of sex determination (TSD and genetic sex determination; GSD), and occur in habitats ranging from exclusively aquatic to arid terrestrial. Although formal

phylogenetic analyses of T_{inc} effects are precluded by the combined broad cross-section and limited sample of taxa represented, I hope that some measure of the variability of thermal effects among turtles can be achieved by these comparisons. This variability may be important from a practical standpoint because it will inform conservation biologists of the degree to which data pertaining to one species may be extrapolated to other species of conservation concern. Included in subsequent chapters are results for the following four species:

1) *Geochelone sulcata* (African Spurred Tortoise; Family Testudinidae) – native to sub-Saharan Africa, this largest of mainland tortoises has likely already experienced shifts in the local climate as the Sahel region has become more arid and stripped of vegetation. Continued warming is likely to compound the effects of vegetational shifts in the region that have contributed to declines in annual rainfall since the 1960s. Interestingly, farmers in parts of this region have initiated large-scale reforestation to improve the landscape for their own habitation. These efforts may have the secondary effect of improving the habitat for *G. sulcata* in parts of its range.
2) *Apalone spinifera* (Spiny Softshell Turtle; Family Trionychidae) – found throughout the eastern two-thirds of the United States, *A. spinifera* belongs to a family that possesses many adaptations to an aquatic lifestyle, including heavily webbed feet, a hydrodynamic shape, and a capacity for extensive aquatic respiration. *Apalone spinifera* typically constructs shallow nests in sandy soil where temperatures closely mirror fluctuations in air temperature. Unlike the

other taxa surveyed, however, this species exhibits GSD; therefore, the temperature-specific differences that are reported are likely not confounded by sex.

3) *Trachemys scripta* (Red-eared Slider Turtle; Family Emydidae) – native to the southeastern United States, but now found inhabiting a wide range of habitats on several continents, *T. scripta* is a freshwater species whose ecology approximates that of many congeners. This species is aquatic, but is known to engage in extensive terrestrial nesting forays and overland migrations. Like all emydids, *T. scripta* lays parchment-shelled eggs, so adequate soil moisture is essential to prevent desiccation. Although nests lie close to the substrate surface and thus are likely to be affected by increased temperatures more than in deeper nests (usually produced by larger species), population differences in nest depth indicate that nests are dug deeper and close to water in drier climates. Unless these differences are strongly genetically influenced, this species may be predicted to exhibit resilience to climate change through changes in nest location and depth selection.

4) *Macrochelys temminckii* (Alligator Snapping Turtle; Family Chelydridae) – this species is notable for two reasons: it is the largest freshwater turtle in North America, and it ranks among the most aquatic. Unlike most turtle species, it does not thermoregulate via basking (heliothermy); rather, it restricts its activities to habitat where water temperatures are within the range of preferred body temperatures (thigmothermy). The result of this constraint on habitat use is a predictable pattern of seasonal movements that correlate with changes in water temperature, an environmental variable likely to be affected by climate change.

The depth at which eggs are deposited is highly variable, a fact that might mute the effects of directional temperature fluctuation.

The applicability of this research to conservation efforts varies among the species studied. The data acquired for *M. temminckii* are already being used to good effect in captive propagation efforts at Tishomingo National Fish Hatchery in southern Oklahoma. The productivity of this program has increased steadily in the last five years, and is well on its way to meeting its goal of re-establishing populations throughout eastern Oklahoma. Similar conservation efforts have been initiated in the last decade for *G. sulcata*; I hope that data presented here will help inform the methods employed for propagating this endangered species.

Results generated for *T. scripta* and *A. spinifera* are less directly applicable, as these species are still widespread and common in the wild. However, there is the potential that generalizations from these species will help inform conservation efforts of some of the highly endangered Trionychids and Emydids for which intensive conservation efforts have been established in the United States and overseas.

Chapters II-V of this manuscript are intended for publication, and are formatted for the journals to which I intend to submit them. Chapter II has been formatted for submission to Physiological and Biochemical Zoology. It addresses T_{inc} effects on embryonic development. Data are reported for just the latter three species listed above because their sympatry makes them a more natural grouping than would be the case had *G. sulcata* data been included.

Chapters III, IV, and V examine post-embryonic temperature effects in *T. scripta* (III), *G. sulcata* (IV), and *M. temminckii* (V), and are formatted for submission to the Journal of Experimental Biology, Canadian Journal of Zoology, and Biological Conservation, respectively. Each species was given its own chapter as a means of breaking up the dataset into units suitable for publication, and because the large ecological and phylogenetic differences among species made direct comparisons unwieldy. It was necessary to preclude a chapter describing post-embryonic effects of T_{inc} in *A. spinifera* because of the small sample size representing this species (one clutch of 16 eggs) and, more importantly, high hatchling mortality rates. It is my hope to readdress T_{inc} effects in *Apalone* in the future.

II. INTERSPECIFIC VARIATION IN TEMPERATURE EFFECTS ON EMBRYONIC METABOLISM AND DEVELOPMENT IN TURTLES

ABSTRACT

The limited materials contained in eggs must be budgeted by embryos to meet requirements for maintenance, organogenesis, and growth. In species that do not incubate their eggs, this allocation of material and subsequent energy use by embryos must be flexible to permit successful development across a range of thermal conditions. This study measured temperature-induced differences in metabolic rates and growth by embryos of three turtle species, *Macrochelys temminckii*, *Trachemys scripta*, and *Apalone spinifera*, at different constant temperatures and examining variation in physiological mechanisms employed to deal with temperature differences during embryonic development, a stage during which behavioral thermoregulation is infeasible. Oxygen consumption rate (VO_2) was measured at least weekly during development, and used to characterize changes in metabolism and calculate total O_2 consumption. Results from eggs incubated at different temperatures were used to calculate Q_{10}s at different stages of development, and to look for evidence of metabolic compensation. Total O_2 consumption over the course of incubation was lowest at high incubation temperatures, and late-term metabolic rate Q_{10}s were < 2 in all three species. Both results are consistent with positive metabolic compensation. However, incubation temperature effects on egg

mass-corrected hatchling size varied among species. Mass of hatchling *A. spinifera* was unaffected by temperature, whereas *T. scripta* hatchling mass was greatest at high temperatures, and *M. temminckii* mass was lowest at high temperatures. Hatchling mass:length relationships tended to correlate negatively with temperature in all three species. Although we cannot reject positive metabolic compensation as an additional factor contributing to the observed VO_2 patterns, there is precedence for drawing the more parsimonious conclusion that differences in yolk-free size alone produced the observed incubation temperature differences without energetic canalization by temperature acclimation during incubation.

Introduction

Although the suite of biochemical activities that contributes to an organism's metabolism is complex and therefore challenging to model (Clarke 2004), strong relationships exist between body temperature and whole-organism metabolic rate (Gillooly et al. 2001). Various thermoregulatory mechanisms are employed by animals to dissociate body temperature from ambient temperature. Endothermy has evolved repeatedly as a physiological means of surviving suboptimal thermal conditions, but ectothermic species often rely primarily on behavioral strategies to regulate body temperature. In addition to behavior, however, ectotherms may exhibit physiological mechanisms to address thermal constraints of their environment. Solutions for surviving inhospitable temperatures may include manipulating biochemical reaction rates by varying enzyme concentrations or receptor densities, production of chaperone proteins to increase the range of temperatures over which target enzymes remain functional (Feder

and Hoffman 1999), changing the composition of cell membranes to affect permeability and (rarely) producing temperature-specific isozymes (Hazel and Prosser 1974; Lin et al. 1996; Somero 2004).

In comparison to other life stages when behavior can play an important role in overcoming thermal constraints, means for maintaining suitable body temperatures of oviparous animals during embryonic development are limited. Three factors contribute minimizing an embryo's exposure to or effects of suboptimal thermal conditions: 1) indirect behavioral temperature selection via maternal thermoregulation or nest-site selection; 2) developmental diapause during periods when thermal conditions are unsuitable; and 3) utilization of one or more of the physiological strategies listed above. These strategies may function alone or in combination to circumvent thermal limitations on development. For example, because the efficacy of maternal nest-site choice may be limited by the stochastic nature of environmental temperatures (Morjan 2003), maternal nest-site selection seems likely to function in combination with complementary physiological compensatory strategies.

Much attention has been paid to the effects of temperature on hatchling traits, particularly among taxa that exhibit temperature-dependent sex determination. In addition to effects on population sex ratios in many species (Bull and Charnov 1989; Crews et al. 1994; Ciofi and Swingland 1997), temperature has been demonstrated to influence hatchling size (Brooks et al. 1991; Spotila et al. 1994; Roosenberg and Kelley 1996; O'Steen 1998; Demuth 2001; Du and Ji 2003), post-hatching growth (Ryan et al. 1990; Brooks et al. 1991; McKnight and Gutzke 1993; Bobyn and Brooks 1994; Spotila et al. 1994; Rhen and Lang 1995; Roosenburg and Kelley 1996; O'Steen 1998; Rhen and Lang

1999; Demuth 2001; Steyermark and Spotila 2001; Du and Ji 2003; Warner and Shine 2005), locomotor performance (Janzen 1993; 1995; Doody 1999; Demuth 2001; Du and Ji 2003), metabolic rate (O'Steen and Janzen 1999; Steyermark and Spotila 2000), agility (Janzen 1995), and crypsis (Janzen 1995). The ways in which temperature experienced during embryonic development affect post-embryonic endpoints have received more attention among reptiles than have endpoints measured during embryonic development (Booth 2006). Thus, some aspects of how differences in the thermal environment experienced by embryos lead to morphological and performance differences remain poorly understood.

The finite quantity of energy and materials packaged in an egg must be budgeted to meet development and growth requirements. Therefore, temperature-induced differences in energy utilization could trickle down to affect post-hatching condition of offspring by directly or indirectly influencing morphology, physiological performance, or postembryonic energy reserves. The effects of temperature on embryo metabolism have been studied in a variety of reptiles (Thompson 1989; 1993; Birchard and Reiber 1995; Angilletta et al. 2000; Booth et al. 2000; Vladimirova et al. 2005a; 2005b). Patterns in the relationship between temperature and embryo energy expenditure have been fairly consistent. For example, Angilletta et al. (2000) found that, in the lizard *Sceloporus undulatus*, energy expenditure over the course of incubation was similar at 30°, 32° and 34° C, but 10-15% lower compared to embryos that developed at 28° C. Similarly, *Crocodylus johnstoni* embryos consumed 10% less oxygen at 31° C than at 29° C (Whitehead and Seymour 1990), and embryonic Nile soft shell turtles (*Trionyx triunguis*) incubated at 27° and 30° C exhibited similar energetic expenditure, but used 5% less

oxygen at 33° C (Leshem et al. 1991). In contrast to this pattern of lower oxygen consumption at higher temperatures, no effect of incubation temperature was observed among *Emydura signata* embryos incubated at 24° and 31° C (Booth 1998) or *Chelonia mydas* incubated at 26° and 30° C (Booth and Astill 2001).

In combination, these studies suggest that embryos of many reptiles utilize less energy during development at higher incubation temperatures, but some species may exhibit a capacity to physiologically correct for sub-optimally high or low body temperatures, commonly referred to as positive compensation (Hochachka and Somero 1973; Hazel and Prosser 1974). The goal of this study was to investigate differences in incubation temperature effects on embryonic development and metabolism among a sympatric but phylogenetically diverse assemblage of freshwater turtles (Ernst et al. 1994), including *Trachemys scripta*, *Apalone spinifera*, and *Macrochelys temminckii*. My objectives were three-fold. The first was to measure the capacity for metabolic compensation during embryonic development by comparing stage-specific VO_2 (oxygen consumption rate) of embryos exposed to different constant incubation temperatures. These measurements were then used to calculate differences in Q_{10} (the rate of change of a physiological process as a consequence of increasing the temperature by 10° C) at different stages of development. Second, I evaluated temperature effects on energetic cost of development by calculating the total volume of oxygen (O_{2total}) used over the course of incubation, and mass-conversion efficiency based on differences in hatchling mass after correcting for variation in initial egg mass. Finally, I qualitatively assessed the degree of variation in different species' responses to temperature.

Material and Methods

All procedures for this research were approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocol #AS023), guaranteeing compliance with animal care guidelines described in The Guide for the Care and Use of Laboratory Animals, 7th edition (1996).

Study Species

The three turtle species included in this study represent three different families and, although sympatric in parts of their range, exhibit substantial ecological differences. *Trachemys scripta* is a medium-sized Emydid that is primarily aquatic but engages in frequent basking and terrestrial migrations between water bodies, and conforms to the Type Ia pattern of temperature-dependent sex determination (Ewert et al. 1994) wherein males are produced at low temperatures and females at high temperatures. *Apalone spinifera* (Family Trionychidae) exhibits many adaptations to a pelagic lifestyle, including a hydrodynamic form and substantial capacity for aquatic respiration. In contrast to most turtles, sex is determined genetically in members of this family. Finally, *Macrochelys temminckii* (Family Chelydridae) is a very large-bodied, primarily bottom-dwelling species that seldom leaves water except to nest. It follows a Type II pattern of temperature-dependent sex determination, characterized by development of females at low and high temperatures, and males or a mixed sex ratio at intermediate temperatures (Ewert et al. 1994).

Egg Collection

Eggs were obtained for all three species in May and June 2004. A single *A. spinifera* nest was excavated from a sand bar at Sequoyah National Wildlife Refuge (SNWR) in eastern Oklahoma, and transported to Oklahoma State University (OSU) within 15 h after deposition. Gravid *T. scripta* were trapped in an oxbow at SNWR using baited hoop nets. These turtles were transported to OSU where oviposition was induced using oxytocin (0.10 IU/kg IM) (Ewert and Legler 1978). Turtles were placed individually in plastic tubs containing approximately 15 cm of water to minimize accidental destruction of eggs by the turtles. After eggs were obtained, the adult females were released at SNWR. Finally, *M. temminckii* eggs were obtained from a captive group maintained at Tishomingo National Fish Hatchery as part of a captive breeding/reintroduction program. The adult turtles by which the eggs for this study were produced originated from SNWR. Eggs were laid naturally and excavated from nests within two d following oviposition. No eggs of any of the three species showed signs of the white banding characteristic of early development (Webb et al. 1987) prior to arriving at OSU.

Incubation

Eggs from each species were measured (±0.1 mm) and weighed (±0.01 g), and then assigned to an incubation treatment in a randomized block design (block = clutch). Within each incubation treatment, eggs were distributed among 1-5 plastic shoeboxes (1.5 L) half-filled with damp vermiculite (1:1 vermiculite:water by mass; ~-150 kPa water tension; Packard et al. 1987). Shoeboxes were then assigned to one of three constant-temperature incubators set at 26.5°, 28.5°, and 30.5° C. Boxes were rotated

within each incubator daily to eliminate the possibility of position effects, and each box was weighed weekly and rehydrated as necessary to maintain its initial mass. Eggs were candled every 2-3 days during early development, and eggs that failed to develop were discarded to eliminate substrate for invasion of mold.

Hatchlings

Upon pipping, each egg was placed in a plastic jar lined with dampened paper towels so that the identity of individuals could be determined after hatching. After emerging from the egg shell, hatchlings were kept in the plastic jars until residual yolks were completely internalized, a period that lasted 0-9 d and varied among species. *T. scripta* and *M. temminckii* then received unique markings to aid future identification, and were removed from the incubator to flow-through raceways.

Metabolic Rate

Metabolic rates were estimated by measuring changes in oxygen concentration in chambers via closed-system respirometry (Vleck 1987) and calculating VO_2 (Peterson 1990). Eggs were placed individually in metabolic chambers constructed from 169-mL plastic jars with screw-top lids. A stopcock was inserted through each lid and sealed in place with silicon. Initial air samples were drawn from each chamber into stopcock-equipped 30-cc syringes, and then sealed. The chambers were then placed into the incubators for 1-1.5 h (longer during early development when VO_2 was expected to be low). Chambers were then removed from the incubator, and final air-samples were drawn

into a second set of syringes. Eggs were weighed at the conclusion of each measurement and returned to the plastic shoeboxes.

Oxygen concentrations of all air samples were analyzed in 10-mL aliquots with a Sable Systems FC-1 oxygen analyzer. A stream of air was drawn from outside the building at a regulated flow rate of 100 mL/min. It passed through serial columns of Drierite and Ascarite to remove water and CO_2, respectively. Each aliquot was injected into the air stream, which passed through a small column of Drierite and Ascarite and then through the oxygen analyzer. VO_2 was calculated for each turtle as the difference between the initial and final volumes of oxygen after correcting for chamber volume (Peterson 1990).

VO_2 of *T. scripta* and *M. temminckii* embryos was measured at 7-d intervals starting 7 d after oviposition. *A. spinifera* embryo VO_2 was measured at 2-3 d intervals early and late in development, and on a 7-d schedule during the middle third of incubation.

The volume of oxygen used over the course of incubation was calculated for each turtle that successfully hatched by summing the trapezoidal areas created by adjacent VO_2 measurements using the equation:

$$O_{2total} = \sum^{i} 24(VO_{2n} + VO_{2n-1})(T_n - T_{n-1}) / 2$$

Where O_{2total} was the oxygen consumed over the duration of incubation, VO_2 was the rate of oxygen consumption at age n measured in mL/h, T was the number of days since the beginning of incubation, n was the days on which VO_2 was measured, and p was day on

which each turtle pipped. Because turtles hatched 0-7 d after the last embryo VO_2 measurement, VO_{2p} at the time of pipping was estimated based on the difference between VO_2 measurements prior to and after hatching. These estimates were calculated as:

$$VO_{2p} = ((VO_{2hatchling} - VO_{2final})(T_{hatchling} - T_{final})^{-1}(T_{pip} - T_{final})) + VO_{2final}$$

Where $VO_{2hatchling}$ was measured at each hatchling's assigned incubation temperature following internalization of the residual yolk, and VO_{2final} was the last measurement prior to pipping. These values were used to calculate the final trapezoidal area of each turtle's O_{2total} to produce a precise estimate of the volume of oxygen used between oviposition and hatching.

Statistics

Statistical analyses were conducted separately for each of the three species. Incubation temperature effects on three characteristics of VO_2 during embryonic development were analyzed: timing of initial VO_2 divergence; maximum VO_2 (VO_{2max}); and developmental stage-specific Q_{10}.

The age at which VO_2 of embryos at different temperatures first diverged was assessed by performing post-hoc comparisons using differences of least-squares means from a repeated measures ANCOVA across all measurements, with individual embryo's VO_2s repeated across measurement intervals and initial egg mass as a covariate.

VO_{2max} was likely affected by timing of measurements; therefore, timing of VO_{2max}, expressed as a percentage of total incubation time, was included as a covariate in analyses comparing VO_{2max} at different incubation temperatures.

Q_{10} values were calculated at four different stages of development, and were based on VO_2 of embryos at 26.5 and 30.5° C. Developmental stages were expressed as a percent of incubation time, and were distributed such that Q_{10} was compared near the beginning, first and second thirds, and end of incubation.

Hatchling size was analyzed three different ways: 1) mass (g) was used in a one-way ANOVA to measure differences among incubation temperature treatments; 2) mass was analyzed in an ANCOVA with initial egg mass as a covariate to assess incubation temperature effects on egg-to-tissue mass conversion efficiency; and 3) differences in body length (carapace length was used for *T. scripta* and *M. temminckii* and plastron length was used for *A. spinifera*) were compared across incubation temperatures with hatchling mass as a covariate to assess differences in body composition (hereafter Body Condition Index, BCI).

In all of the above analyses, homogeneity of slopes among treatments was tested by comparing interaction terms that included the covariate. In all cases homogeneity was confirmed, and interaction terms were removed prior to final analyses. Also, non-significant covariates ($P>0.05$) were removed to increase degrees of freedom for the error term. All statistical tests were conducted using SAS v. 9.1 Proc Mixed after testing the homogeneity of variance assumption using Proc GLM (SAS Institute 2002). All metabolism, mass, and length values were $\log_{10}$-transformed prior to analysis to improve data distribution. Results are expressed as mean±1SE.

Results

Species Comparisons

Eggs used in this study comprised one *A. spinifera*, five *T. scripta*, and four *M. temminckii* clutches (Table 1). Egg mass varied among clutches in the latter two species ($P < 0.0001$), but because a randomized block experimental design was employed, did not vary among incubation temperatures (*A. spinifera*: $P = 0.507$; *M. temminckii*: $P = 0.145$; *T. scripta*: $P = 0.953$)

Temperature affected incubation duration (Table 1), embryo VO_2 (Figures 1, 2) and total O_2 consumed (Figure 3) in all three species. Incubation temperature affected incubation duration differently among the three species. The 4° C difference experienced by eggs incubated at 26.5° compared to 30.5° C produced 31% and 28% differences in incubation time in *A. spinifera* and *T. scripta*, respectively, compared to just 15% difference in *M. temminckii*. The effect of incubation temperature on *M. temminckii* incubation duration was an even smaller 4% across the 2° C span between 28.5-30.5° C, compared to 19% and 12% in *A. spinifera* and *T. scripta*, respectively.

Despite low VO_2 values during early development, precision of measurements was sufficient to detect differences among all three incubation temperatures at the first measurement in *A. spinifera* and *T. scripta* (day 2 and 5, respectively). Among *M. temminckii* embryos, mean VO_2 at 26.5° differed from that at 28.5° and 30.5° C by day 7, and differed among all three temperatures by day 14 ($P < 0.001$; Figure 1).

Patterns in the magnitude of VO_{2max} among different incubation temperatures varied among species. *Macrochelys temminckii* embryos maintained at 26.5° and 28.5° had similar VO_{2max} ($P = 0.353$), and were both greater than those incubated at 30.5°C (P

< 0.0001; Figure 4A). In contrast, VO_{2max} among *T. scripta* embryos was lowest at 26.5° (P < 0.0001) and did not differ between 28.5° and 30.5° C (P = 0.054; Figure 4B). Finally, *A. spinifera* VO_{2max} was greatest at the intermediate incubation temperature (28.5°: P < 0.0003), and did not differ at the extremes (26.5-30.5° C: P = 0.157; Figure 4C).

The timing of VO_{2max} (expressed as % total incubation duration), was unaffected by temperature for all three species (*M. temminckii*: 87.5%, range = 82-97%, P = 0.521; *T. scripta*: 91%, range = 84-97%, P = 0.661; *A. spinifera*: 87%, range = 83-96% P = 0.060). These values were no doubt dependent on the timing of VO_2 measurements, however. It should be noted that whereas *M. temminckii* and *A. spinifera* exhibited distinctly peaked embryo VO_2 patterns, VO_{2max} among *T. scripta* tended to occur at the last measurement prior to hatching (Figure 1). The exceptions to this pattern were five out of 18 embryos incubated at 26.5° C that peaked at the penultimate VO_2 measurement.

Total O_2 consumption over the course of embryonic development was similar at 26.5° and 28.5° C and lower at 30.5° C in *A. spinifera* and *M. temminckii*. By comparison, total O_2 consumption in *T. scripta* was highest at 28.5° C, intermediate at 26.5° C and lowest at 30.5° C (Figure 3).

Metabolic Compensation

VO_2 measurements used for calculating Q_{10} values were selected based on their timing (targets: 0, 33, 67, and 100% of incubation) and degree of overlap in the timing of 26.5° and 30.5° C measurements when expressed as a proportion of incubation duration in order to closely match the developmental stages compared among embryos at different

incubation temperatures (Figure 2, Table 2). Among *A. spinifera*, Q_{10} was 4.0-69.5 for the first three stages. However, at approximately 95% development Q_{10} = 1.2, lower than expected in the absence of positive compensation. In comparison, *T. scripta* exhibited Q_{10}'s between two and three during the initial two-thirds of development, and a similar decrease close to hatching (Q_{10} = 1.4 at approximately 88% development). In contrast, *M. temminckii* registered Q_{10}'s less than two throughout development. VO_2 measurements at 89% development produced a Q_{10} = 0.7.

Cost of Development

Although hatchling mass was not significantly different among incubation temperatures, (*A. spinifera*: P = 0.983; *T. scripta*: P = 0.065; *M. temminckii*: P = 0.200; Figure 5), temperature-induced differences were evident in mass conversion efficiency (Figure 6) and hatchling BCI (Figure 7) in *T. scripta* and *M. temminckii*, but not *A. spinifera*.

Hatchling mass scaled positively to egg mass in all three species. *Trachemys scripta* hatchling mass fit the equation $M_{hatchling} = -0.04M_{egg}^{0.93}$ ($r^2 = 0.32$, $P < 0.0001$), *M. temminckii* fit the equation $M_{hatchling} = -0.05M_{egg}^{0.93}$ ($r^2 = 0.50$, $P < 0.0001$), and *A. spinifera* fit the equation $M_{hatchling} = -0.13M_{egg}^{0.92}$ ($r^2 = 0.25$, P = 0.04). Mass conversion efficiency was unaffected by incubation temperature in *A. spinifera* (P = 0.93), was reduced at high temperatures in *M. temminckii* (P = 0.03), and was elevated at high temperatures in *T. scripta* (P = 0.02; Figure 6).

Hatchling BCI, expressed as the relationship between $\log_{10}$-transformed length and mass, did not differ across incubation temperatures in *A. spinifera* (Figure 7A). BCI of hatchlings was not different at 26.5° and 28.5° C in *T. scripta*, and was higher at both

these temperatures in comparison to hatchlings from 30.5° C (Figure 7B). Among *M. temminckii*, BCI was greatest at 26.5°, intermediate at 28.5° and lowest at 30.5° C (Figure 7C).

Discussion

Interspecific variation was evident in the effects temperature had on stage-specific Q_{10} values, O_{2total}, VO_{2max}, hatchling size and hatchling composition (BCI). However, broad scale consistency in the direction and timing of observed patterns suggest that incubation temperature influenced embryo energetics and growth similarly among the three species.

Chemical reaction rates typically exhibit Q_{10}'s of 2-3. Biological rates that fall below this range are often interpreted to be indicative of temperature compensation, whereas higher values indicate inverse compensation, or hypersensitivity to temperature (Gatten 1978). Progressively decreasing Q_{10}s over the course of embryonic development have been observed in several poikilotherms, including a turtle (*Emys orbicularis*; Vladimorova et al. 2005a), and two fishes (*Danio rerio* and *Salmo gairdneri*; Alekseeva 1987; Barrionuevo and Burggren 1999). This pattern frequently has been proposed to reflect greater thermal compensation as development progresses.

Q_{10} values were highly variable both within and among species, and did not neatly fit a negative correlation with embryonic development. However, as in previous studies, Q_{10} was consistently lower near the conclusion of embryonic development than during earlier stages in all three species.

Macrochelys temminckii incubated at 30.5° C consumed less oxygen than those at lower temperatures and exhibited VO_{2max} lower than that of turtles at 28.5° C and similar

to those at 26.5° C. Such results could stem from metabolic compensation to temperature, ultimately resulting in greater efficiency in the conversion of yolk to metabolically active tissue. Although it was infeasible in this study to sacrifice embryos or hatchlings to directly measure yolk:tissue ratios, evidence from Q_{10}s and hatchling size support an alternative conclusion: though incubation temperature produced minimal differences in hatchling mass, those from the highest temperature exhibited lower BCIs, and were therefore morphometrically smaller after correcting for variation in mass, than hatchlings from lower temperatures. Thus, I conclude that high temperatures negatively affected embryo growth, but that those smaller hatchlings may have emerged with large quantities of unmetabolized yolk, thus accounting for the lack of large differences in mass. The combination of these factors suggests that less metabolically active tissue was present in high incubation temperature hatchlings, resulting in lower VO_2 even in the absence of metabolic compensation.

The effects of high incubation temperature on VO_2 and O_{2total} in *T. scripta* and *A. spinifera* were similar to those in *M. temminckii*. At 30.5° C, VO_{2max} was lower than at 28.5° C (though not significantly so among *T. scripta*), and hatchlings at 30.5° C exhibited lower O_{2total} than conspecifics at lower temperatures. Whereas *M. temminckii* hatchlings from 30.5° C incubation temperature were slightly lighter than from lower temperatures, no such negative mass-incubation temperature relationship was evident in the two other species, and *T. scripta* from the highest temperature were actually slightly heavier. However, BCIs were lowest at 30.5° C in all three species (though not significantly so in *A. spinifera*), suggesting that, as appears likely in *M. temminckii*, body length was smaller and the proportion of mass composed of metabolically inactive yolk

was higher among hatchlings that developed at high temperatures. Evidence supports such a negative correlation between quantity of residual yolk and the length-to-mass relationship in another turtle, *Chelonia mydas*, in which post-hatching yolk mass and hatchling yolk-free mass were measured (Booth and Astill 2001).

These results suggest, albeit indirectly, that incubation temperature produced differences in yolk-free mass. This conclusion is consistent with a number of turtle and lizard studies that have shown a negative correlation between incubation temperature and yolk-free tissue mass (Packard et al. 1987; Whitehead and Seymour 1990; Booth and Astill 2001; Diaz-Paniagua and Cuadrado 2003; Angilletta et al. 2006). Although we cannot reject positive metabolic compensation as an additional factor contributing to the observed VO_2 patterns, there is precedence for drawing the more parsimonious conclusion that differences in yolk-free size alone produced the observed incubation temperature differences without a compensatory response to temperature acclimation during incubation (Angilletta et al. 2006).

The differences in the effects of temperature on hatchling size and yolk reserves suggest that the costs and benefits of developing at relatively high temperatures differ among the three species studied. It has been demonstrated in several lizard species that large hatchling size increases survival, presumably by increasing foraging efficiency and decreasing susceptibility to predation (Ferguson and Bohlen 1978; Fox 1978; Swingland and Coe 1979; Ferguson and Fox 1984; Packard 1991; Van Damme et al. 1992; Brana and Ji 2000; Warner and Andrews 2002). Studies suggest that size affects aquatic turtle hatchling survival, as well (Haskell et al. 1996; Janzen et al. 2000a; 2000b; Tucker 2000; but see Janzen 1995; Kolbe and Janzen 2001). However, hatchling size may affect fitness

in subtler ways. It is also generally assumed that large post-embryonic yolk reserves increase fitness, either by increasing the time a hatchling can survive before eating or, similarly, increasing winter survival in species that delay emergence until spring (Congdon et al. 1983; Filoramo and Janzen 1999).

Trachemys scripta exhibited evidence of retaining more residual yolk at 30.5° C compared to hatchlings at lower incubation temperatures. Therefore, development at high incubation temperature may confer the benefits of additional energy reserves, even at the cost of smaller body length. Because females are produced exclusively at 30.5° C, and female *T. scripta* attain sexual maturity at a larger size than do males, this difference in hatchling composition lends support to the hypothesis that temperature-dependent sex determination evolved due to asymmetrical benefits to the sexes of developing at different temperatures. Similar patterns were observed in *A. spinifera*, and so the benefits of developing at high incubation temperatures are likely similar. However, males and females do not differentially benefit, as this species exhibits genetic sex determination.

As with *T. scripta* and *A. spinifera*, evidence suggests that *M. temminckii* that were incubated at high temperatures retained relatively large yolk residuals. This potential benefit to survival was offset by the fact that hatchlings were morphometrically smaller at 30.5° C. Additionally, *M. temminckii* embryo mortality was high at 30.5° C (see Chapter V), whereas hatching success was unaffected by incubation temperature in the two other species. Therefore, it is unlikely that the phenotype expressed at high temperatures by *M. temminckii* hatchlings enhances fitness, but is instead the result of limitations on growth at high temperatures.

Future research investigating the ways that incubation temperature affects embryo energetics and hatchling body composition is needed to differentiate costs associated with growth and maintenance during development, and to assess the relative benefits to hatchlings of maximizing body size versus retaining yolk for sustaining post-hatching metabolism. A large body of literature exists on the effects of incubation temperature on hatchling turtle size and growth (Brooks et al. 1991; Spotila et al. 1994; Roosenberg and Kelley 1996; O'Steen 1998; Demuth 2001), but more detailed analyses of temperature's effect on the proportions of tissue and yolk that comprise hatchling mass will lend greater insight into the role incubation temperature plays in affecting survival and, ultimately, fitness.

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Table 1. Clutch, egg and hatchling data for turtles included in this study. Mean incubation periods are reported for eggs incubated at 26.5, 28.5 and 30.5° C, respectively.

Species	No. Clutches	No. Eggs*	Hatching success (%)	Incubation period (d)	Egg mass (g)‡	Hatchling mass (g)‡	Hatchling length (mm)†‡
A. spinifera	1	18 (18)	89	77, 61, 53	6.7±0.06	4.4±0.06	25.3±0.25
M. temminckii	4	109 (17-33)	66	93, 82, 79	26.1±0.18	16.4±0.17	35.2±0.17
T. scripta	5	58 (10-14)	93	71, 59, 52	11.5±0.08	8.9±0.09	32.1±0.11

*Number of eggs of each species used in this study, with clutch size range in parentheses.
†Plastron length reported for *A. spinifera*, carapace length reported for *M. temminckii* and *T. scripta*.
‡Mean±1SE

Table 2. VO_2 Q_{10} values calculated at different stages of embryonic development in three turtle species. Stages are expressed as % of total incubation duration, followed by the number of days since oviposition. All Q_{10} values were calculated based on VO_2's of turtles incubated at 26.5 and 30.5° C.

		Stages (% incubation duration, day of incubation)							
Species	T_{inc}	1		2		3		4	
A. spinifera	26.5° 30.5°	2.6%, 2 3.8%, 2	Q_{10}=69.54	30.0%, 23 30.2%, 16	Q_{10}=3.98	66.6%, 51 69.8%, 37	Q_{10}=5.57	94.0%, 72 96.2%, 51	Q_{10}=1.24
T. scripta	26.5° 30.5°	7.1%, 5 9.7%, 5	Q_{10}=2.14	36.7%, 26 36.7%, 19	Q_{10}=2.30	66.3%, 47 63.7%, 33	Q_{10}=2.37	86.1%, 61 90.7%, 47	Q_{10}=1.39
M. temminckii	26.5° 30.5°	7.6%, 7 8.9%, 7	Q_{10}=1.38	37.5%, 35 35.7%, 28	Q_{10}=1.72	67.5%, 63 71.1%, 56	Q_{10}=1.98	89.8%, 84 88.9%, 70	Q_{10}=0.67

FIGURE LEGENDS

Figure 1. Changes in VO_2 during embryonic development of A) *Apalone spinifera*, B) *Trachemys scripta* and C) *Macrochelys temminckii* at three constant temperatures. Circles = 26.5° C, triangles = 28.5° C and squares = 30.5° C. Asterisks indicate first measurement at which VO_2 diverged across all three incubation temperatures. Error bars = ±1 SE.

Figure 2. Comparison of changes in VO_2 of A) *Apalone spinifera*, B) *Trachemys scripta* and C) *Macrochelys temminckii* embryos maintained at different temperatures, with time expressed as a percentage of total incubation duration. Ovals superimposed on each figure indicate samples used to calculate developmental stage-specific Q_{10} values (see text). Symbols are as in Figure 1.

Figure 3. Comparison of the total volume of O_2 consumed over the duration of incubation by A) *Apalone spinifera*, B) *Trachemys scripta* and C) *Macrochelys temminckii* embryos incubated at different constant temperatures. Lower case letters indicate treatment differences ($P < 0.05$). Error bars are as in Figure 1.

Figure 4. VO_{2max} of turtle embryos incubated at three temperatures. A) *Apalone spinifera*, B) *Trachemys scripta*, C) *Macrochelys temminckii*. Lower case letters indicate treatment differences ($P < 0.05$). Error bars are as in Figure 1.

Figure 5. Mass of hatchling A) *Apalone spinifera*, B) *Trachemys scripta*, C) *Macrochelys temminckii* incubated at different constant incubation temperatures. Lower case letters indicate treatment differences ($P < 0.05$). Error bars are as in Figure 1.

Figure 6. Differences in hatchling mass, corrected for differences in initial egg mass. Values were calculated from L-S regression residuals from a log-log plot of

hatchling mass against initial egg mass. A) *Apalone spinifera*, slope = 0.917, r^2 = 0.25; B) *Trachemys scripta*, slope = 0.934, r^2 = 0.32; C) *Macrochelys temminckii*, slope = 0.868, r^2 = 0.46. Lower case letters indicate treatment differences ($P < 0.05$). Error bars are as in Figure 1.

Figure 7. Hatchling body composition indices, calculated as residuals from a L-S regression from a log-log plot of body length on mass. A) *Apalone spinifera*, slope = 0.314, r^2 = 0.14; B) *Trachemys scripta*, slope = 0.270, r^2 = 0.58; C) *Macrochelys temminckii*, slope = 0.384, r^2 = 0.59. Lower case letters indicate treatment differences ($P < 0.05$). Error bars are as in Figure 1.

Figure 1

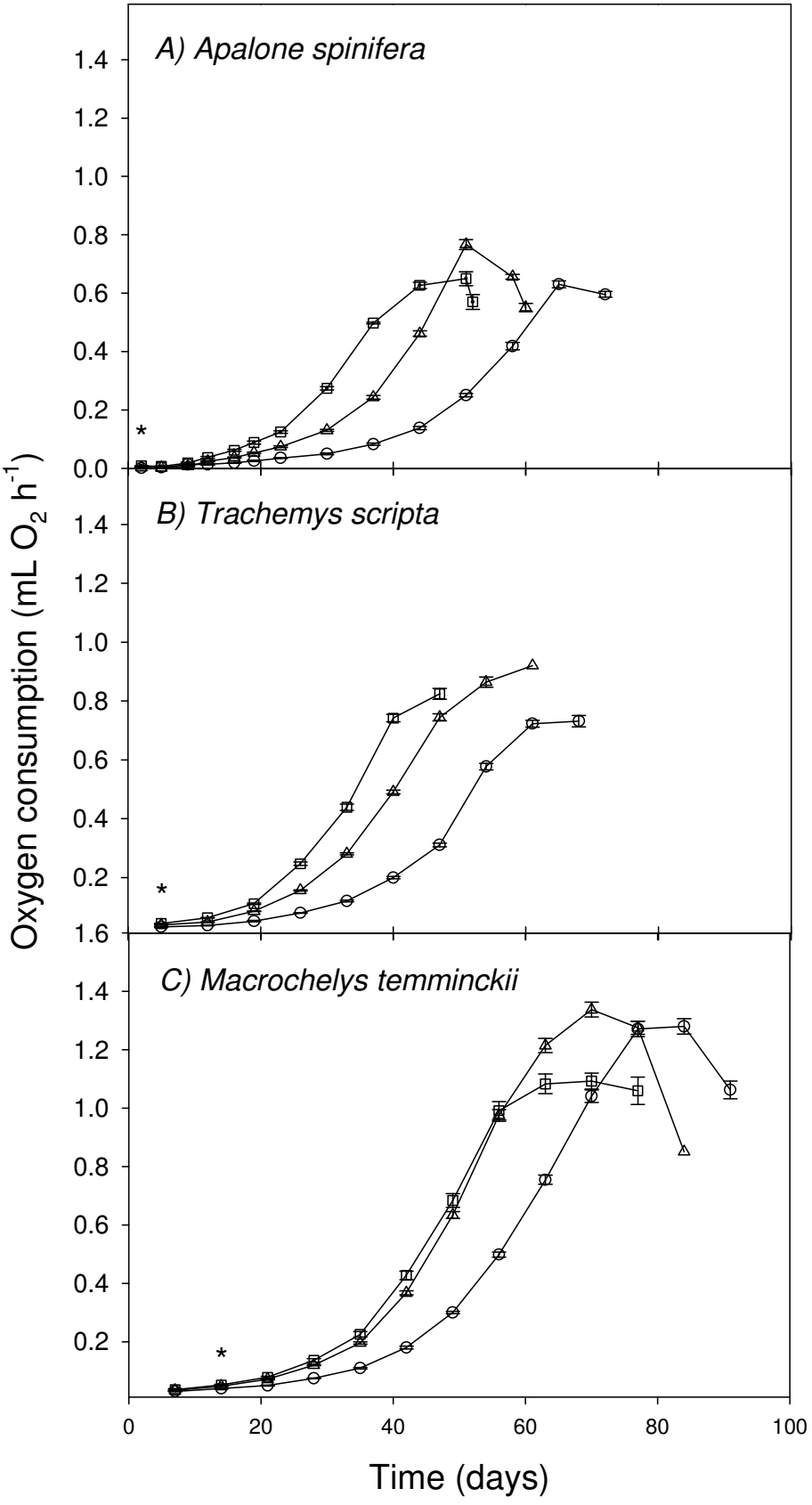

A) Apalone spinifera
B) Trachemys scripta
C) Macrochelys temminckii
Oxygen consumption (mL O_2 h^{-1})
Time (days)
0
20
40
60
80
100
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
*

Figure 2

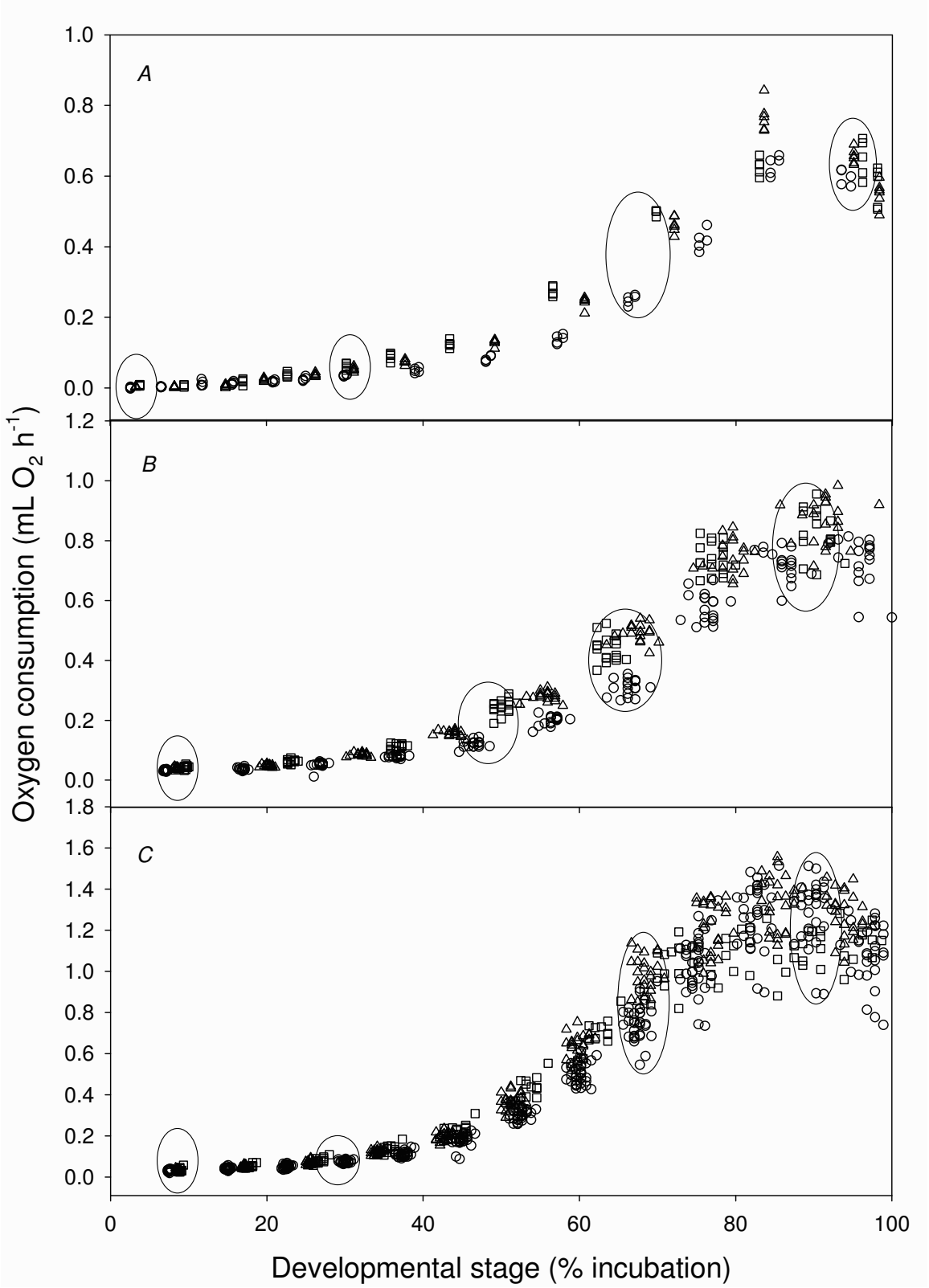

A
B
C
Oxygen consumption (mL O_2 h^{-1})
Developmental stage (% incubation)
0
20
40
60
80
100

Figure 3

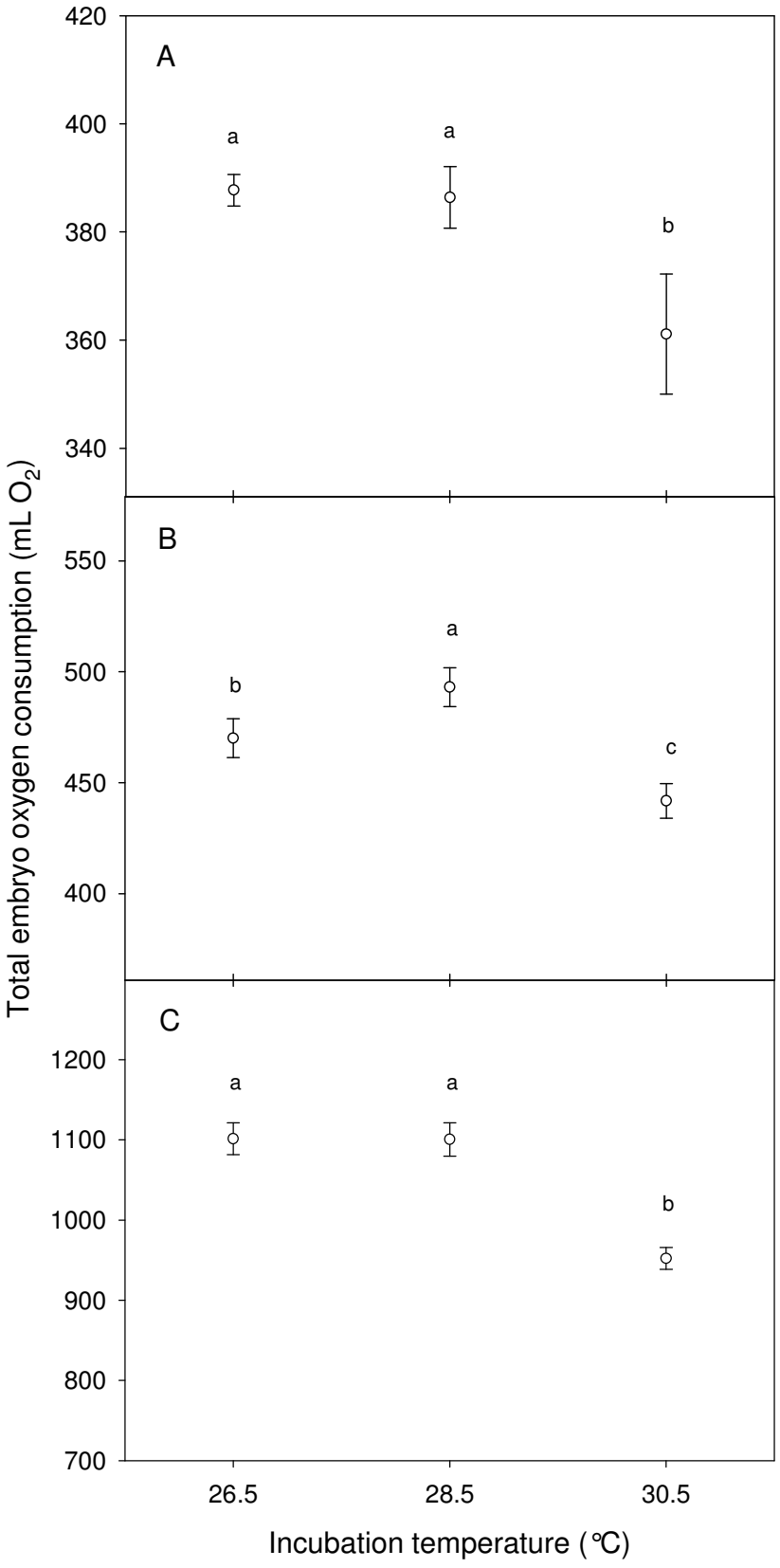

A
B
C
a
a
b
a
b
c
a
a
b
420
400
380
360
340
550
500
450
400
1200
1100
1000
900
800
700
Total embryo oxygen consumption (mL O_2)
26.5
28.5
30.5
Incubation temperature (°C)

Figure 4

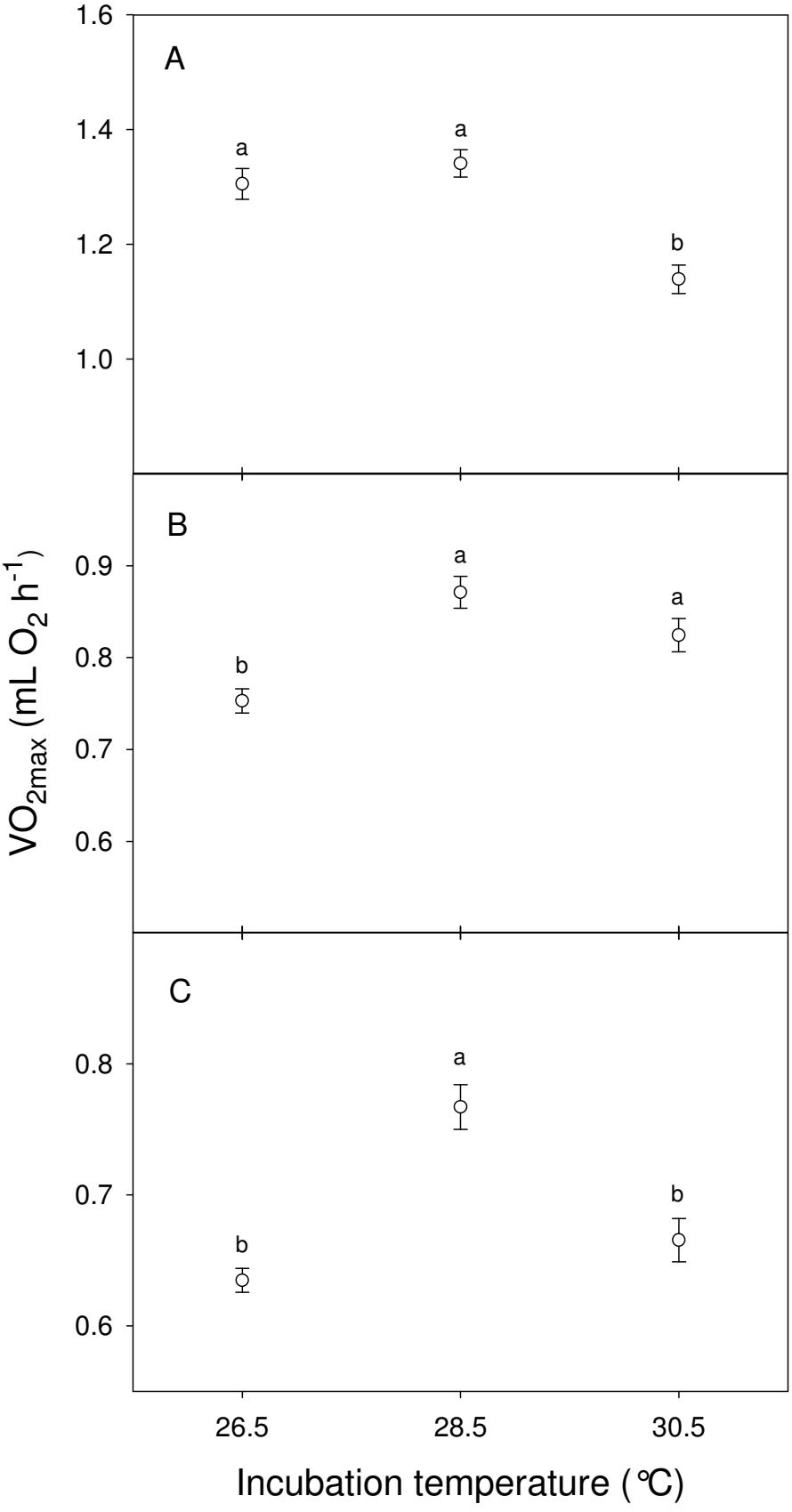

A
B
C
a
b
1.6
1.4
1.2
1.0
0.9
0.8
0.7
0.6
VO_{2max} (mL O_2 h^{-1})
26.5
28.5
30.5
Incubation temperature (℃)

Figure 5

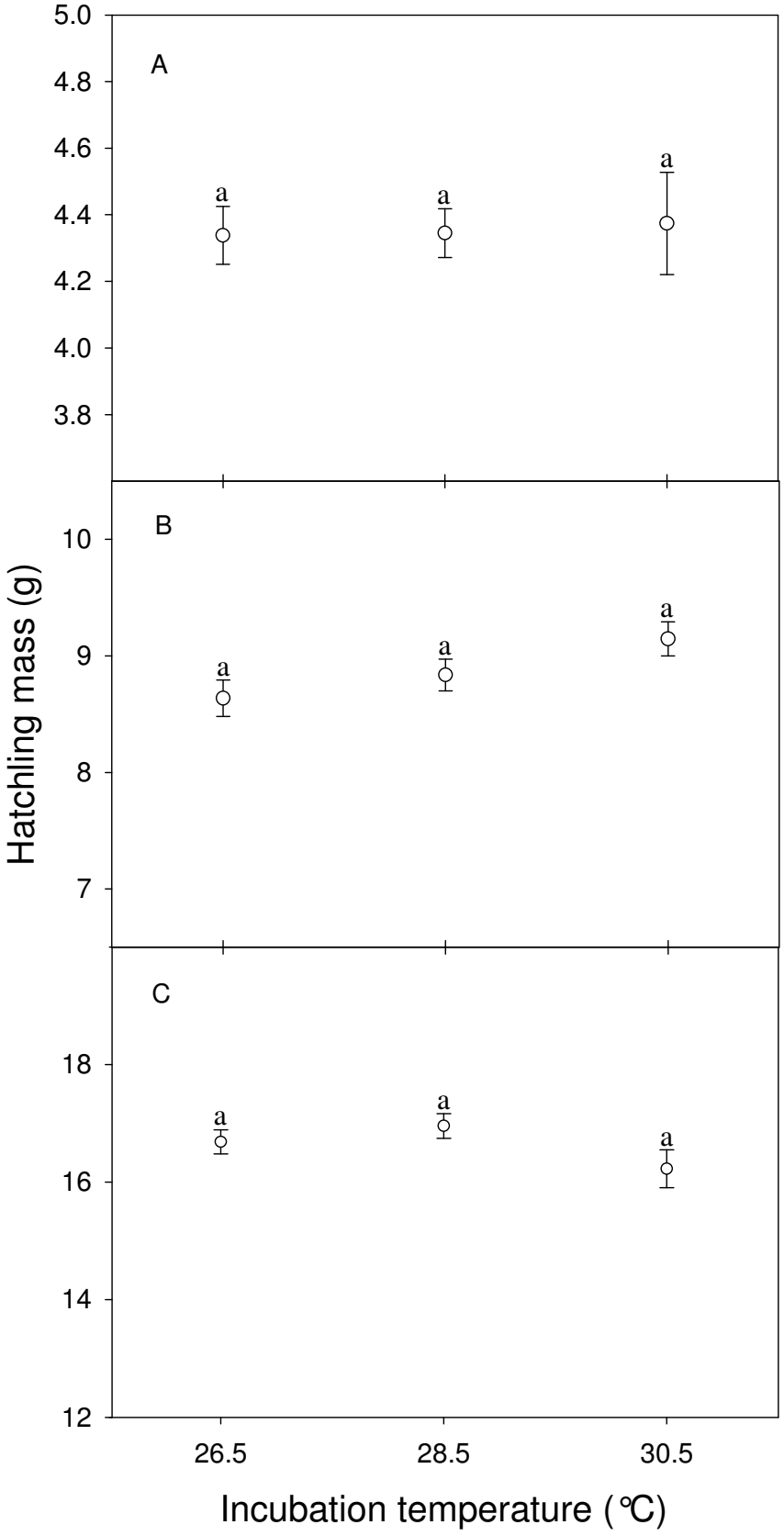

A
B
C
a
a
a
a
a
a
a
a
a
5.0
4.8
4.6
4.4
4.2
4.0
3.8
10
9
8
7
18
16
14
12
26.5
28.5
30.5
Hatchling mass (g)
Incubation temperature (℃)

Figure 6

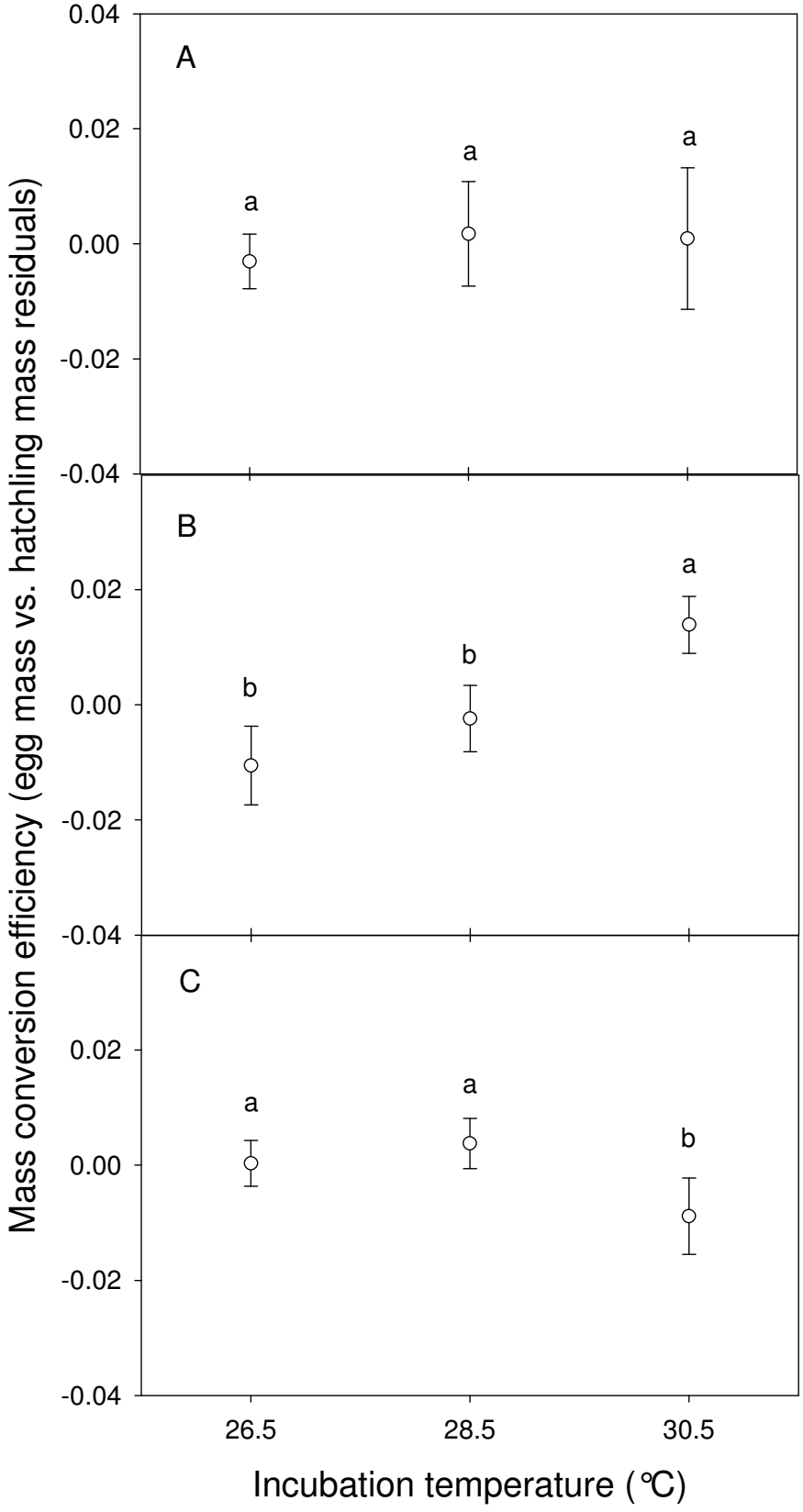
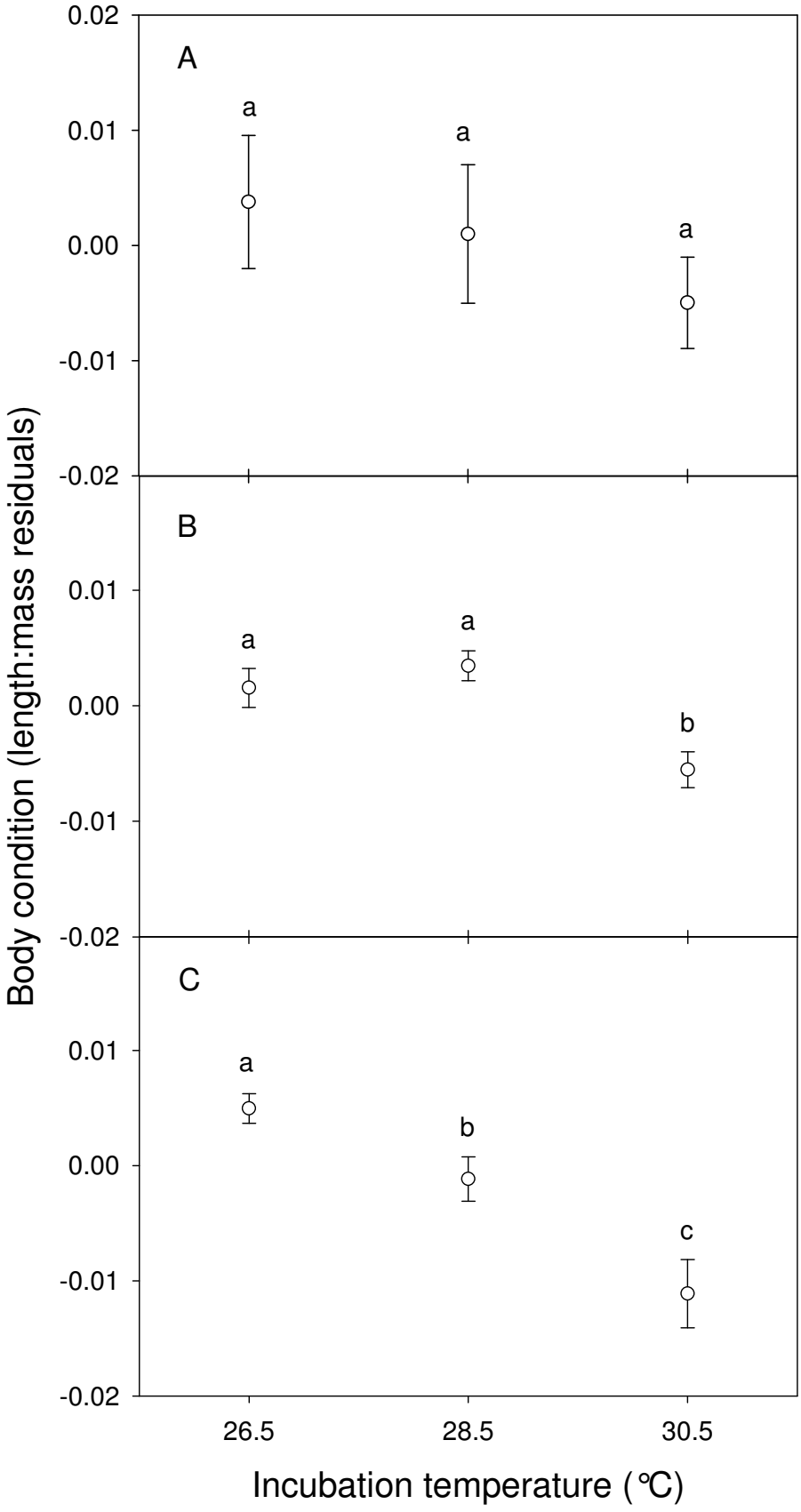

A
B
C
0.04
0.02
0.00
-0.02
-0.04
a
a
a
b
b
a
a
a
b
Mass conversion efficiency (egg mass vs. hatchling mass residuals)
26.5
28.5
30.5
Incubation temperature (℃)

Figure 7


A
B
C
a
a
a
a
a
b
a
b
c
0.02
0.01
0.00
-0.01
-0.02
Body condition (length:mass residuals)
26.5
28.5
30.5
Incubation temperature (°C)

III. Acute and persistent effects of incubation and post-embryonic thermal environments on hatchling growth and metabolism in the red-eared slider turtle, *Trachemys scripta elegans*

Summary

Many ectotherms possess the capacity to survive a wide range of thermal conditions. However, long-term exposure to different temperatures can induce acclimational and/or organizational temperature effects, and the developmental stage at which temperature exposure occurs may affect the type, degree, and persistence of these effects. We incubated red-eared slider turtle embryos at different constant temperatures (T_{inc}; 26.5, 28.5, 30.5° C), then divided the resulting hatchlings among two different water temperatures (T_{water}; 25, 30° C). We calculated growth rates 11 weeks and six months after hatching to assess the short- and long-term effects of thermal experience on a metabolically costly process. We also measured acute response to temperature of resting metabolic rate (RMR) at three body temperatures (T_{body}) shortly after hatching and six months post-hatching to characterize the degree and persistence of acclimation to T_{inc} and T_{water}. Hatchling RMRs were highest among turtles incubated at 26.5°, intermediate among those from 28.5°, and lowest among those from 30.5° C at all T_{body}'s, a pattern consistent with positive but incomplete metabolic compensation to T_{inc}. Average growth rates over the first 11 weeks post-hatching were strongly affected by T_{water} but only marginally by T_{inc}, and only at T_{water} = 30° C. Six-month RMR's exhibited strong

acclimation to T_{water} consistent with positive metabolic compensation. However, within each T_{water} treatment, RMR patterns among T_{inc} treatments fit patterns indicative of inverse metabolic compensation. Average growth rates calculated over six months continued to show a strong effect of T_{water}, and the previously weak effect of T_{inc} observed within the 30° C T_{water} treatment became more pronounced. My results suggest that metabolic compensation was reversible regardless of the life stage during which exposure occurred, and therefore is more appropriately considered acclimational than organizational. Growth patterns indicate that T_{inc} can induce differences that are small in comparison to T_{water} effects. However, small, non-persistent T_{inc}-induced growth advantages may compound over time to produce significant differences.

Introduction

Environmental temperatures affect nearly all biological processes in ectothermic organisms (Huey, 1982; Lillywhite, 1987; Bennett, 1990). The rates of biological processes correlate positively with temperature; rates of individual biochemical reactions were described by Arrhenius (1915), and typically increase two- to three-fold over a 10° C range (Q_{10} = 2 to 3). However, whole-organism resting metabolic rate (RMR), which is an integrative measure of developmental and anabolic processes, often exhibits more variable temperature sensitivity than those typical of individual reactions (e.g., Ikeda, 1985). This increased variability may result from the combination of Arrhenius effects and differences in the suite of physiological processes that occur at different temperatures (Heldmaier and Ruf, 1992). This conclusion is supported by the fact that acute responses to temperature generally exhibit higher Q_{10} values at lower temperatures (Litzgus and

Hopkins, 2003), presumably because of suppression of physiological processes that in turn results in higher Q_{10}s than would be predicted by Arrhenius effects alone. For example, heat production often demands a significant proportion of energy consumption in endotherms, but may be suspended as body temperature decreases during hibernation (Michenfelder and Milde, 1991; Heldmaier and Ruf, 1992).

In addition to acute responses to proximate conditions, large scale temperature patterns such as those occurring across circadian cycles or seasons can produce reversible acclimational changes that influence physiological processes and performance. Resting metabolic rate (RMR) responses to long-term temperature exposure can conform to a variety of patterns collectively referred to as metabolic acclimation (Angilletta et al., 2006; Deere and Chown, 2006). In general, acclimation produces some degree of positive metabolic compensation which, whether 'perfect' or 'incomplete', dampens the acute effects of temperature (i.e., the immediate effects of changes in body temperature), thereby meliorating effects of different past thermal exposure among individuals (Hazel and Prosser, 1974; O'Steen and Janzen, 1999). In contrast, 'negative' or 'inverse' compensation (Precht, 1958; Hazel and Prosser, 1970) enhances acute response to temperature, a phenomenon that may be beneficial during low-temperature dormancy (Thorp, 1978; Bailey and Lazaridou-Dimitriadou, 1991; St. Pierre and Boutilier, 2001).

The effects of long-term exposure to specific temperatures may differ among stages of development. For example, the temperature profile to which oviparous reptiles are exposed during embryonic development differs in many ways from that experienced after leaving the nest. Temperatures experienced during incubation are determined largely by ambient temperature, maternal nest-site selection and soil conductivity

(Packard et al., 1987; Roosenburg and Kelley, 1996; Valenzuela and Janzen, 2001; Kolbe and Janzen, 2002), and in contrast to post-hatching life-stages, embryos lack the capacity for behavioral thermoregulation. Importantly, the thermal profile during embryogenesis—when many irreversible organizational aspects of development are determined—could produce either reversible acclimational responses or permanent organizational differences among individuals (Wilson and Franklin, 2002). If the latter, incubation temperature (T_{inc}) effects should persist beyond the embryonic stage.

Three studies have looked at how T_{inc} effects metabolic compensation in turtles. The first (Booth, 1998) tested the hypothesis proposed by Birchard and Reiber (1995) that turtle embryos have the capacity for metabolic compensation early in development when organization of the body plan is occurring and growth is slow, but then lose that capacity during later stages of development when growth dominates. This was done by exposing *Emydura signata* embryos to different temperatures during early and late incubation and comparing their RMRs to those of turtles maintained at constant temperatures for the duration of incubation. There was no evidence of metabolic compensation during the late stages of development, and it is therefore likely that no evidence of acclimation to T_{inc} persisted after hatching in this species. A second study reported an inverse correlation between T_{inc} and RMR among hatchling *Chelydra serpentina* following incubation at different constant temperatures (O'Steen and Janzen, 1999), a pattern consistent with positive metabolic compensation because the effect of increasing temperature was dampened. In contrast, RMRs of *C. serpentina* measured six months after incubation found a positive correlation between RMR and T_{inc} (Steyermark and Spotila, 2000). Such a pattern accentuates differences among T_{inc} treatments. The

variation among these three studies may be due to methodological peculiarities, but more likely resulted from differences in the timing of RMR measurements.

In addition to the previous studies examining relationships between T_{inc} and metabolic compensation, one study measured RMR of adult turtles following prolonged exposure to constant temperatures (Gatten, 1978). Two groups of *C. serpentina* were maintained at 10 or 25° C for several months, after which RMR was measured at 10, 20, and 30° C. The animals exhibited a consistent positive correlation between acclimation temperature and RMR, and Q_{10} values were higher between 10 and 20° C than between 20 and 30° C in both acclimation treatments.

While RMR is a useful integrative measure of pre- and post-hatching temperature effects, measurements of growth have dominated investigations of T_{inc} effects on hatchling turtle phenotype (Rhen and Lang, 2004). Growth rates correlate positively with acute body temperature (Avery et al., 1993), and can demand a large proportion of individuals' energy budget. Moreover, many studies cite differences in post-hatching growth rate as a persistent (i.e., organizational) effect of T_{inc} that may contribute to the evolutionary adaptiveness of temperature-dependent sex determination (review: McCue, 2004).

We gauged the short- and long-term effects of temperature exposure, as well as the interactive effects of embryonic and post-embryonic temperatures, on growth and RMR of red-eared slider turtles (*Trachemys scripta elegans*) by assigning eggs to different T_{inc} treatments and then rearing the resulting hatchlings at different constant water temperatures in a randomized-block experiment. Mass was measured after 11 weeks and six months, and RMR was measured at three temperatures immediately after

hatching and six months post-hatching. Acute responses to temperature were compared among treatment combinations. This design allowed us to determine: 1) the degree to which *T. scripta* is sensitive to acute temperature changes immediately after hatching and six-months post-hatching; 2) whether T_{inc} effects on RMR and growth rate are evident and, if so, whether they are reversible (acclimational) or persistent (organizational) in nature; and 3) to what degree and in what direction chronic exposure to constant water temperatures after hatching affects metabolic compensation and growth rate.

Materials and methods

All procedures for this research were approved by the Oklahoma Department of Wildlife Conservation and the Oklahoma State University Institutional Animal Care and Use Committee (protocol #AS023), assuring compliance with animal care guidelines described in The Guide for the Care and Use of Laboratory Animals, 7th edition (1996).

Eggs

Gravid *Trachemys scripta* were collected at Sequoyah National Wildlife Refuge and transported to Oklahoma State University in June 2004. Oviposition was initiated with oxytocin (0.10 IU/kg IM) (DeNardo, 1996). Eggs were measured, weighed and numbered, then assigned to incubation treatments (T_{inc} = 26.5, 28.5, and 30.5 °C) in a randomized block design with clutch serving as the blocking variable to minimize the confounding effects of maternal identity. Eggs within each T_{inc} treatment were distributed among three plastic shoeboxes containing damp vermiculite (water potential ≈ -150 kPa; 1:1 water:vermiculite by mass) (Packard et al., 1987). Incubator temperatures were

checked daily via calibrated thermocouple wires inserted into one egg container in each incubator. Each container was weighed weekly and, when necessary, distilled water was added to compensate for evaporation. Additionally, egg containers were rotated within incubators daily and eggs redistributed within each box weekly to minimize effects of thermal or moisture gradients.

Hatchlings

Eggs were removed from the plastic shoeboxes after pipping and placed individually in plastic jars lined with damp paper towel to retain individual identification. Hatchlings remained in their individual containers until residual yolk was internalized, at which time they were weighed (±0.1 g) and midline carapace length was measured (±0.1 mm). RMR of each hatchling was measured (described below). Following RMR measurements, unique combinations of marginal scutes were notched to facilitate long-term identification of individuals, and each turtle was assigned to one of two T_{water} treatments maintained at 25° and 30° C. These temperatures are within the range of temperatures in which *T. scripta* can survive and grow (Avery et al., 1993). Within each T_{water} treatment, turtles were evenly distributed among six flow-through enclosures (45 × 45 × 35 cm, L × W × D), and were redistributed weekly to avoid confounding group or container effects. No basking platforms were provided, so body temperature (T_{body}) remained at or near T_{water}. Turtles were fed a 52% protein pellet diet ad libitum and weighed and measured 11 weeks and six months after hatching.

All turtles were sexed via laparoscopy 7-8 months after hatching, after all growth and RMR measurements were completed to avoid potentially adverse effects of surgery

on these measurements. Male and female gonads were readily distinguishable; testes were highly vascularized but otherwise uniform in color and texture, whereas oviducts and white prefollicles on ovaries distinguished females. Two observers verified each turtle's gonads, and agreement was 100%.

Metabolic rate

Oxygen consumption (VO_2) was measured via closed-system respirometry (Vleck, 1987) and used to calculate RMR (Peterson, 1990). RMR of each turtle was measured at body temperatures (T_{body}) equal to each of the three T_{inc}s (26.5°, 28.5°, and 30.5°C) twice: first, 1-2 d after internalizing yolk, and second approximately six months (range = 180-195 d, mean = 186 d) post-hatching. Furthermore, because activity while in the metabolic chambers can elevate oxygen consumption above resting rates, the lower of two serial measurements conducted at each T_{body} was used at each period to represent RMR.

Metabolic chambers were constructed from 169- 322- and 959-mL cylindrical plastic jars with threaded lids. A stopcock was inserted through the lid of each jar, and a thin film of vacuum grease was applied on the inside of each lid to ensure an airtight seal when the stopcock was closed.

Prior to the six-month RMR measurements, turtles were fasted for 4 d to minimize metabolic costs associated with specific dynamic action and growth. Hatchling measurements were conducted as soon as residual yolk was internalized, and therefore digestion and growth likely contributed to RMR during this early measurement. On the day of measurement, each turtle was weighed and placed into a metabolic chamber.

Chambers were then placed inside an environmental chamber for 1.5-2 h to allow body temperatures to stabilize and equilibrate to ambient temperature. With the overhead lights turned off to minimize disturbance to the turtles, chambers were carefully removed from the environmental chamber. After sealing lids to create an airtight chamber, pre-trial air samples were drawn into 20-mL syringes, also equipped with stopcocks. The stopcocks on the syringe and chamber were then closed, the syringes removed, and the time of sampling recorded. Chambers were placed back into the dark environmental chamber and removed after approximately 1 h, when post-trial samples were drawn from the stopcock after pumping each syringe several times to ensure mixing of the air inside the chamber. After the final measurement at a given T_{body}, turtles were moved to an environmental chamber set at another temperature and the process was repeated. Duplicate measurements at all three temperatures were made on the same day.

Oxygen concentrations of all air samples were analyzed in 10-mL aliquots with a Sable Systems FC-1 oxygen analyzer. Air was drawn from outside the building at a regulated flow rate of 100 mL/min and through serial columns of Drierite and Ascarite to remove water and CO_2, respectively. Each sample was injected into the air stream, which passed through a small column of Drierite and Ascarite and then through the oxygen analyzer. Oxygen consumption by each turtle was calculated as the difference between the initial and final volumes of oxygen after correcting for chamber volume (Peterson, 1990).

Statistics

Mass and average growth rate of each turtle was calculated twice: once 11 weeks post-hatching and again at six months of age. Rates were corrected for differences in initial hatchling mass using the equation $Growth\ rate = (mass_n - mass_0) \times (mass_0)^{-1} \times n^{-1}$, where n = age in days since hatching and $mass_0$ = mass at hatching. Mass and growth rate were then analyzed separately at each age in 2-way ANOVAs with T_{inc} and T_{water} as fixed effects. Disparities in embryo mortality among clutches and T_{inc}s precluded using clutch identity as a factor in the model.

Metabolic compensation following acclimation to a constant temperature—both during incubation and after prolonged exposure to constant T_{water} —was measured shortly after hatching and six months post-hatching by measuring RMR at T_{body} = 26.5°, 28.5°, and 30.5° C. Analyses of data from both stages were performed in a repeated-measures ANCOVA, with T_{inc} and T_{body} as fixed effects, turtle ID repeated over each temperature, and mass as a covariate. Six-month measurements were analyzed similarly, but with T_{water} included as a third fixed effect.

Q_{10} values were calculated for each turtle after hatching and at six-months post-hatching from RMR measurements obtained at 26.5° and 30.5° C. T_{inc} effects on hatchling Q_{10}, and T_{inc} and T_{water} effects on Q_{10} of six-month-old juveniles were analyzed in two separate ANOVAs.

Results

Growth

Short- and long-term effects of T_{inc} and T_{water} on growth rates were assessed by calculating average mass and growth rates of turtles 11 weeks (77 days) and six months (180-191 days) after hatching (Figure 1). No $T_{water} \times T_{inc}$ interaction or T_{inc} main effect on growth rate was evident after 11 weeks (interaction: $F_{2, 123} = 2.58$, $P = 0.080$; T_{inc}: $F_{2, 123} = 2.54$, $P = 0.083$ (Figure 1B). However, T_{water} strongly influenced growth rates during the first 11 weeks ($F_{1, 123} = 153.9$, $P < 0.0001$). Pooled across incubation temperatures, after 11 weeks *T. scripta* reared in 30° C water had increased in mass an average 392%, whereas those maintained at 25° C increased just 90% (size at 11 weeks: 30° = 43.09±1.89 g; 25° = 16.37±0.53g) (Figure 1A).

Differences in turtle size among T_{water} treatments were of even greater magnitude after six months (Figure 1C), and growth rates calculated over six months continued to show a strong T_{water} effect ($F_{1, 71} = 112.7$, $P < 0.0001$). Additionally, T_{inc} effects on growth rate emerged among turtles maintained at $T_{water} = 30°$ but not at 25° ($T_{water} \times T_{inc}$: $F_{2, 71} = 4.27$, $P = 0.018$). Specifically, growth rates among turtles maintained at $T_{water} = 30°$ C was lower among turtles from $T_{inc} = 26.5°$ C compared to those from $T_{inc} = 28.5$ or 30.5° C treatments (Tukey's post-hoc tests: $P < 0.01$) (Figure 1D).

Sex

All turtles incubated at 26.5 and 28.5° C had readily identifiable testes, and ovaries were observed in all turtles from $T_{inc} = 30.5°$ C. Because no level of the factor T_{inc} produced a mixed sex ratio, sex was excluded as a factor in statistical analyses.

Metabolic rate

Prior to analyzing RMR data, mass-corrected VO_2 measurements that were > 2 standard deviations higher than the mean within each T_{body} treatment were eliminated from the dataset because such uncharacteristically high values are generally indicative of high activity levels while confined in the metabolic chambers. The resulting dataset included data from 96 hatchlings (26.5°: n = 31; 28.5°: n = 30; 30.5°: n = 31), but just 49 juveniles (reared at 25° C: 26.5°: n = 11; 28.5°: n = 9; 30.5°: n = 14; reared at 30° C: 26.5°: n = 5; 28.5°: n = 0; 30.5°: n = 10).

For both hatchlings and 6-month-old juveniles, RMR scaled to body mass similarly at each T_{body} (Hatchlings: slopes = 0.50-0.56, r^2 = 0.08-0.11, $P < 0.0001$, Figure 2A; Juveniles: slopes = 0.86-0.90, r^2 = 0.70-0.76, $P < 0.0001$, Figure 2B) and exhibited a positive acute response to temperature. Among hatchlings, exposure to constant T_{inc} induced positive metabolic compensation, such that turtles incubated at 26.5° C had the highest and those incubated at 30.5° C the lowest RMR's at each of the three T_{body} ($F_{2,92}$ = 7.72, P = 0.0008; Figure 3A).

In addition to body mass ($F_{1,43}$ = 113.16, $P < 0.0001$) and T_{body} ($F_{2,88}$ = 76.79, $P < 0.0001$), RMR among 6-month-old juveniles was influenced by T_{inc} ($F_{2,43}$ = 8.37, $P < 0.0009$), and T_{water} ($F_{1,43}$ = 4.57, P = 0.0383). Juveniles demonstrated positive metabolic compensation to T_{water} (Figure 3B). After correcting for body size, turtles reared at 25° C had higher RMRs than those reared in 30° C water. However, within each T_{water} treatment, the effect of T_{inc} on RMR was the reverse of that observed in hatchlings: among juveniles reared at T_{water} = 30° C, those incubated at 26.5° C had lower RMRs at all T_{body} than those incubated at 30.5° C (Tukey's post-hoc tests: all $P < 0.0001$).

Differences among turtles reared in T_{water} = 25° C water were similar but less pronounced: those incubated at 26.5° had lower RMR at all T_{body} compared to those incubated at 30.5° (Tukey's post-hoc tests: all $P < 0.0001$). The acute response to temperature of turtles from T_{inc} = 28.5° was intermediate to, but not significantly different from, the response of those from T_{inc} = 26.5 and 30.5° (Tukey's post-hoc tests: $P = 0.095$ and 0.221, respectively).

RMR Q_{10} averaged 2.52±0.07 among hatchlings and was unaffected by T_{inc} ($F_{2,92} = 2.83$, $P = 0.064$). Juvenile Q_{10} = 2.53±0.13, and was also unaffected by either T_{inc} or T_{water} (T_{inc}: $F_{2,44} = 0.50$, $P = 0.612$; T_{water}: $F_{1,44} = 0.68$, $P = 0.413$), and did not differ significantly from that observed at hatching (Repeated Measures ANOVA: $F_{1,86} = 1.16$, $P = 0.285$).

Discussion

Growth

Turtles reared in 30° C water grew faster than did turtles at 25° C. However, differences in post-hatching growth rates among T_{inc} treatments were subtle; only at six months of age and only at T_{water} = 30° C did turtles from the lowest T_{inc} exhibit significantly slower growth than others at the same T_{water}. The divergence occurred between two male-producing temperatures (26.5 and 28.5° C); therefore T_{inc} rather than sex emerges as the clear source of these growth differences.

Several studies have tested the effects of T_{inc} on post-hatching growth in turtles because the presence of such differences has been hypothesized to provide a selective advantage for temperature-dependent sex determination in species that exhibit sexual size

dimorphism. Results from these studies have been variable, even within individual species. For example, post-embryonic changes in mass in *C. serpentina* have been variously described as highest at low temperatures (Brooks et al., 1991; Rhen and Lang, 1995; Rhen and Lang, 1999; O'Steen, 1998), highest at high temperatures (Ryan et al., 1990), highest at intermediate temperatures (Bobyn and Brooks, 1994; McKnight and Gutzke, 1993), and equivalent across T_{inc}s (Steyermark and Spotila, 2001). These studies differed in duration, feeding regimen, and whether or not individuals were permitted to thermoregulate, but none of these variables consistently explains the variability among studies. Importantly, the degree of T_{inc}-induced growth difference reported in these and other turtle studies (Spotila et al., 1994; Roosenberg and Kelley, 1996; Du and Ji, 2003) and presented here, is generally small in comparison to differences produced by the post-hatching thermal environment; therefore, the evolutionary significance of such differences may be minimal.

Metabolic rate

RMR scaled weakly to mass and exhibited high variability among hatchling *T. scripta*. In contrast, there was a strong correlation between RMR and mass among juveniles. This difference among age classes is due in large part to the low variability in mass among hatchlings, and probably also reflects differences in turtles' digestive state. Food was withheld from juveniles for several days prior to measuring oxygen consumption. Measurements at that stage therefore closely approximate a true 'resting' state. Hatchlings, on the other hand, had internalized but not metabolized the entirety of their yolk reserves. Therefore, measurements at the earlier life stage likely deviated from a true RMR due to contributions from specific dynamic action and growth.

Q_{10}s calculated across a 4° C range of body temperature within individual turtles did not vary among T_{inc}s at hatching or among T_{water}s after six months, indicating that temperature *sensitivity* was unaffected by long-term temperature exposure at either life stage. Furthermore, Q_{10} values were between two and three in all treatments, suggesting that Arrhenius effects alone likely account for the observed patterns. This is not surprising given that acute RMR measurements were conducted within the range of temperatures at which these turtles are normally active.

RMR measurements among hatchling *T. scripta* indicated that T_{inc} produced incomplete metabolic compensation between exposure temperatures of 26.5 and 28.5° C, and near-perfect compensation between those of 28.5 and 30.5° C. There are at least two reasons why organisms might fail to achieve perfect compensation. First, physiological constraints could limit compensatory adjustments at lower levels of organization (e.g., enzymatic activity, membrane structure, organ function), such that completely offsetting acute temperature differences is infeasible (Angilletta et al., 2006). Alternatively, perfect compensation may be obtainable, but the energetic costs associated with it simply outweigh the benefits (Guderley and St. Pierre, 1999). This second reason likely accounts for the pattern observed in this study in light of the modest temperature range utilized. No T_{inc} deviated from the pivotal temperature for the species (29° C) (Ewert et al., 1994) by more than 3° C, and all were well within the range of temperatures experienced in nests locally (D. Ligon, unpublished data). The two T_{water} treatments to which turtles were assigned were also conservative in relation to the range of active-season temperatures normally experienced by *T. scripta*. Therefore, the likelihood that the temperature

differences exceeded the physiological limits of metabolic compensation in the species is low.

Metabolic compensation to T_{inc} was similar to that reported in *C. serpentina* (O'Steen and Janzen, 1999), but within six months, and possibly much sooner, shifted to a pattern of compensation dominated by the effects of T_{water}. Thus, metabolic compensation to T_{inc} remains reversible and is therefore acclimational rather than organizational in nature. However, within each T_{water}, T_{inc} differences remained evident but fit the patterns consistent with inverse compensation that have been reported in 6-month-old *C. serpentina* (Steyermark and Spotila, 2000). Turtles that were incubated at 30.5° C were relatively unaffected by T_{water}, whereas those incubated at 26.5° C exhibited substantial acclimational adjustments. To my knowledge, this is the first study to measure the interactive effects on acclimation of embryonic and post-hatching thermal environments in a reptile. However, it is unclear either how (physiologically/ biochemically) or why (ecologically/evolutionarily) such persistent yet reversed differences that are secondary to positive compensation to more proximal temperature acclimation might occur.

Our observations of positive acclimation to T_{water} contradict the inverse compensation reported among adult *C. serpentina* (Gatten, 1978). Although it is possible that results differ because of age-specific differences in physiological response to long-term temperature exposure, or to species-specific acclimational responses, it seems more likely that the difference in the range of temperatures used by Gatten (1978) and this study account the different results. I limited my focus to temperatures typically

encountered by active *T. scripta*, whereas the adult *C. serpentina* were exposed to temperatures likely to induce torpor.

The capacity for metabolic acclimation to changing thermal conditions has potential implications for the mechanisms by which *T. scripta* respond to seasonal and longer-term climate changes (Janzen, 1994). Because behavioral thermoregulation via basking is well-documented in this species (Moll and Legler, 1971; Spotila et al., 1990; Dreslik, 2000), it is probable that the precision with which animals thermoregulate would be unaffected during the majority of the active season. However, the duration of the active season and the proportion of individuals' activity budget dedicated to thermoregulation could be affected. Physiological adjustments to shifting temperatures could offset the need for behavioral adjustments. Finally, physiological compensation as was observed in this study has the potential to provide much faster and more plastic responses to climate changes than do population-level genetic shifts. Given the fast rate at which global temperatures are expected to rise in the next century, combined with the protracted life history characteristic of most turtle species, capacity for temperature compensation could play a crucial role in the ecology of these animals.

Acknowledgments

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Figure Legends

Figure 1. Eleven-week (A and B) and six-month (C and D) mass and growth rates of *Trachemys scripta* incubated at three temperatures and subsequently reared at 25° or 30° C. * Denotes differences among T_{inc}s ($p < 0.05$).

Figure 2. Log-log regressions of RMR on body mass measured at three acute temperatures of A) hatchling and B) 6-month-old *Trachemys scripta*. Slopes of lines within each age group were similar: A) slopes = 0.50-0.56, r^2 = 0.08-0.11; B) slopes = 0.86-0.89, r^2 = 0.70-0.76. Circles = 26.5° C, triangles = 28.5° C, and inverted triangles = 30.5° C.

Figure 3. RMR of A) hatchling and B) juvenile *Trachemys scripta* from three different incubation treatments measured at three temperatures (x-axis). After hatching, turtles were assigned to one of two T_{water} treatments (25 or 30° C). RMR's were calculated as residuals from L-S means regressions of $\log_{10}$-transformed oxygen consumption rates and body mass. Turtles that exhibited RMR's indicative of high and/or variable activity rates during measurements were removed from the dataset. Consequently, no turtles from T_{inc} = 28.5° C and subsequently reared at T_{water} = 30° C were represented. Incubation temperatures: circles = 26.5° C, triangles = 28.5° C, and inverted triangles = 30.5° C. Water temperatures: closed symbols = 25° C, open symbols = 30° C. Error bars = ±1 SE. Dotted lines connect points where body temperature = incubation temperature.

Figure 1

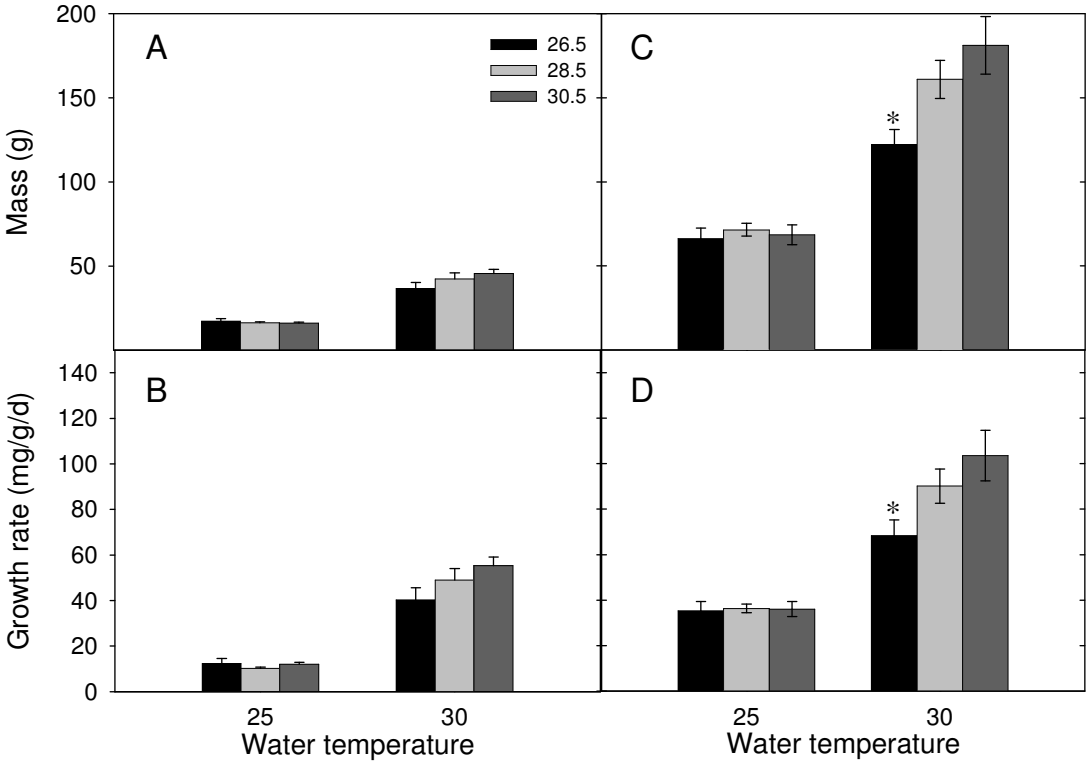
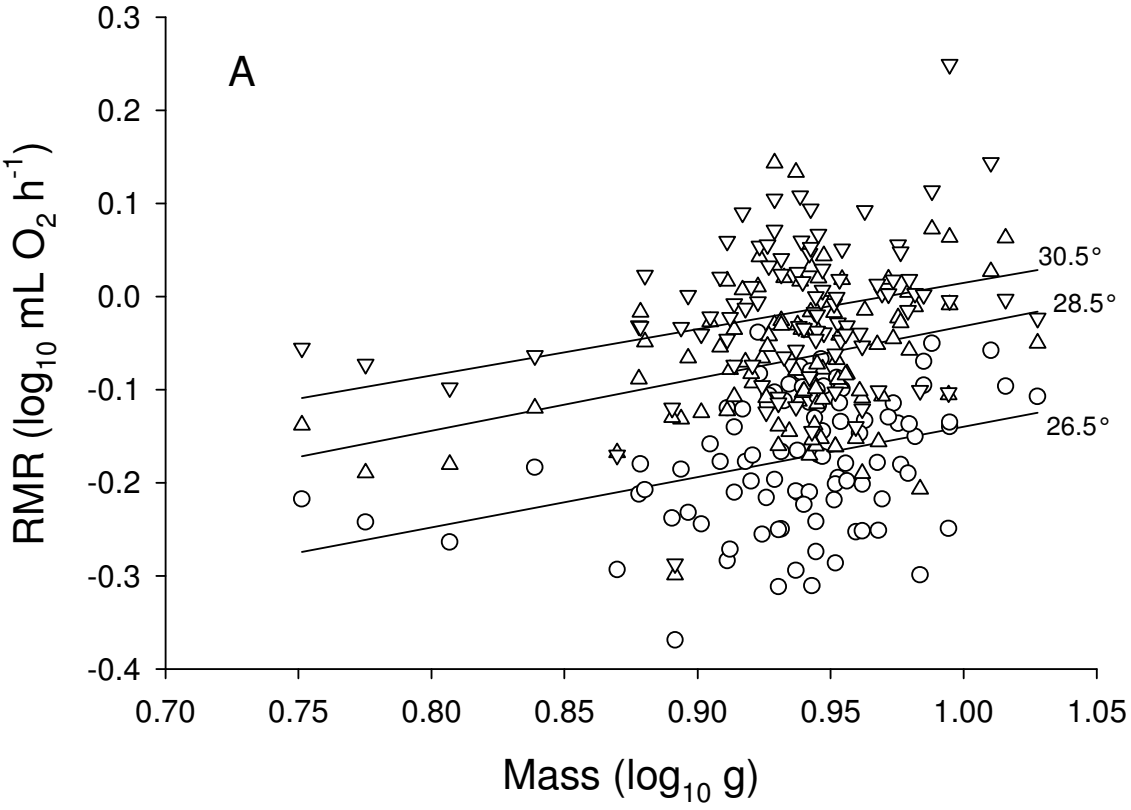

A
B
C
D
26.5
28.5
30.5
Mass (g)
Growth rate (mg/g/d)
200
150
100
50
140
120
100
80
60
40
20
0
*
*
25
30
25
30
Water temperature
Water temperature

Figure 2


A
RMR (log_{10} mL O_2 h^{-1})
Mass (log_{10} g)
30.5°
28.5°
26.5°
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
0.70
0.75
0.80
0.85
0.90
0.95
1.00
1.05

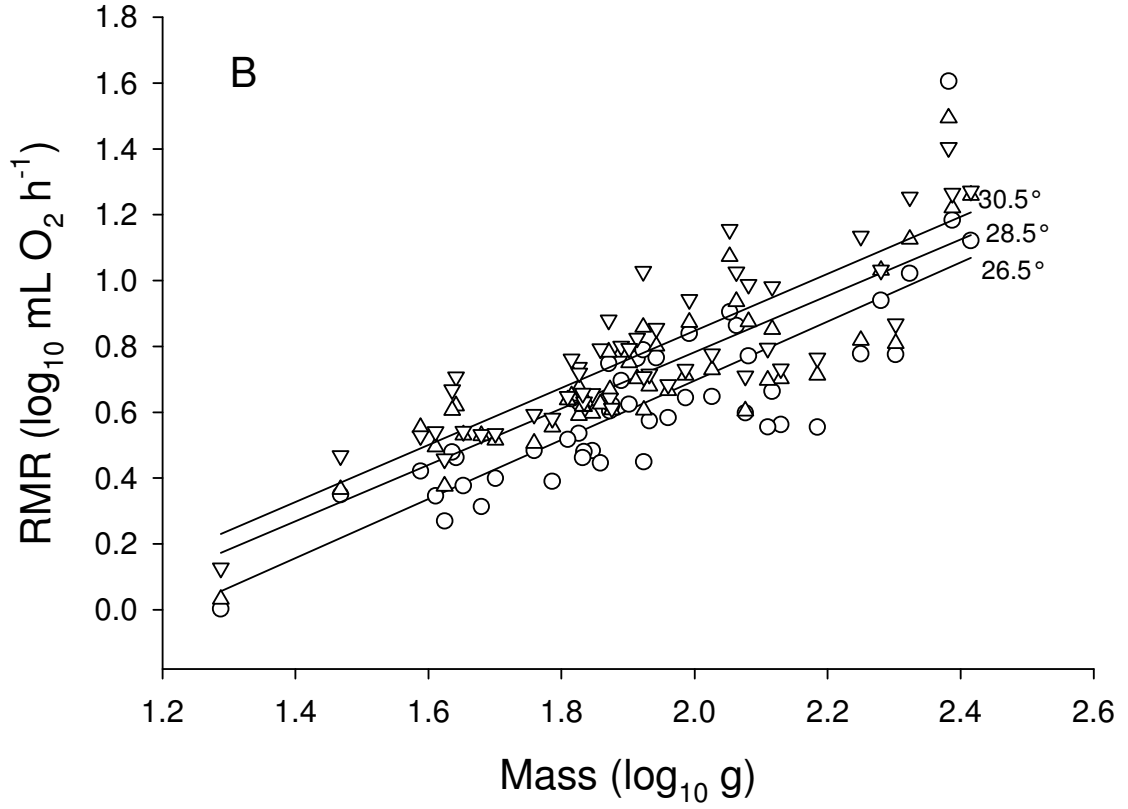
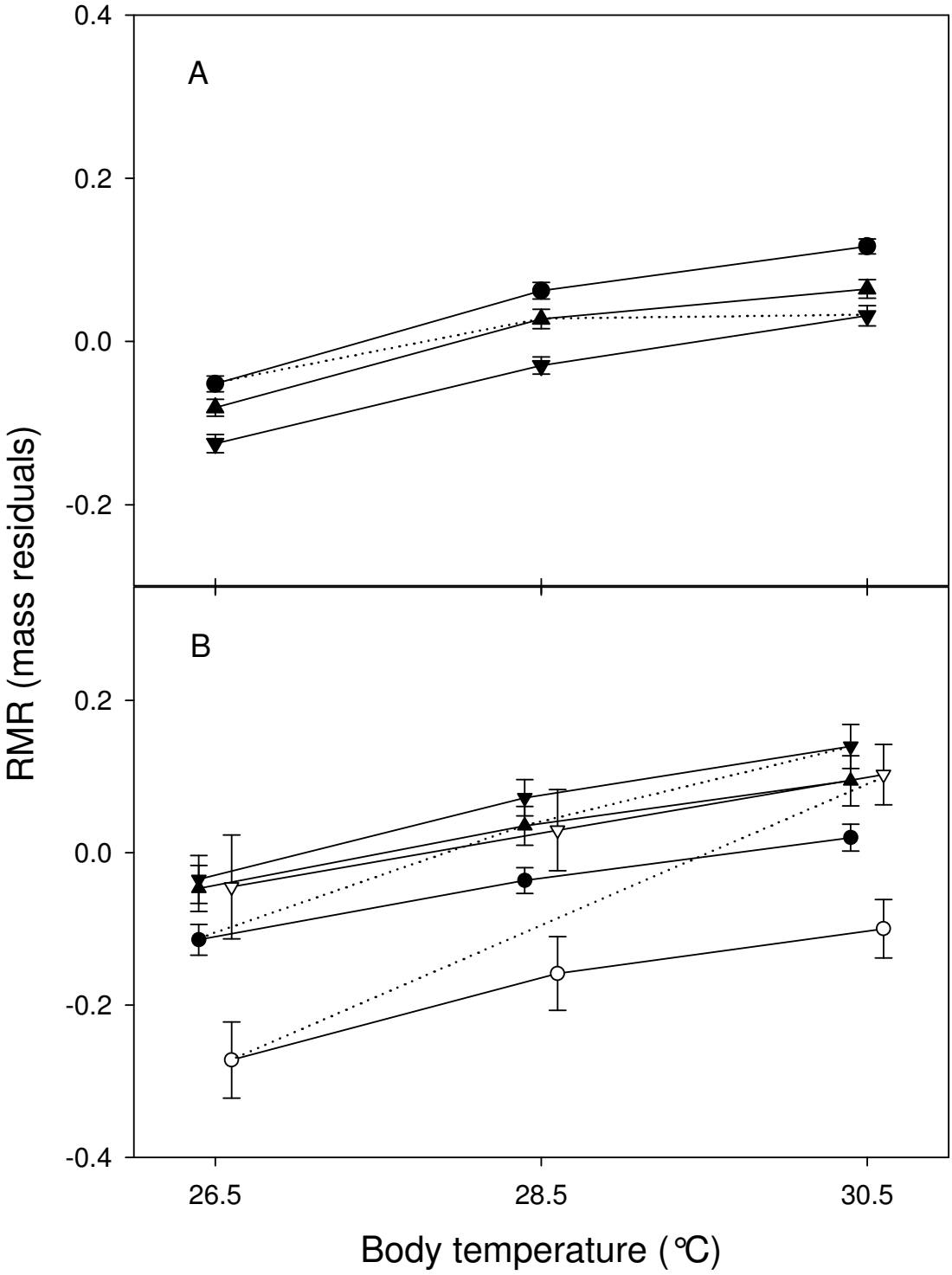

B
RMR (log_{10} mL O_2 h^{-1})
Mass (log_{10} g)
30.5°
28.5°
26.5°
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6

Figure 3


A
B
0.4
0.2
0.0
-0.2
-0.4
RMR (mass residuals)
26.5
28.5
30.5
Body temperature (°C)

IV. INCUBATION TEMPERATURE EFFECTS ON HATCHLING GROWTH AND METABOLIC RATE IN THE AFRICAN SPURRED TORTOISE, *GEOCHELONE SULCATA*

Abstract: We tested competing hypotheses regarding the persistence of temperature-dependent sex determination (TSD) in the African spurred tortoise, *Geochelone sulcata*, by measuring effects of incubation temperature (T_{inc}) on a suite of characteristics, including resting metabolic rate, yolk-to-tissue conversion efficiency, post-hatching growth and temperature preference. Correlations with T_{inc} would be interpreted as potentially supporting the evolutionarily adaptive value of TSD, whereas absence of T_{inc} effects support the hypothesis that TSD persists because selection favoring alternate sex determining mechanisms is weak or absent. Several traits exhibited temporal variation. Metabolic rate Q_{10} was higher immediately after hatching than 40 or 100 days post-hatching, and mass conversion efficiency varied among clutches. However, T_{inc} did not influence size, growth, or resting metabolic rate. The results suggest that TSD is not evolutionarily adaptive in this species under present conditions.

Introduction

Offspring phenotype is determined by genotype, maternal investment and environment. Whereas genotype is fixed at fertilization, the latter two factors often vary throughout embryonic development. Maternal investment can be highly variable among

oviparous ectotherms, ranging from differential allocation of nutrients during egg production to variation in the degree of post-laying care. Among environmental variables, effects of temperature and hydric conditions are the best studied. Temperature has been shown to affect duration of embryonic development, pre- and post-hatching growth rate and survival (Yntema 1976, 1978; Bull and Vogt 1979; Wilhoft et al. 1983; Gutzke and Packard 1987; Brooks et al. 1991). The hydric conditions experienced by eggs can affect development rates of embryos, oxygen uptake and size at hatching (Gutzke and Paukstis 1983; Gustzke et al. 1987; Morris et al. 1983; Gettinger et al. 1984; Miller et al. 1987; Packard and Packard 1988).

Perhaps the most striking (and most studied) environmental effect on phenotype is its influence on gonadal differentiation. Temperature-dependent sex determination (TSD) is the most prevalent and best-studied form of environmental sex determination (Bull 1980), a phenomenon known to occur in a variety of invertebrates and vertebrates, including some crustaceans, fishes, saurians, chelonians, and crocodilians (Bull 1980; Conover and Kynard 1981; Conover 1984; Naylor et al. 1988; Bull and Charnov 1989; Ciofi and Swingland 1997).

Among turtles (and in sauropsids generally), TSD is the ancestral sex-determining mechanism, from which genetic sex-determination has evolved at least six times (Ewert and Nelson 1991; Janzen and Paukstis 1991; Janzen and Krenz 2004), whereas no shifts from genetic sex determination to TSD are evident (Janzen and Krenz 2004). Partly for this reason, turtles have been popular model organisms for studying both mechanisms and evolutionary consequences of TSD. A benefit of much of this TSD research has been concomitant investigations of incubation temperature (T_{inc}) effects on many other traits in

chelonians, such as size at hatching (Brooks et al. 1991; Spotila et al. 1994; Roosenberg and Kelley 1996; O'Steen 1998; Demuth 2001; Du and Ji 2003), growth rate (Ryan et al. 1990; Brooks et al. 1991; McKnight and Gutzke 1993; Bobyn and Brooks 1994; Spotila et al. 1994; Rhen and Lang 1995; Roosenburg and Kelley 1996; O'Steen 1998; Rhen and Lang 1999; Demuth 2001; Steyermark and Spotila 2001; Du and Ji 2003), locomotor performance (Janzen 1993, 1995; Doody 1999; Demuth 2001; Ashmore and Janzen 2003; Du and Ji 2003), and thermoregulation (O'Steen 1998). The purpose of much of this work has been to identify T_{inc}-affected traits that are differentially beneficial to males and females in an effort to identify selective mechanisms for the evolution and persistence of TSD (Bull and Vogt 1979). Plausible mechanisms have been described involving some, but by no means all, of these traits (Janzen and Paukstis 1991; Shine 1999). For example, it has been hypothesized that males of species that exhibit male-biased sexual size dimorphism could benefit from hatching at temperatures that produce relatively large or fast-growing hatchlings, if such temperatures exist.

In comparison to many of the endpoints described above, the effects of T_{inc} on hatchling resting metabolic rate (MR) have been investigated in relatively few turtles (O'Steen and Janzen 1999; Steyermark and Spotila 2000). MR is an integrative measure of biochemical activity necessary to meet basic maintenance requirements (i.e., absent activity, growth or digestion). Therefore, MR represents a non-lethal whole-animal measure that may identify the presence of physiological differences (e.g., differences in organ size or enzyme activity), but not the underlying biochemical or physiological nature of the differences. If MR is low and constitutes a relatively small fraction of an individual's total energy budget, then a greater proportion of available energy can be

allocated to other processes that may increase evolutionary fitness such as those related to growth and activity. This difference in allocation, if differentially beneficial to males and females, would lend support to the hypothesis that TSD is an adaptive trait. Conversely, a lack of MR differences between the sexes would suggest that this unusual sex-determining mechanism persists in a given species due simply to genetic inertia.

My study organism was *Geochelone sulcata* (African spurred tortoise; Miller, 1779), a testudinid once common in Africa at latitudes coincident with the Sahel region. Recent population declines have been steep, and likely are attributable to climatic and vegetational changes, as well as human exploitation, which collectively have resulted in inclusion of the species on Appendix II (controlled trade), and petitioned for elevation to Appendix I (threatened with extinction) of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2007). This species is particularly suitable for studying TSD because, like all testudinids that have been examined (Spotila et al. 1994; Eendeback 1995; Burke et al. 1996; Demuth 2001; Ewert et al. 1994, 2004), TSD follows a relatively simple dichotomous patterns wherein males are produced at low temperatures and females at higher temperatures. In addition, *G. sulcata* exhibits extreme male-biased sexual size dimorphism, and is therefore suitable for testing the oft-hypothesized hatchling size and/or growth T_{inc} effect. Although TSD patterns have been examined in a small fraction of tortoise species, the uniformity of available TSD data suggests either that it is actively maintained by natural selection among testudinids or that current selection on sex-determining mechanisms in tortoises is relatively neutral.

We measured T_{inc} effects on RMR as a potentially integrative measure of TSD-sustaining mechanisms, as well as hatchling size, post-hatching growth and temperature

preference (T_{pref}) of juvenile *G. sulcata*. We hypothesize that if TSD maintains a selective advantage over genetic sex determination, then differences among T_{inc}s in MR, size, growth, and/or activity patterns would be evident, and that differences would be greater between male- and female-producing temperatures than among temperatures producing each sex. Absence of differences that correlate with T_{inc} and sex, on the other hand, provide evidence that strong selection favoring genetic sex determination has not occurred among those taxa in which TSD persists.

Materials and methods

All procedures for this research were approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocol #AS023), guaranteeing compliance with animal care guidelines described in The Guide for the Care and Use of Laboratory Animals, 7th edition (1996).

Eggs

We obtained *Geochelone sulcata* eggs from a commercial tortoise breeder (Riparian Farms, Hereford, AZ). Eggs were collected from nests produced by captive females between 19 January and 31 March 2003 and shipped to arrive at Oklahoma State University within 48 h following oviposition. Upon arrival, the eggs were weighed (± 0.1 g) and diameter was measured (± 0.1 mm). Eggs were then distributed among 15 plastic shoeboxes filled with damp vermiculite (1:1 vermiculite:water by mass; -150 kPa) in a randomized block design with clutch serving as the blocking variable. Three shoeboxes were assigned to each of five incubators programmed to maintain constant temperatures (28.5°, 29.5°, 30.5°, 31.5°, 32.5° C). Shoeboxes were rotated daily within each incubator

to minimize potential effects of temperature gradients. Thermocouple wires placed in each incubator were used to monitor the temperature of each incubator daily. Each box was weighed weekly, and water was added when necessary to maintain a consistent hydric environment. Beginning on day 20 of incubation, MRs (oxygen consumption) of eggs comprising two of the clutches were measured at 10-d intervals until pipping (see below). After pipping, eggs were placed into individual containers and maintained in the incubators to retain the identification of individual hatchlings until residual yolks were internalized. Individual identification numbers were then written on each tortoise's carapace with acrylic paint.

Hatchlings

MR of each hatchling was measured at each of the five incubation temperatures 9-14 d after pipping (after residual yolk was internalized) to test for metabolic compensation to long-term temperature acclimation during incubation. Because activity in the chambers can elevate MR above resting levels, measurements at each temperature were made in duplicate, and the lower of each pair of measurements was used to estimate activity-free MR. Tortoises were likely still subsisting on stored yolk and thus not post-absorptive. Therefore, these measurements do not meet the criteria for measuring standard metabolic rate. After completing these measurements, hatchlings were removed from the incubators and housed in groups of five in plastic boxes (57x41x15 cm). Each box was equipped with an overhead 60-W incandescent light for basking and a box of moist peat moss into which the tortoises could retreat. Lights were on a 12:12 light cycle; at night ambient temperature dropped to 25° C.

Tortoises were fed a commercial tortoise diet (Mazuri Tortoise Diet 5M21) daily. Food pellets were saturated with water to soften them. In addition, tortoises were soaked for 30 min. daily in distilled water, after which individuals were randomly reassigned to enclosures to avoid confounding cage and social effects among cage mates.

Each tortoise was weighed and measured (midline carapace and plastron lengths) at 10-d intervals for 110 d after hatching. A final set of mass and length measurements were recorded 330 d after hatching, after which tortoises were returned to Riparian Farms.

Metabolic rate

Oxygen consumption was measured via closed-system respirometry (Vleck 1987) and used to calculate MR (Peterson 1990). Oxygen consumption rates of each tortoise were measured three times at all five T_{inc} (28.5°, 29.5°, 30.5°, 31.5°, and 32.5° C): 1-3 d after internalizing yolk and on days 40 and 100 post-hatching.

Metabolic chambers were constructed from 169- 322- and 959-mL plastic jars with screw-on lids. A stopcock was inserted through the lid of each jar, and a thin film of vacuum grease was applied on the inside of each lid to ensure an airtight seal when the stopcock was closed.

Prior to each MR measurement, tortoises were fasted for 4 d to minimize metabolic costs associated with specific dynamic action and growth. On the day of measurement, each hatchling was weighed and placed into a chamber. Chambers were then placed inside an incubator for 1.5-2 h to allow body temperatures to stabilize. With the overhead lights off to minimize disturbance to the tortoises, chambers were removed

from the incubator. After screwing lids on to create an airtight seal, pre-trial air samples were drawn into 30-mL syringes, also equipped with stopcocks. The stopcocks on the syringe and chamber were then closed, the syringes removed, and the time of sampling recorded. Chambers were then placed back into the dark incubator and removed after 56-85 min, when post-trial samples were drawn from the stopcock after pumping each syringe several times to ensure mixing of the air inside. After the final measurement at a given temperature, the tortoises were moved to another temperature and the process was repeated. Measurements at all five temperatures were made in 1-2 d.

Oxygen concentrations of all air samples were analyzed in 10-mL aliquots with a Sable Systems FC-1 oxygen analyzer. Air was drawn from outside the building at a regulated flow rate of 100 mL/min and through serial columns of Drierite and Ascarite to remove water and CO_2, respectively. Each aliquot was injected into the air stream, which passed through a small column of Drierite and Ascarite and then through the oxygen analyzer. Oxygen consumption by each turtle was calculated as the difference between the initial and final volumes of oxygen after correcting for chamber volume (Peterson 1990).

Thermoregulation

Preferred temperature of tortoises from two clutches (clutch 5: n=15; clutch 6: n=17) were measured in a three-lane linear thermal gradient. Each lane measured 124 cm x 25 cm x 63 cm (LxWxH). The floor of the gradient was constructed from a 0.6 cm thick aluminum plate. The plate was bent down at a right angle 20 cm from one end and the resulting vertical tab was inserted into an insulated box of ice water. Three 250-W

incandescent lights were placed under the opposite end of the plate to create a 14-44°C surface gradient. Fluorescent lights set on a 12L:12D cycle were placed above each lane to produce an evenly lit environment.

All measurements were conducted 136-144 d post-hatching so that disturbance to the animals did not coincide with growth measurements. After fasting for four days to eliminate postprandial effects on temperature preference, tortoises were outfitted with a thermocouple (see below) and placed individually in a lane of the gradient between 12:00 and 14:00 h. They then remained in the gradient for ~46 h. The first 6-8 h of data were discarded to provide a period of acclimation to the gradient. Data from the first dark cycle (20:00h-08:00) were analyzed separately from the proceeding light cycle (08:30-20:00).

Body temperature (T_b) was measured by inserting a precalibrated 18-G thermocouple 2-3 cm into the cloaca of each tortoise. Thermocouples were secured with epoxy applied to the posterior carapace and then connected to temperature data loggers (Onset Comp. Corp., Pocasset, MA) that were allowed to slide on hooks along monofilament strung longitudinally above each lane of the gradient, thereby permitting each tortoise free movement without risk of getting entangled in the thermocouple. Loggers were programmed to record temperature at one-minute intervals. After data were downloaded from the loggers, average temperatures were calculated for each 30-min interval, and average T_b was calculated for each turtle during each cycle.

Sex determination

Individuals were sexed when 152-235 d old via laparoscopic surgery (Rostal et al. 1994). Food was withheld for 4 d prior to the procedure and tortoises were soaked daily

to stimulate elimination of feces. The tortoises were transferred to the veterinary facilities at the Tulsa City Zoo on the mornings that surgeries were performed. General anesthesia was achieved with a cocktail of 10 mg/kg Ketamine and 0.1 mg/kg Medetomidine. After cleaning the site with Chlorhexidine, a 3-4 mm incision was made posterior to the plastral bridge. A laparoscope probe was inserted into the incision, and the gonads were identified visually. Two observers examined each individual, and agreement was 100%. The gonads of *G. sulcata* in this age range were very distinct; ovaries appear as long ribbons of white follicles, whereas testes are darker, less textured, and highly vascularized. Incisions were closed with a suture, and Atipamezole was used as a reversing agent for the Medetomidine in three cases where individuals did not show signs of activity within 1 h after surgery.

Statistics

All mass and length data were $\log_{10}$-transformed prior to analyses to improve the distribution of the data and the homogeneity of variances. Appropriate assumptions of parametric statistics were verified for all tests, and non-parametric alternatives were used when assumptions of normality and homogeneity of variances were not supported (see Results).

We analyzed size using two different methods: first, by comparing $\log_{10}$-transformed mass, and second by comparing length-mass residuals among treatments. These size measurements, along with maximum growth rate, were analyzed using analysis of covariance (ANCOVA), with T_{inc} as a fixed factor and clutch as a random factor. Egg mass was used as a covariate for hatchling mass and mass at hatching was

used as a covariate for analyzing maximum growth rate. Interactions between the covariate and main effects were tested to ensure homogeneity of slopes among treatments ($P > 0.05$). These interaction terms were then removed for final analyses.

Metabolic compensation to incubation temperature was tested in a repeated-measures analysis, with T_{inc} as a fixed effect, mass as a covariate and MR repeated on each tortoise over the five measurement temperatures. Additionally, Q_{10} values (using MR data from 28.5 ° and 32.5 ° C) were calculated for each tortoise at each of the three MR measurement intervals. Q_{10} was then analyzed in a repeated measures design with T_{inc} as a fixed effect, clutch as a random effect and Q_{10} repeated for each tortoise over the three measurement periods.

Similarly, effects on growth of T_{inc} and clutch were analyzed in a repeated-measures ANCOVA model, with mass repeated over 11 time intervals and egg mass as a covariate.

T_{pref} was analyzed by first calculating day-time and night-time mean T_{pref} for each tortoise. Day- and night-time means were then used as response variables in separate repeated-measures ANOVAs with Clutch and T_{inc} as independent variables and T_{pref} repeated for each tortoise.

Results

Eggs

We randomly distributed 107 eggs comprising six clutches (clutch size range = 15-21; Table 1) among the five incubation treatments. Egg mass was 51.4 ± 0.6 g (mean ± 1SE) (range = 37.2-62.4 g) and varied among clutches (Kruskal-Wallis One-Way

ANOVA on Ranks: $H = 89.64$, $P \leq 0.001$; Table 1). Of the 107 eggs incubated, 82 (76.6%) hatched; hatching success varied among clutches ($\chi^2 = 39.049$, df = 5, $P < 0.001$) but was unaffected by T_{inc} ($\chi^2 = 0.658$, df = 4, $P = 0.956$, respectively). Clutches 2 and 4 exhibited low hatching success relative to the other four clutches (Table 1) and therefore were not included in subsequent analyses. Three tortoises that hatched but refused to eat—and ultimately died—were also excluded from subsequent analyses.

Incubation duration was 72-116 d and was inversely correlated with T_{inc} (time = $1.54(T_{inc})^2 - 101.56(T_{inc}) + 1754$; Fig. 1). The relationship best fit a quadratic curve, indicating that one-unit increases at higher temperatures influenced duration less than increases of similar magnitude at lower temperatures.

Hatchlings

Hatchling mass varied among clutches (Table 1). Log_{10} hatchling mass correlated positively with log_{10} egg mass (Fig. 2, 3a), and mass-conversion efficiency varied among clutches (Figure 3). This pattern remained significant for up to (and likely beyond) 330 d, but the coefficient of determination decreased with time ($P < 0.001$, r^2 range = 0.10-0.85; Fig. 2). Hatchlings from different clutches variedTortoises grew 0.471± 0.014 $g \cdot d^{-1}$. Incubation temperature had no effect on or growth rate ($F_{4, 50} = 1.54$, $P = 0.205$; Fig. 4), whereas clutch did, even after controlling for differences in initial egg mass ($F_{5, 51} = 11.49$, $P < 0.001$). The maximum growth rate calculated over a 10-d interval was 1.082 ± 0.038 $g \cdot d^{-1}$, and was unaffected by T_{inc} or clutch.

Sex determination

Sex was verified for all hatched tortoises. As has been observed in other testudines (Spotila et al. 1994; Eendeback 1995; Burke et al. 1996, Demuth 2001; Ewert et al. 2004), males were produced at low temperatures and females at high temperatures. A mixed sex ratio was observed at two intermediate temperatures, and although no single temperature in the study produced a balanced sex ratio, interpolation from the data suggests a pivotal T_{inc} of 30.8° C (Fig. 5).

Metabolic rate

Hatchling MR exhibited a positive correlation with ambient temperature ($F_{4, 304}$ = 175.12, $P < 0.001$). However, hatchlings did not exhibit metabolic compensation following acclimation to a constant incubation temperature ($F_{16, 304} = 1.31$, $P = 0.1861$; Fig. 6). Q_{10} was unaffected by T_{inc} ($F_{4,135} = 0.30$, $P = 0.8749$) but was higher 5 d after hatching than at 40 or 100 d post-hatching (2.75±0.07, 2.38±0.08, 2.32±0.07, respectively; $F_{2,143} = 9.64$, $P < 0.0001$). There was a maternal effect ($F_{3,135} = 3.91$, $P = 0.0102$), due to consistently higher Q_{10} values among Clutch 3 hatchlings compared to other clutches.

Temperature regulation

T_{inc} had no effect on either daytime or nighttime T_{pref}, or on the precision with which tortoises maintained a consistent body temperature (Table 2). However, tortoises from Clutch 5 consistently selected lower body temperatures than did Clutch 6 tortoises

(daytime: 23.5 vs. 30.2°C, nighttime: 23.6 vs. 31.0°C), and tortoises from Clutch 6 tended to maintain more consistent body temperatures then did those from Clutch 5.

DISCUSSION

T_{inc} strongly affected incubation duration and gonad differentiation. However, within the range of temperatures tested T_{inc} had no discernable effect on egg viability, hatchling mass, post-hatching growth, RMR, Q_{10}, or T_{pref}. The effects of T_{inc} on hatchling size and growth reported in previous chelonian studies have been variable, even within individual species, ranging from approximately linear positive relationships between temperature and size (Packard et al. 1987; Rhen and Lang 1995) to no observed temperature effect (Janzen 1995; Steyermark and Spotila 2001). A number of studies have reported little effect of T_{inc} on hatchling size or growth rate except at extreme temperatures that also negatively affected egg viability, indicating that those temperatures pushed the limits that embryos can physiologically tolerate (Brooks et al. 1991; McKnight and Gutzke 1993; Bobyn and Brooks 1994; Spotila et al. 1994; Du and Ji 2003). We attempted to select temperatures that straddled the pivotal temperature for *G. sulcata*, but limited the range to that expected to produce high hatching rates (pers. comm.; Richard Fife).

Fewer studies have examined T_{inc} effects on RMR in reptiles, but there is still wide variation in observed results. *Chelydra serpentina* has been the subject of two such studies. Measurements conducted shortly after hatching found a negative correlation between T_{inc} and RMR (O'Steen and Janzen 1999), whereas the effect of T_{inc} on RMR produced inconsistent patterns among six-month-old juveniles (Steyermark and Spotila

2000). I share Steyermark and Spotila's (2000) view that T_{inc} may act in a similar fashion to acclimation temperature, so that T_{inc} effects can be interpreted as acclimation effects. Therefore, it is not surprising that in their study hatchling *C. serpentina* exhibited a MR pattern consistent with positive compensation, but that juveniles six months removed from the acclimation period did not.

G. sulcata showed a predictable positive correlation between MR and ambient temperature in this study, but there was no evidence of metabolic compensation to T_{inc} immediately after hatching, or 40-100 d post-hatching. Q_{10} values at all ages fell within the generally-predicted 2-3 range. Q_{10} was consistent at 40 and 100 d, but was lower at both later stages compared to that measured at hatching. This temporal difference may stem from differences in digestive state: hatchling tortoises were subsisting on stored yolk reserves at the time of initial MR measurements, whereas they were in a post-absorptive state following a period of fasting during subsequent measurements. These results suggest that biochemical processes associated with digestion may be affected by temperature to a greater degree than those contributing to maintenance metabolism.

Differences due to maternity were more prevalent than T_{inc} effects. Hatching success was low for two out of six clutches, and egg and hatchling mass differed significantly among clutches. There was a consistent correlation among clutches between egg and hatchling mass, and Clutch 3, which was composed of the largest eggs, produced the largest hatchlings. However, Clutch 3 did *not* exhibit the greatest egg mass-to-hatchling mass conversion efficiency, indicating that, in addition to the quantity of material allocated to each egg, females may vary the ratios or quality of egg components, and thereby influence hatchling size.

Finally, clutch, but not T_{inc}, strongly influenced T_{pref}. The magnitude of difference between Clutches 5 and 6 were dramatic and consistent during both daytime and nighttime measurement intervals. It is surprising that, despite a nearly 7°C difference in T_{pref}, tortoises in the two clutches for which this endpoint was measured did not differ in other respects such as growth rate or temperature-specific metabolic rate. Unfortunately, without data from additional clutches it is impossible to know whether Clutch 6 tortoises exhibited preferences dramatically higher than typical, or if Clutch 5 tortoises selected unusually low temperatures. Based on what little is known about *G. sulcata* natural history and the climate to which it is presently accustomed, it seems likely that daytime temperatures of 30°C are more commonly encountered *in situ* than temperatures in the low to mid 20°C range .

Our results do not lend support to the hypothesis that TSD is actively maintained in this species due to males and females benefiting differently from development at different temperatures. It is possible that more extreme temperatures would have produced measurable temperature differences; however, the range that we used extended 2-3°C on either side of the pivotal temperature for the species, and thus should have been sufficiently broad to reveal any ecologically relevant effects that existed. This leaves two possibilities: 1) that TSD in *G. sulcata* is no longer evolutionarily adaptive, but persists because selection against it and in favor of other SDM's is weak, or 2) that TSD is an adaptive trait, but for reasons not elucidated by this study. Differentiating between these two competing hypotheses remains the challenge for investigators of TSD in this and other chelonian species.

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Table 1. Clutch size (n), hatching success, and egg and hatchling size for six *Geochelone sulcata* clutches. Mass and length values expressed as mean(SE).

Clutch	n	Hatching success (%)	Egg mass (g)	Hatchling mass (g)	Carapace length (mm)	Plastron length (mm)
1	16	94	40.46(0.33)	32.87(0.53)	46.52(0.25)	42.30(0.31)
2	15	40	46.36(0.81)	37.19(1.21)	49.00(0.75)	46.37(0.75)
3	20	85	57.38(0.30)	33.25(0.28)	46.45(0.16)	42.13(0.19)
4	15	34	53.42(0.26)	43.38(0.50)	51.68(0.21)	47.58(0.34)
5	21	95	50.14(0.43)	38.50(0.46)	51.50(0.26)	48.20(0.36)
6	20	95	55.74(0.77)	47.15(0.72)	52.31(0.40)	48.98(0.53)

Table 2. Incubation temperature and clutch effects on preferred body temperature of juvenile *Geochelone sulcata*. Standard deviations calculated from data acquired at 15-minute intervals were used as measures of the precision with which tortoises thermoregulated. Shown are F-values (degrees of freedom, P-values). Bold text indicates statistically significant relationships.

Light cycle		**T_{inc}**	**Clutch**	**T_{inc}xClutch**
Daytime	T_{pref} (mean)	0.61(4,21,0.657)	**9.23(1,21,0.006)**	1.08(4,21,0.390)
	T_{pref} (precision)	2.56(4,21,0.069)	**4.57(1,21,0.045)**	1.70(4,21,0.187)
Nighttime	T_{pref} (mean)	1.26(4,21,0.317)	**8.90(1,21,0.007)**	1.36(4,21,0.279)
	T_{pref} (precision)	1.78(4,21,0.168)	**7.85(1,21,0.010)**	0.85(4,21,0.510)

FIGURE LEGENDS

Figure 1. Relationship between constant incubation temperatures and incubation duration in *Geochelone sulcata*. Error bars = ± 1SE.

Figure 2. Changes with age in the relationship between initial egg mass and hatchling mass. Closed circles = age 0 d (r^2 = 0.85), open circles = age 50 d (r^2=0.53), closed inverted triangles = age 110 d (r^2=0.34), and open inverted triangles = age 330 d (r^2=0.10). Note $\log_{10}$ axes.

Figure 3. A, Least-squares regression between initial egg mass and hatchling mass, fitting the equation $\log_{10}$ hatchling mass = $-0.008(\log_{10}$ egg mass$)^{0.952}$. Different symbols identify four different clutches. Dotted lines = 95% Confidence Intervals. Note $\log_{10}$ axes. B, differences in hatchling mass among different clutches. Values were calculated from log-log residuals to correct for differences in initial egg mass. Lower case letters signify differences among clutches (α = 0.05). Error bars = ± 1SE.

Figure 4. A, Growth of juvenile tortoises incubated at different temperatures. B, Growth rate calculated over 10-d intervals. Lower growth rates at 40 and 100 d correspond with fasting periods prior to measuring MR. Closed circle = 28.5°C, open circle = 29.5°C, open square = 30.5 °C, open diamond = 31.5°C, closed diamond = 32.5°C. Error bars = ±1SE.

Figure 5. Relationship between incubation temperature and sex ratio, presented as percent male. Parenthetical numbers = sample sizes; dotted lines indicate interpolated pivotal temperature.

Figure 6. Changes in resting metabolic rates (O_2 consumption) with temperature of hatchling tortoises incubated at five different temperatures. T_{inc} symbols as in Fig. 5. Error bars = ±1SE.

Figure 1

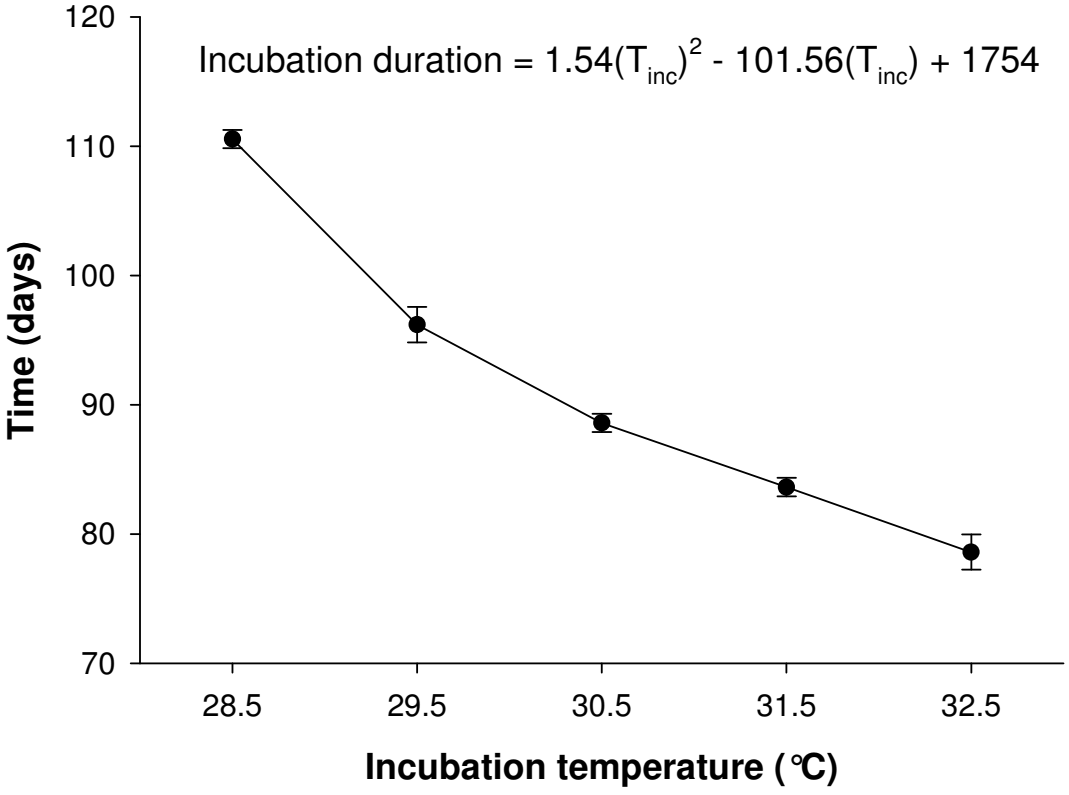

Incubation duration = 1.54(T_inc)^2 - 101.56(T_inc) + 1754
Time (days)
120
110
100
90
80
70
28.5
29.5
30.5
31.5
32.5
Incubation temperature (°C)

Figure 2

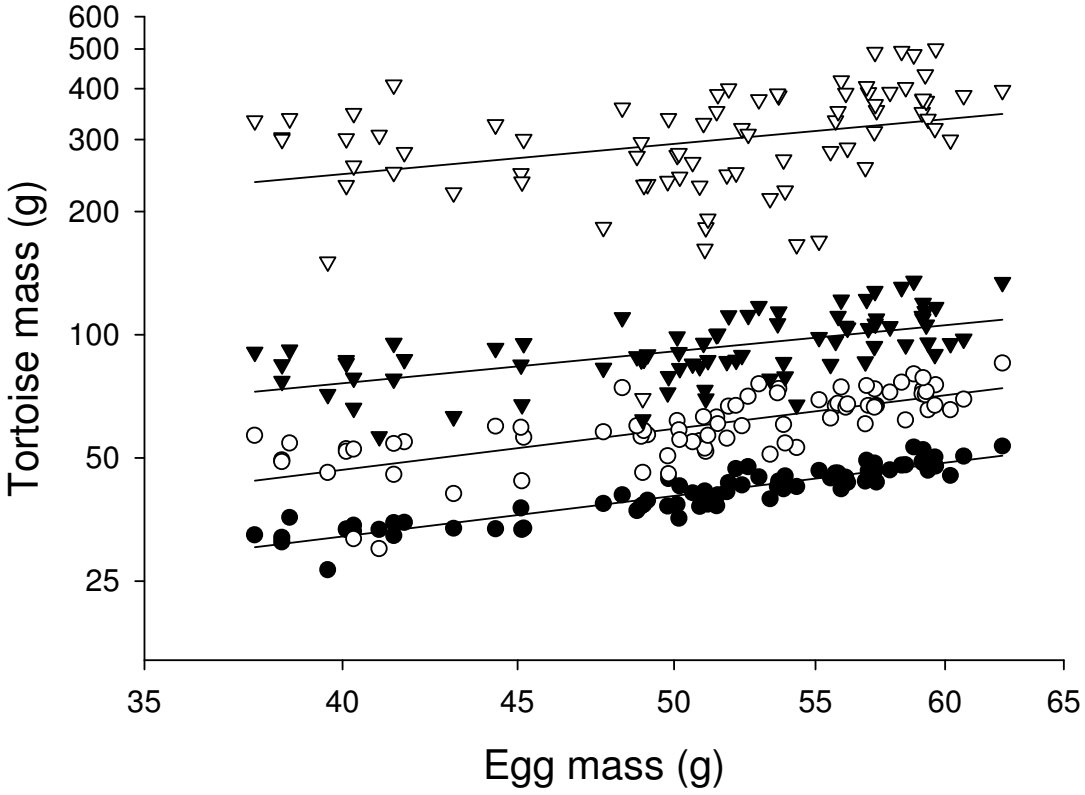

600
500
400
300
200
100
50
25
Tortoise mass (g)
35
40
45
50
55
60
65
Egg mass (g)

Figure 3

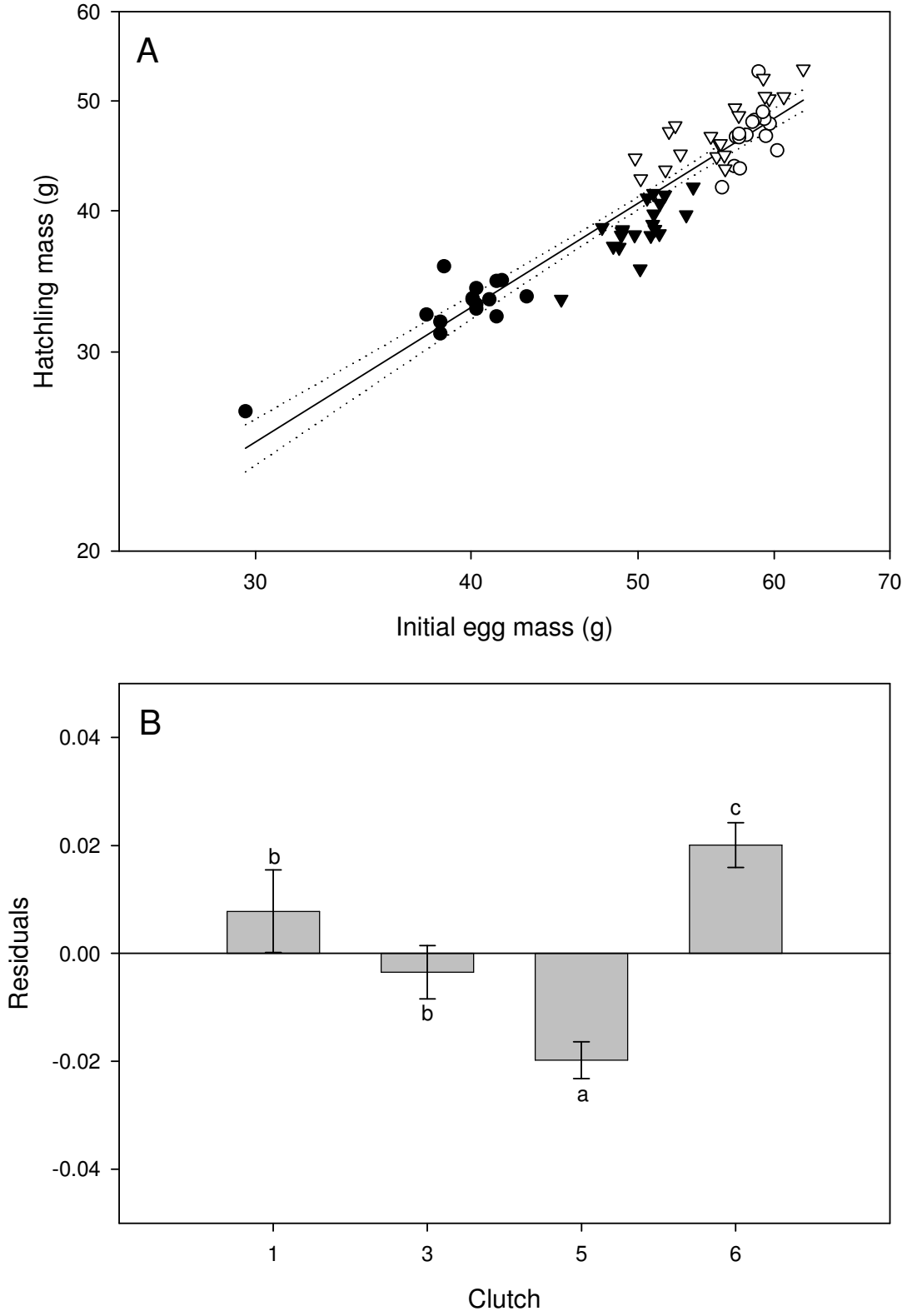

A
Hatchling mass (g)
60
50
40
30
20
30
40
50
60
70
Initial egg mass (g)
B
Residuals
0.04
0.02
0.00
-0.02
-0.04
b
b
a
c
1
3
5
6
Clutch

Figure 4

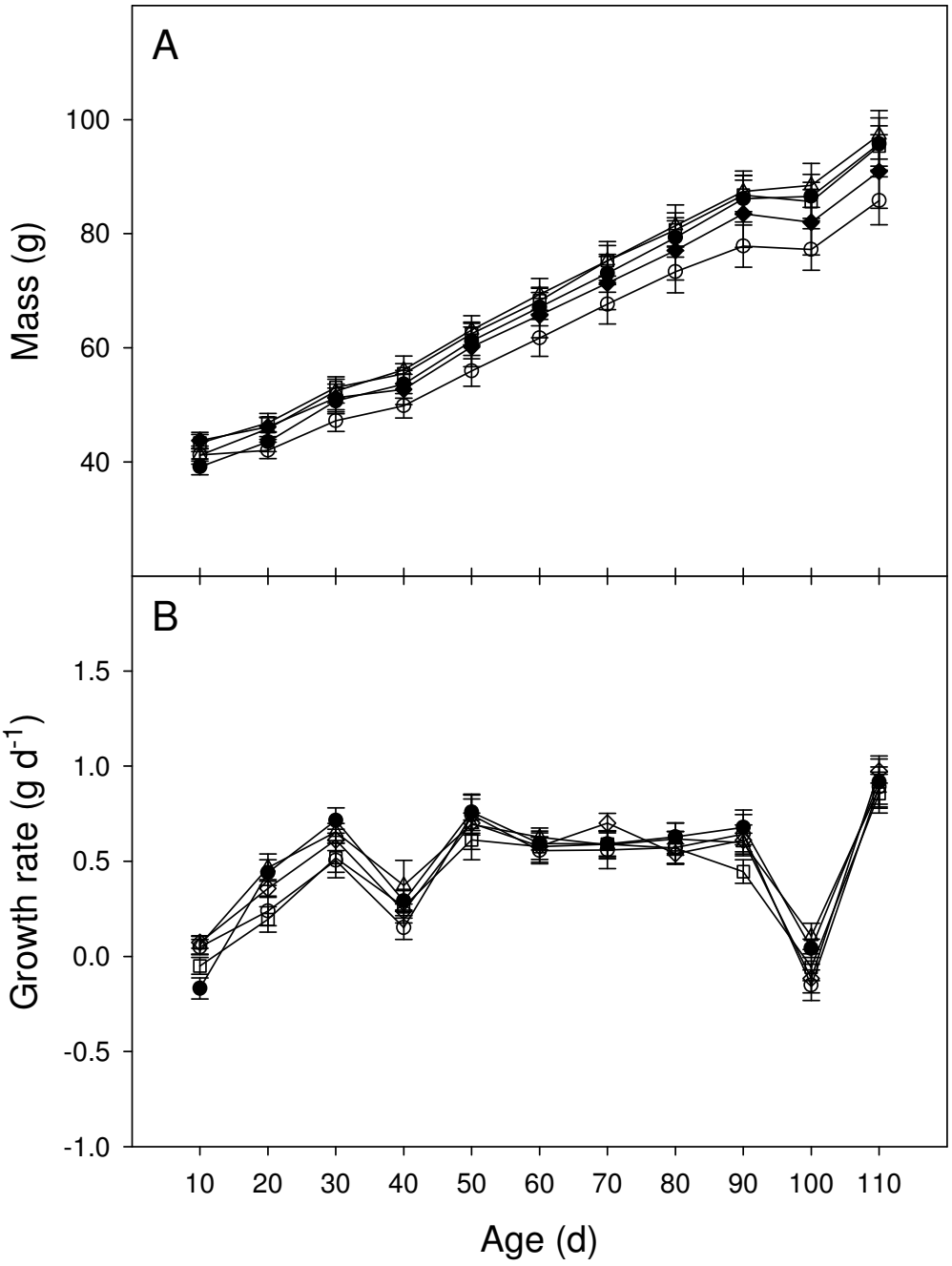

A
B
Mass (g)
Growth rate (g d-1)
Age (d)
100
80
60
40
1.5
1.0
0.5
0.0
-0.5
-1.0
10
20
30
40
50
60
70
80
90
100
110

Figure 5

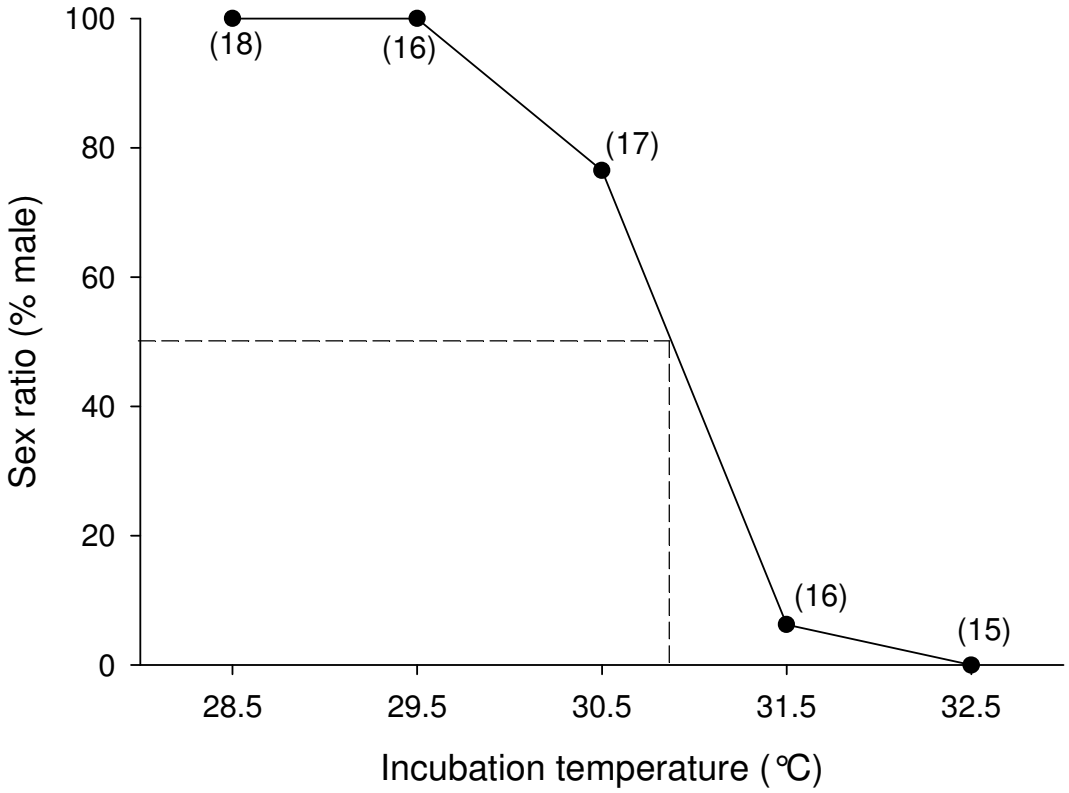
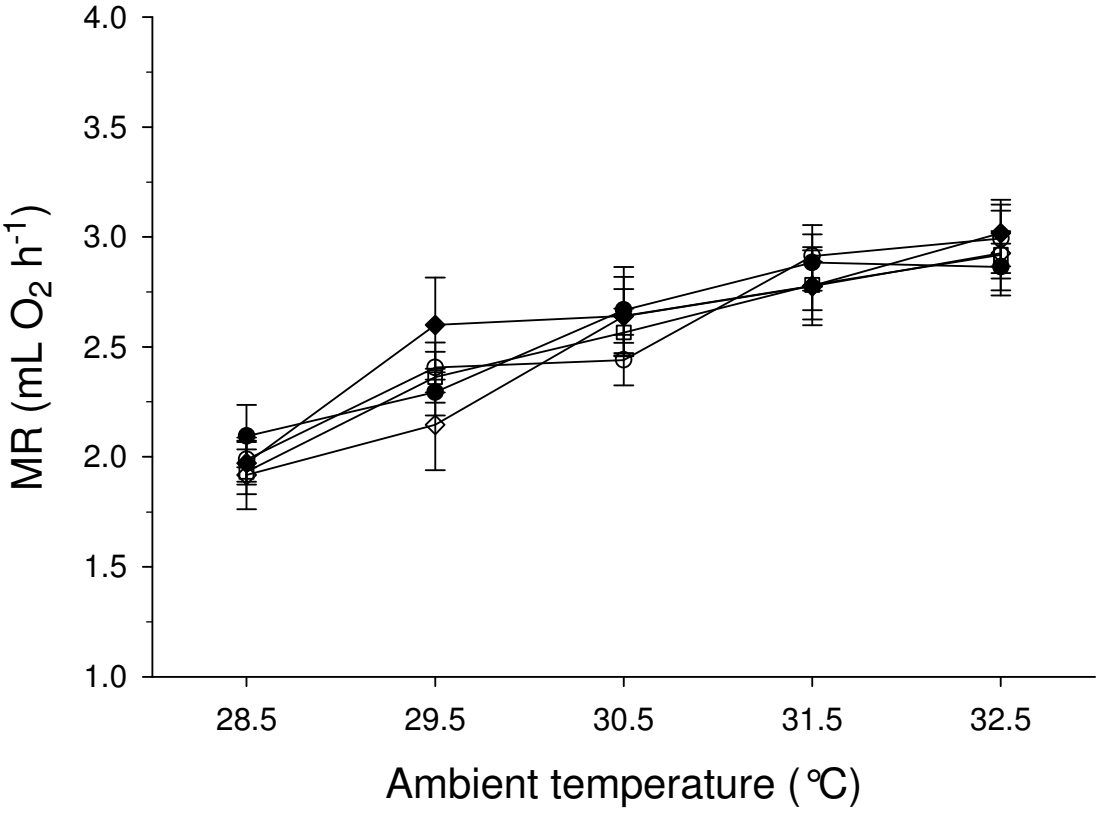

100
80
60
40
20
0
Sex ratio (% male)
(18)
(16)
(17)
(16)
(15)
28.5
29.5
30.5
31.5
32.5
Incubation temperature (°C)

Figure 6


4.0
3.5
3.0
2.5
2.0
1.5
1.0
MR (mL O_2 h^{-1})
28.5
29.5
30.5
31.5
32.5
Ambient temperature (℃)

V. Captive propagation and rearing of a threatened river turtle, *Macrochelys temminckii*

ABSTRACT

Alligator snapping turtle (*Macrochelys temminckii*) populations have declined across much of the southeastern United States in recent decades, due at least in part to strong demand for turtle meat. Recently, however, legal protection from large-scale harvesting has been granted to the species in all of the states where it is native, thereby drastically reducing one of the greatest threats to its survival. There is growing interest in propagating alligator snapping turtles in captivity to provide stock to reintroduce in rivers where the species has been extirpated. In response, we analyzed the physiological effects of temperature during embryonic and post-hatching development to provide information about optimal egg and hatchling husbandry. We found that extreme high and low incubation temperatures negatively affected embryo survival and that high incubation temperatures corresponded with shorter incubation time but also produced smaller hatchlings. The effects of temperature on gonadal differentiation indicated that the upper pivotal temperature was ~27.5° C. Post-hatching growth was faster at warmer water temperatures, and there was little-to-no acclimation of metabolic rate to exposure to either incubation or water temperature. I conclude that intermediate (27.5-28.5° C) incubation temperatures produce a female-biased mixed sex ratio and maximize hatching

success and hatchling size while increasing incubation duration only slightly over that at the higher temperatures. In addition, post-hatching growth was influenced by water temperature; therefore, warmer water temperatures (~30° C) decrease the time required to raise turtles to a size suitable for reintroduction.

1. Introduction

Turtles are long-lived organisms, and as such are characterized by delayed maturity (resulting in a long generation time), low fecundity, low nest survival, and high adult survival (Congdon et al., 1993, 1994). Survivorship in turtles is directly proportional to body size (Haskell et al., 1996; Wilbur, 1975; Frazer et al., 1990; Iverson, 1991), and as a result tends to increase dramatically during the juvenile life stages when growth rates are highest (Cagle, 1946; Dunham and Gibbons, 1990; Bobyn and Brooks, 1994).

Presumably, the benefits associated with delayed maturity yield higher average lifetime fitness than that of individuals that mature earlier. However, the long generation time exhibited by most turtles makes them susceptible to chronic environmental disturbances that impact egg or juvenile survival (Congdon et al., 1993, 1994). Similarly adverse effects on population demographics can result from increased adult mortality (Deevey, 1947; Congdon et al., 1993, 1994). Commercial collection of turtles for the pet and meat trades represents an important anthropogenic source of disturbance that disproportionately impacts larger size classes (review in van Dijk et al., 1999). Collection for consumption, in particular, targets larger turtles, which represent the reproductively mature age classes that otherwise enjoys high annual survivorship. Although still a serious problem in many parts of the world, collection of a number of

threatened and endangered turtle species in the United States has been curtailed in recent years because of increased state and federal protection.

Embryo and juvenile age classes are more likely to be affected by abiotic environmental disturbances than are adults. Such events include fluctuations in water levels that can result in flooded nests and long-term temperature fluctuations that can in turn affect a number of variables, ranging from food availability to the incubation conditions experienced by embryos (Janzen, 1994). For example, whereas most vertebrates have genetically fixed sex determination, most turtles exhibit temperature-dependent sex determination (TSD) (Bull, 1980; Bull and Vogt, 1981). As a result, rising global temperatures resulting from the emission of greenhouse gasses and other factors present a potentially serious threat to turtles by skewing population sex ratios (Fisher, 1930; Janzen and Paukstis, 1991).

In addition to sex, several other physiological and morphological characteristics have been identified that may be influenced by incubation temperature (T_{inc}). Among these are size at hatching (Brooks et al., 1991; Rhen and Lang, 1995), post-hatching growth rate (Etchberger et al., 1990; Ryan et al., 1990; Brooks et al., 1991; Bobyn and Brooks, 1994; Etchberger, 1993; McKnight and Gutzke, 1993; Paez et al., 1995; Roosenburg and Johnson, 1995), and temperature acclimation (O'Steen and Janzen, 1999; Steyermark and Spotila, 2000). Because these variables can reasonably be expected to influence survivorship among hatchling and juvenile age classes, it follows that incubation conditions can dramatically affect average lifetime fitness (Miller, 1993; Janzen, 1993, 1995; Bobyn and Brooks, 1994).

Although legal protection has been granted to some turtles, such actions are often taken after a species has already experienced declines sufficient to compromise hatchling and juvenile recruitment. In response to severe population declines, a number of efforts have been made—with some success—to rehabilitate populations of a few species through captive rearing of hatchlings to a size that is expected to substantially reduce mortality prior to being released (Iverson, 1991; Haskell et al., 1996; Caillouet, 1998). Unfortunately, due to a lack of critical data, such efforts are often made without the benefit of a basic knowledge of the factors affecting reproduction and fitness (Morreale et al., 1982; Spotila et al., 1987; Wibbels et al., 1989; Spotila et al., 1994; Moll and Moll, 2000). Although such 'headstart' programs have the potential for improving conservation of threatened and endangered turtles, it will become increasingly important to maximize the success of such programs as more turtle species face critical population declines. The objective of this study was to determine conditions that optimized embryonic development, hatching success, and post-embryonic growth.

Alligator snapping turtles (*Macrochelys temminckii*) (Figure 1) have experienced population declines in recent decades, and have been extirpated in many parts of the species' historical range (Wagner et al., 1996; Pritchard, 1989; Ernst et al., 1994; Heck, 1998; Riedle et al., 2005). This species is found in river systems throughout much of the southeastern United States; it is the largest freshwater turtle in North America, and ranks among the most highly aquatic species as well (Pritchard, 1989; Ernst et al., 1994). Among the many turtle species throughout the region for which population declines have been documented, *M. temminckii* has been identified by several state and federal agencies

as a particularly promising candidate for conservation via captive propagation and release.

A recent three-year survey in eastern Oklahoma found *M. temminckii* in restricted portions of just four rivers within the historical range of the species (Riedle et al., 2005), and reports of incidental catch by fishermen at two additional locations were reported to me 2002 and 2007 (Figure 2). Of these, apparently robust populations were found in tributaries feeding Kerr Reservoir and nearby Lake Eufala in eastern Oklahoma. While river impoundments have likely affected alligator snapping turtle populations in the state by fragmenting populations and creating barriers to migration (Pritchard, 1989; Moll and Moll, 2000), the most devastating impact has likely stemmed from the harvesting of adult turtles to meet the demands of the turtle meat market (Pritchard, 1989; Heck, 1998).

In recent years, *M. temminckii* has been awarded some level of protection in all states in which it occurs (Roman and Bowen, 2000). The United States Fish and Wildlife Service was petitioned in 1983 to list the alligator snapping turtle as threatened, but the petition failed due to insufficient information on the species (Heck, 1998).

In Oklahoma, *M. temminckii* is currently listed as a species of special concern (Ramus, 1998), a status that prohibits possession and export from the state. It is hoped that this ban will curb further population declines. In response to the extirpation of the species across much of the state, Tishomingo National Fish Hatchery, in collaboration with Sequoyah National Wildlife Refuge, initiated a pilot captive-breeding study in 1999 to assess the merit of using captive bred stock to repopulate rivers where alligator snapping turtles were extirpated.

Here, I report T_{inc} effects on *M. temminckii* sex, size at hatching, and tail morphology. Additionally, I measured T_{inc} and water temperature (T_{water}) effects on post-hatching growth rates, metabolic compensation, and metabolic thermal sensitivity. Finally, I evaluated the potential effects of these variables on survival of captive-reared turtles. These objectives were designed to be relevant to current captive propagation/release programs for the species, and to provide insights into the viability of similar programs for other threatened and endangered turtle species.

2. Materials and Methods

All procedures for this research were approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocol #AS023), assuring compliance with animal care guidelines described in The Guide for the Care and Use of Laboratory Animals, 7th edition (1996).

2.1. *Eggs*

Alligator snapping turtle eggs were obtained in 2002 and 2004 from nests laid by females housed at Tishomingo National Fish Hatchery. Eggs were removed from nests and numbered, then transported to Oklahoma State University within 24 h of oviposition. There, they were weighed (±0.1 g) and distributed among plastic shoeboxes containing damp vermiculite (1:1 water:vermiculite by mass) in a randomized block design with clutch serving as the blocking variable. Eggs were distributed among six incubation temperatures ranging from 23–31 °C in 2002. Due to high egg failure at the upper and lower temperatures used, incubation was restricted to three temperatures—26.5°, 28.5°,

and 30.5°C—in 2004. Incubator temperatures were monitored daily with calibrated thermocouple wires inserted into one egg container in each incubator. Each egg box was weighed weekly and, when necessary, distilled water was added to compensate for evaporation. Additionally, egg boxes were rotated daily and eggs were redistributed within each box weekly to meliorate potential effects of thermal or moisture gradients.

2.2 *Hatchlings*

Eggs were removed from the plastic shoeboxes and placed individually in plastic jars lined with damp paper towel after pipping to retain individual identification. Hatchlings remained in their individual containers until residual yolk was internalized, at which time they were weighed (±0.1 g) and midline carapace length and post-cloacal tail length were measured (±0.1 mm). Metabolic rate (oxygen consumption; MR) of each hatchling in 2004 was measured (detailed below). Following MR measurements, unique combinations of posterior marginal scutes were marked by tying loops of dental floss through needle holes to facilitate long-term identification of individuals (O'Steen, 1998), and each turtle was assigned to one of two water temperature (T_{water}) treatments maintained at 25° and 30°C. Turtles were fed a commercially produced fish-based pellet diet *ad libitum* and weighed and measured weekly for 11 weeks.

2.3 Metabolic rate

Oxygen consumption was measured via closed-system respirometry (Vleck, 1987) and used to calculate MR (Peterson, 1990). Oxygen consumption rate of each turtle was measured at two different times at all three T_{inc} (26.5°, 28.5°, and 30.5°C): first, 1-2 d

after internalizing yolk, and second approximately six months post-hatching (mean = 187 d, range = 185-198 d).

Metabolic chambers were constructed from 169- 322- and 959-mL cylindrical plastic jars with screw-on lids. A stopcock was inserted through the lid of each jar, and a thin film of vacuum grease was applied on the inside of each lid to ensure an airtight seal when the stopcock was closed.

Prior to the six-month MR measurements, turtles were fasted for 4 d to minimize metabolic costs associated with specific dynamic action and growth. Hatchling measurements were conducted as soon as residual yolk was internalized, and therefore digestion and growth likely still contributed to MR during this early measurement. On the day of measurement, each turtle was weighed and placed into a metabolic chamber. Chambers were then placed inside an environmental chamber for 1.5-2 h to allow body temperatures to stabilize and equilibrate to ambient temperature. With the overhead lights off to minimize disturbance to the turtles, chambers were carefully removed from the environmental chamber. After screwing on lids to create an airtight seal, pre-trial air samples were drawn into 20-mL syringes, also equipped with stopcocks. The stopcocks on the syringe and chamber were then closed, the syringes removed, and the time of sampling recorded. Chambers were placed back into the dark environmental chamber and removed after approximately 1 h, when post-trial samples were drawn from the stopcock after pumping each syringe several times to ensure mixing of the air inside. After the final measurement at a given temperature, turtles were moved to an environmental chamber set at another temperature and the process was repeated. Measurements at all three temperatures were conducted on the same day.

Oxygen concentrations of all air samples were analyzed in 10-mL aliquots with a Sable Systems FC-1 oxygen analyzer. Air was drawn from outside the building at a regulated flow rate of 100 mL/min and through serial columns of Drierite and Ascarite to remove water and CO_2, respectively. Each aliquot was injected into the air stream, which passed through a small column of Drierite and Ascarite and then through the oxygen analyzer. Oxygen consumption by each turtle was calculated as the difference between the initial and final volumes of oxygen after correcting for chamber volume (Peterson 1990).

2.4 *Sex determination*

Individuals were sexed 267-278 (mean = 273) d after hatching via laparoscopic surgery (Rostal et al. 1994). Food was withheld for 6 d prior to the procedure to minimize the volume of gut contents. The tortoises were transferred to the veterinary facilities at the Tulsa Zoo on the mornings that surgeries were performed. General anesthesia was achieved with a cocktail of 10 mg/kg Ketamine and 0.1 mg/kg Medetomidine. After cleaning the site with Chlorhexidine, a 3-4 mm incision was made posterior to the plastral bridge. A laparoscope probe was inserted into the incision, and the gonads were identified visually. Small tissue biopsies were taken from four individuals and examined histologically to validate macroscopic determinations. The gonads of *M. temminckii* at this age were distinct; ovaries appeared as gray tissue with varying numbers of primordial follicles lying ventral to the oviducts, whereas testes were cream colored and highly vascularized (Figure 3). Following gonad identification, incisions were closed with a

suture and surgical adhesive. Turtles were maintained under moist conditions but out of water for 48 h following surgery to ensure recovery from anesthesia.

2.5 *Statistics*

Mass and MR values were $\log_{10}$ transformed prior to statistical analyses to improve the distribution and homogeneity of variance among treatments. After transformation, assumptions of parametric statistics were met for all subsequent analyses.

Hatchling mass and tail length were analyzed using ANCOVAs, with T_{inc} as a fixed effect and clutch as a random effect. Initial egg mass was included as a covariate for mass analyses, and carapace length was used as a covariate for analyzing tail length.

Growth rate was calculated for each 7-d interval for 77 d after hatching using the equation Growth rate = $(mass_n - mass_{n-1}) \times (mass_{n-1})^{-1} \times [n - (n - 1)]^{-1}$, where n = the last day of each measurement interval. These growth rate values were then analyzed over 77 d that growth was monitored using a repeated-measures analysis in which T_{inc} and T_{water} were treated as fixed effects, clutch was treated as a random effect, and hatchling ID was repeated over the 11 measurement periods. Additionally, the effects of T_{inc}, T_{water} (fixed effects) and clutch (random effect) on the maximum growth rate over any 7-d interval of each turtle was analyzed using ANCOVA.

Metabolic compensation following acclimation to a constant temperature—either during incubation or prolonged exposure to constant T_{water} —was measured shortly after hatching and six months post-hatching by measuring MR at all three incubation temperatures: 26.5°, 28.5°, and 30.5°C. Analyses of data from both stages were performed in a repeated-measures ANCOVA, with T_{inc} and ambient temperature as fixed

effects, turtle ID repeated over each temperature, and mass as a covariate. Six-month measurements were analyzed similarly, but with T_{water} included as a third fixed effect.

Q_{10} values were calculated for each turtle after hatching and at six-months post-hatching using MR measurements obtained at 26.5° and 30.5° C. Separate ANCOVAs were used to analyze clutch and T_{inc} effects on hatchling Q_{10}, and clutch, T_{inc} and T_{water} effects on six-month-old juveniles.

3. Results

3.1 *Eggs*

A total of 88 alligator snapping turtle eggs comprising three clutches were obtained from Tishomingo National Fish Hatchery in 2002. Clutch size ranged from 15-37, but eggs in the smallest clutch proved infertile (Table 1). One hundred eighty-six alligator snapping turtle eggs were obtained from six nests in 2004. Clutches varied in size from 17-42 eggs (mean = 31.2), and hatching success was extremely variable among clutches, again including one infertile clutch (Table 1).

T_{inc} strongly influenced hatching success (Fig. 4). Turtles at 23.0° and 24.5° C in 2002 appeared fully-formed, but failed to pip, whereas embryos at 31.0° C initiated development but died in the first three weeks of incubation. Hatching success also varied within the narrower T_{inc} range in 2004; 26.5° and 28.5° C exhibited 85% and 73% hatching success, respectively, whereas hatch rate fell to 40% at 30.5° C.

Incubation duration varied non-linearly with T_{inc}. There was substantial overlap in incubation duration among eggs incubated at 28.5° and 30.5° C, but no overlap between those incubated at 26.5° and 28.5° C (26.5° range = 90-99 d; 28.5° range = 80-87 d; 30.5°

= 75-85 d). In 2004, embryonic development took an average 11 d longer at 26.5° compared to 28.5° C, but took an average 3 d longer at 28.5° compared to 30° C (Figure 5).

Variation in egg mass was analyzed separately for 2002 and 2004 due to the likelihood that individual females contributed clutches in both years. Clutch strongly influenced egg mass (2002, 2004: $P < 0.0001$); however, because each clutch was distributed randomly among treatments, mean egg mass did not differ among T_{inc}s ($P > 0.05$).

3.2 *Hatchlings*

There was a positive correlation between $\log_{10}$ egg and $\log_{10}$ hatching mass $\log_{10}$ hatchling mass = $0.9151(\log_{10}$ egg mass$) - 0.0716$, $r^2 = 0.495$; Figure 6A). Eggs that incubated at 30.5° C produced smaller hatchlings than those incubated at 26.5° or 28.5° C (ANOVA: $F_{2,94} = 4.84$, $P = 0.010$); this relationship remained consistent after correcting for differences in initial egg mass (ANCOVA: $F_{2,93} = 3.72$, $P = 0.028$; Figure 6B). However, the relationship between mass and T_{inc} became progressively weaker, and had disappeared by the third week post-hatching (7 d: $F_{2,86} = 4.60$, $P = 0.011$; 14 d: $F_{2,86} = 4.00$, $P = 0.022$; 21 d: $F_{2,86} = 1.35$, $P = 0.264$).

Growth rates during the first 11 weeks after hatching were affected by T_{inc}, T_{water} and age (Repeated Measures ANOVA—T_{inc}×age interaction: $F_{20,950} = 2.44$, $P = 0.0004$; T_{water}×age interaction: $F_{10,950} = 4.76$, $P < 0.0001$). Tukey's post-hoc tests indicated that turtles reared in warm water grew consistently faster than those maintained in cool water in weeks 6-11 (Figure 7A). In contrast to this consistent pattern, differences among T_{inc}

treatments were variable, with no regular pattern emerging across multiple weeks (Fig. 7A). However, over time, these modest and variable differences in mass-specific growth rates translated into consistent differences in mass among T_{inc} treatments (Figure 7B). At T_{water} = 30°C, turtles incubated at 26.5° and 30.5°C were larger than those incubated at 28.5°C beginning eight weeks after hatching, whereas at T_{water} = 25°C turtles from the two lower incubation temperatures were larger than those from 30.5°C in weeks 7-11 (Figure 7B). Averaged across incubation temperatures, after 11 weeks turtles raised in 30°C water had gained more than twice the mass as had those maintained at 25°C (30°C: +31.04±1.47 g; 25°C: +14.92±0.55 g).

Maximum growth rate (independent of age) was strongly influenced by T_{water} but not affected by T_{inc} (T_{water}: $F_{1,87} = 9.04$, $P = 0.004$; T_{inc}: $F_{2,87} = 0.89$, $P = 0.416$; Figure 8A). The time at which maximum growth occurred also varied with T_{water} but not T_{inc} (T_{water}: $F_{1,87} = 4.89$, $P = 0.029$; T_{inc}: $F_{2,87} = 2.68$, $P = 0.075$; Figure 8B). Turtles that were maintained at 25°C exhibited average maximum growth rates of 19.23±0.72 $mg \cdot g^{-1} \cdot d^{-1}$, compared to maximum growth rates of 28.27±0.95 $mg \cdot g^{-1} \cdot d^{-1}$ among turtles raised in 30°C water.

Average post-vent tail length was shorter among hatchlings from T_{inc} = 30.5° than from lower T_{inc}s, and was not significantly different between 26.5° and 28.5° C (Type 3 ANOVA: $F_{2,82} = 12.22$, $P < 0.0001$).

Gonadal differentiation followed a pattern consistent with previously published data (Ewert et al. 1994; Figure 9). A high proportion of males (81.4%) was produced at 26.5°C, whereas males constituted 3.3% and 0% of hatchlings at 28.5° and 30.5°C, respectively.

3.3 *Metabolic rate*

Hatchling oxygen consumption rates showed a positive correlation with body temperature ($\log_{10}$ [oxygen consumption] = 0.047 [body temperature] – 1.180; $r^2 = 0.549$; $P < 0.0001$). Among hatchlings, T_{inc} induced metabolic compensation ($F_{2,84} = 11.47$, $P < 0.0001$; Figure 10A), though the effect was subtle. Prolonged exposure to constant T_{inc} resulted in slightly higher MR among turtles incubated at 26.5° C compared to those incubated at 28.5° and 30.5° C (Tukey's post-hoc tests: $P < 0.01$). MR of turtles incubated at 28.5° and 30.5° C did not differ (Tukey's post-hoc test: $P = 0.185$). Among six-month-old juveniles, the previously observed effects of T_{inc} on metabolic compensation had disappeared, and no effect of recent exposure to constant T_{water} was apparent (T_{inc}: $F_{2,85} = 0.76$, $P = 0.471$; T_{water}: $F_{1,85} = 0.02$, $P = 0.884$; Figure 10A, B).

MR Q_{10} values did not differ among hatchlings incubated at different temperatures ($F_{2,17} = 2.94$, $P = 0.079$; Figure 11A), and neither T_{inc} nor T_{water} affected Q_{10} among six-month-old juveniles (T_{inc}: $F_{2,86} = 0.15$, $P = 0.865$; T_{water}: $F_{1,86} = 0.90$, $P = 0.346$; Figure 11A, B).

4. Discussion

4.1 Incubation duration

The inverse relationship between the duration of embryonic development and temperature that was evident in this study is not surprising. However, at any given T_{inc} incubation duration is longer than in many other sympatric species. For example, *Trachemys scripta* and *Apalone spinifera* took an average of 59 and 61 days to hatch at 28.5° C, respectively (Chapter 2), compared to 82 days for *M. temminckii*. This

prolonged incubation time could limit the northern extent of the species' range because of shorter summers and lower average temperatures at northern latitudes. If this hypothesis is true, it could account for the fact that there are records of adult *M. temminckii* in Illinois (the northern-most extent of the range) but no records of hatchlings or juveniles (Galbreath 1961; S. Ballard, Illinois Department of Natural Resources, pers. comm.).

4.2 Sex

In contrast to many turtle species that exhibit TSD, the pivotal temperature range within which a mixed sex ratio is produced in *M. temminckii* spans a wide range (Ewert et al. 2004). My results support previously published data suggesting that no constant temperature results in 100% male production (Ewert et al. 1994). It has been hypothesized that the production of females at all T_{inc}s is due to interactions between TSD and GSD mechanisms (Ewert et al. 1994). This hypothesis has not been explicitly tested in *M. temminckii*, but more recent data from another turtle with TSD suggests that among-clutch variation in the concentrations of maternally-derived sex steroids deposited in the yolks of eggs present a more parsimonious explanation for the phenomenon (Bowden et al. 2000; Elf 2004).

4.3 Hatchling survival, size, growth, and tail length

Hatchling survival, mass, and tail length were reduced at the highest incubation temperatures used in this study. Survival has obvious direct effects on average lifetime fitness, but even in the absence of embryo mortality, it is generally assumed that the probability of survival increases with size among age classes that are at risk of predation

(Janzen 1995). Therefore, high T_{inc}s may be detrimental to the average fitness of *M. temminckii*, even when embryo mortality is not affected. However, this effect could be dampened to some degree by the fact that turtles from high T_{inc}s develop and hatch faster, and therefore would have greater opportunity to grow and acquire resources prior to winter torpor.

The effects of having an abnormally short tail are not immediately clear, and its impact on overall fitness may in fact be minimal. However, possessing a short tail could reduce lateral stability when swimming, or be indicative of other, less apparent, physiological defects. Tests of hypotheses regarding tail utility should be conducted.

4.4 Temperature effects on MR

The metabolic rates of hatchling turtles from all three T_{inc}s scaled positively with body temperature. T_{inc} did not affect sensitivity to temperature, but the long-term exposure to a single temperature during development did induce incomplete metabolic compensation that could dampen the effects of incubation at extreme temperatures. However, this effect was short lived, and *M. temminckii* exhibited no compensatory response following periods of exposure to different temperatures after hatching. Therefore, it appears likely that this species has only limited capacity to physiologically dampen temperature effects. This leaves the species two options: it might simply tolerate a wide range of non-optimal temperatures, or could restrict activity to areas and seasons in which suitable temperatures prevail. Although the first option is certainly a possibility, one study has described seasonal movements from shallow water in spring to deeper water during the

heat of the summer, suggesting that at least some seasonal temperature selection occurs (Riedle et al. 2006).

4.5 Conclusion

Much remains to be discovered about the thermal ecology of *M. temminckii*. However, there is sufficient information to make informed recommendations for conservation programs that incorporate captive hatching and rearing into their protocols. Intermediate temperatures (27.5-28.5° C) will produce a female-biased mixed sex ratio. Although bias is not predicted in natural populations where individual selection dominates (Fisher 1930), it is reasonable to produce more females than males when manipulating population demographics to maximize reproduction. Additionally, female-biased sex ratios have been observed in several natural populations of turtles with TSD (Bull and Charnov 1989). The same temperatures that produce a desirable sex ratio also result in high embryo survivorship, normal development, efficient yolk-to-tissue conversion that results in relatively large hatchlings, and substantially shorter development times than occurred at lower T_{inc}s.

After hatching, turtles grew much more quickly at 25° than at 30° C. Because fast growth minimizes the time that juvenile turtles need to be maintained in captivity, and/or maximizes the size at which turtles are released, warmer water temperatures should be utilized when it is feasible. Other studies have measured effects of diet on growth and shown that high protein (45-55%) will also increase the rate at which turtles grow (Harrell 1998). These results, in combination with studies that have addressed causes of population decline (Roman and Bowen 2000), population genetics (Roman et al. 1999;

Hackler 2004), and behavior and population demographics (Riedle et al. 2005) should be instrumental in maximizing the success of *M. temminckii* conservation.

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Table 1. Clutch size (n), hatching success, and egg and hatchling size for nine *Macrochelys temminckii* clutches produced in 2002 and 2004. Mass and length values expressed as mean±1SE.

	Clutch	n	Hatching success (%)	Egg mass (g)	Hatchling mass (g)	Carapace length (mm)
2002	1	37	30	26.38±0.19	16.46±0.23	36.51±0.17
	2	36	22	24.37±0.18	14.68±0.28	35.11±0.27
	3	15	0	24.32±0.49	---	---
2004	1	33	79	27.26±0.20	17.17±0.17	35.93±0.19
	2	28	75	23.76±0.18	14.72±0.27	33.62±0.29
	3	35	0	27.99±0.13	---	---
	4	31	65	26.59±0.17	16.97±0.14	35.61±0.24
	5	42	69	25.99±0.24	17.37±0.14	36.40±0.18
	6	17	29	26.83±0.51	16.92±0.55	35.40±0.45

FIGURE LEGENDS

Figure 1. A representative juvenile Alligator Snapping Turtle. Where abundant, this species likely fills important functions as a top predator. Adult males commonly exceed 50 kg.

Figure 2. Oklahoma map depicting the four locations at which Alligator Snapping Turtles were recorded during a 1997-99 statewide survey (circles) and two subsequent localities of turtles caught and reported by fishermen (triangles).

Figure 3. Alligator Snapping Turtle A) female, and B) male gonads at approximately 9-months post-hatching. Females were identifiable by the presence of the oviduct (i) and prefollicles on the ovaries (ii). In contrast to ovaries, testes (iii) were highly vascularized.

Figure 4. Alligator snapping turtle hatching success in A) 2002 and B) 2004. Bar height indicates the number of eggs incubated at each incubation temperature, and the black portions of each bar indicate the proportion that successfully hatched. Hatching success was zero at temperatures represented by solid gray bars.

Figure 5. Incubation duration among *M. temminckii* incubated in 2004 at three constant temperatures. Error bars: ± 1SE.

Figure 6. Mass conversion efficiency of yolk to tissue at three incubation temperatures. A) Regression line fitted to the scatter plot fits the equation: $\log_{10}$ [hatchling mass] = 0.9151($\log_{10}$ egg mass) − 0.0716, r^2 = 0.4952. Closed circles = 26.5 °C, triangles = 28.5 °C, and inverted triangles = 30.5 °C incubation temperatures. Dashed lines = 95% C.I. B) Least-squares residuals from log-log plot. Error bars = ± 1SE.

Figure 7. Changes in A) growth rate and B) mass over time of alligator snapping turtles incubated at three temperatures and maintained at two different water temperatures. Circles = 26.5°, triangles = 28.5° and inverted triangles = 30.5° C incubation temperatures. Closed symbols = 25° and open symbols = 30° C water temperatures. Error bars = ± 1SE.

Figure 8. A) Peak growth rates of alligator snapping turtles from three incubation temperatures and reared at two water temperatures. Rates calculated over 7-d intervals. Error bars = ± 1SE. B) Timing of peak growth during the first 11 weeks post-hatching. Black bars = 25° C water, gray bars = 30° C water.

Figure 9. Sex ratio of alligator snapping turtles incubated in 2004 (open circles). Closed symbols are data adapted from Ewert et al. (1994).

Figure 10. Metabolic response to changes in body temperature following prolonged exposure to A) constant incubation temperature and B) constant water temperatures. Symbols in (A) are as in figure 7. B: open squares = 25° C, closed squares = 30° C water temperatures.

Figure 11. Metabolic rate Q_{10} values calculated from O_2 consumption measurements at 26.5° and 30.5° C. A) Hatchlings (black bars) and 6-month-old juveniles (gray bars) from three constant incubation temperatures, and B) juveniles after prolonged exposure to either 25° or 30° water temperatures. Error bars = ± 1SE.

Figure 1

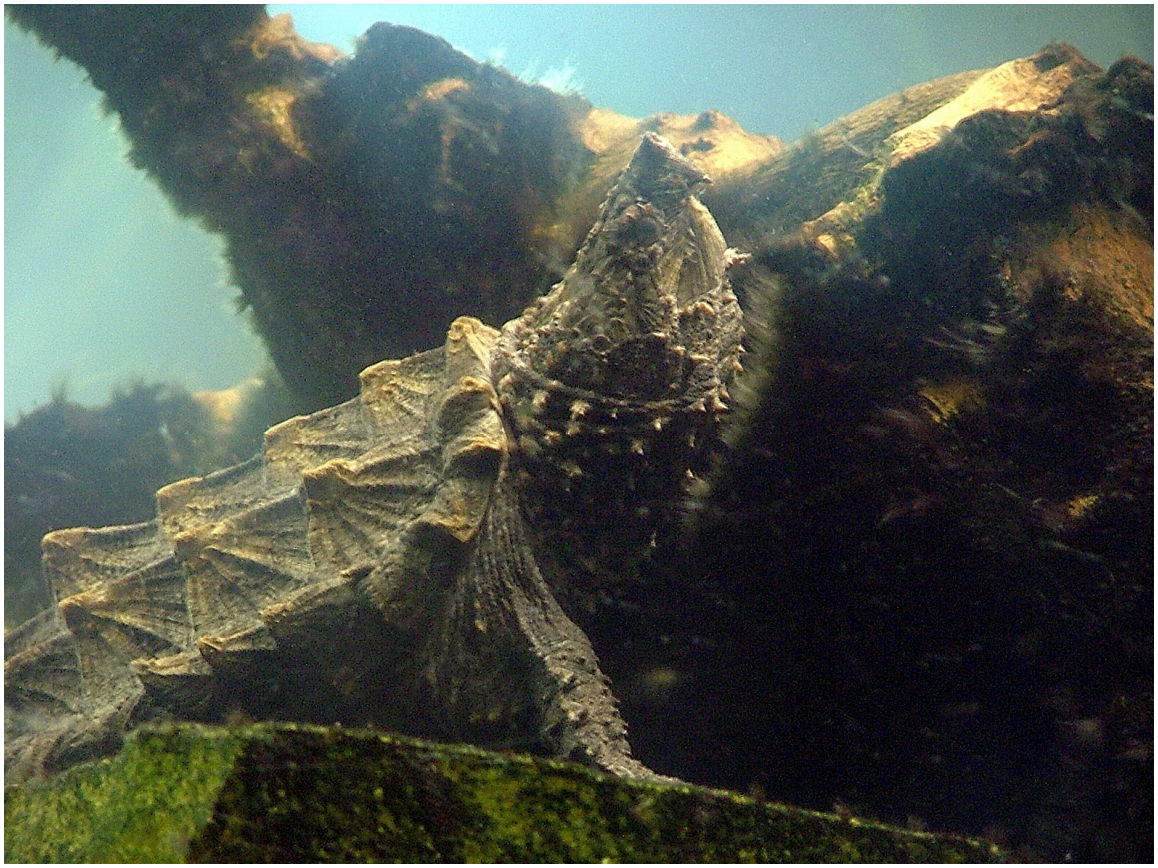


Figure 2

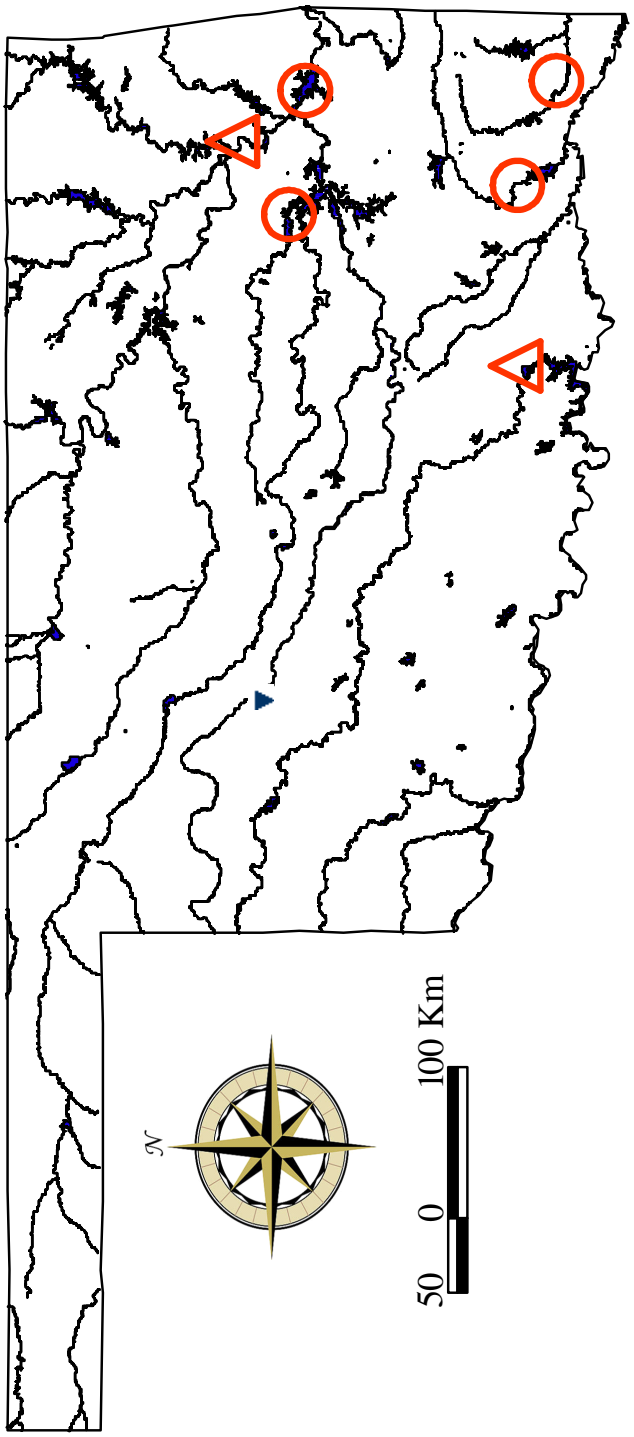
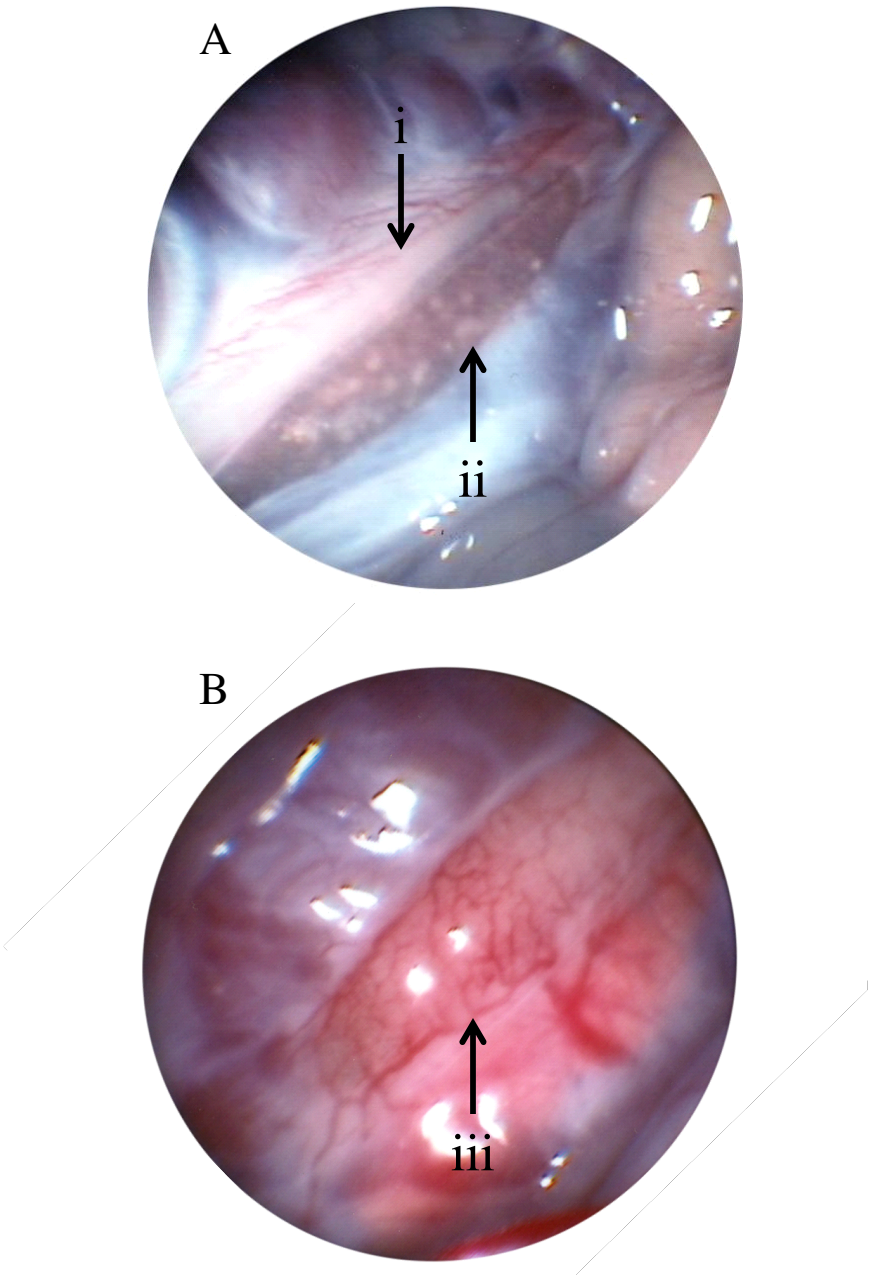


Figure 3



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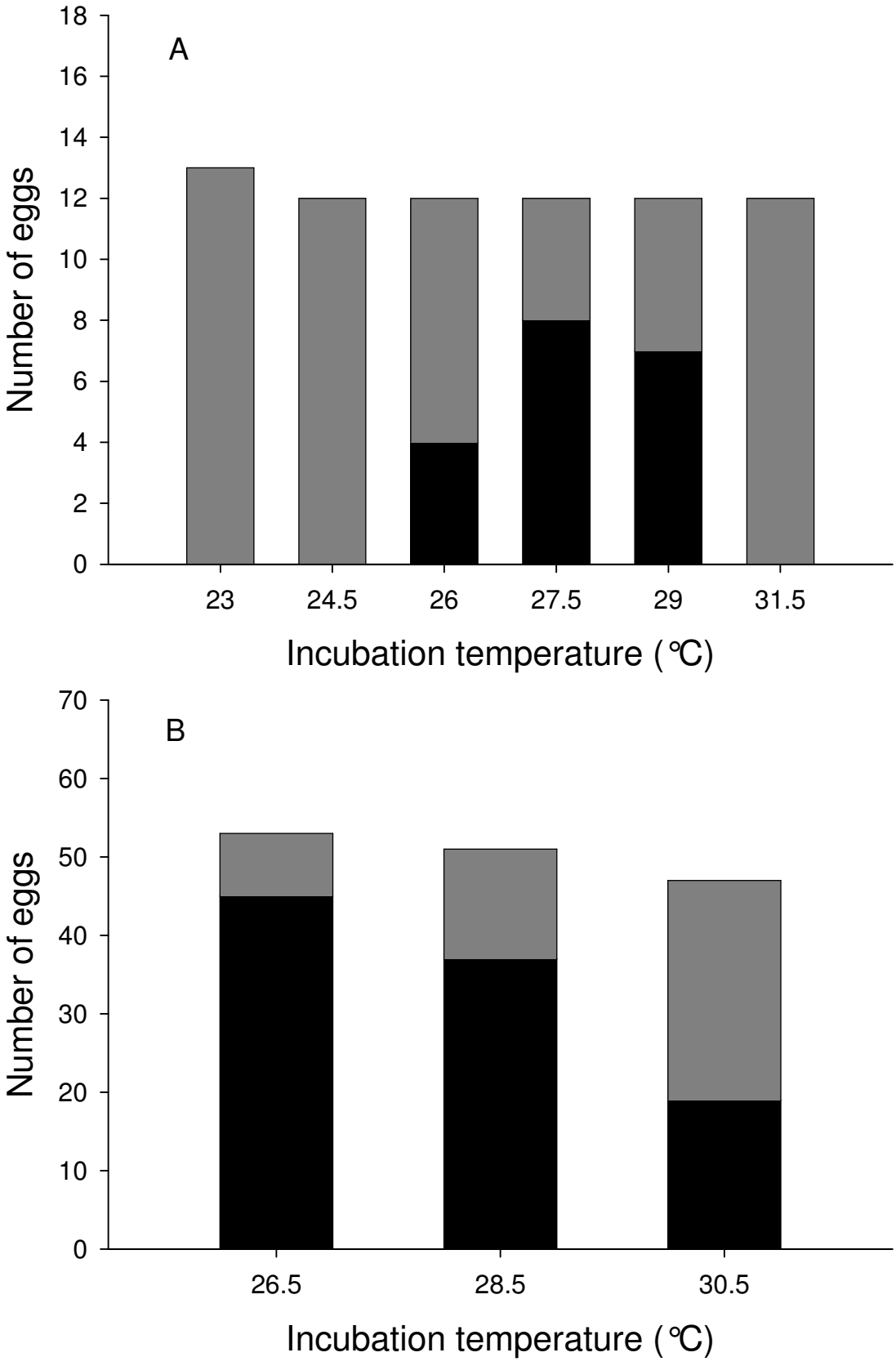

A
Number of eggs
0
2
4
6
8
10
12
14
16
18
23
24.5
26
27.5
29
31.5
Incubation temperature (°C)
B
Number of eggs
0
10
20
30
40
50
60
70
26.5
28.5
30.5
Incubation temperature (°C)

Figure 5

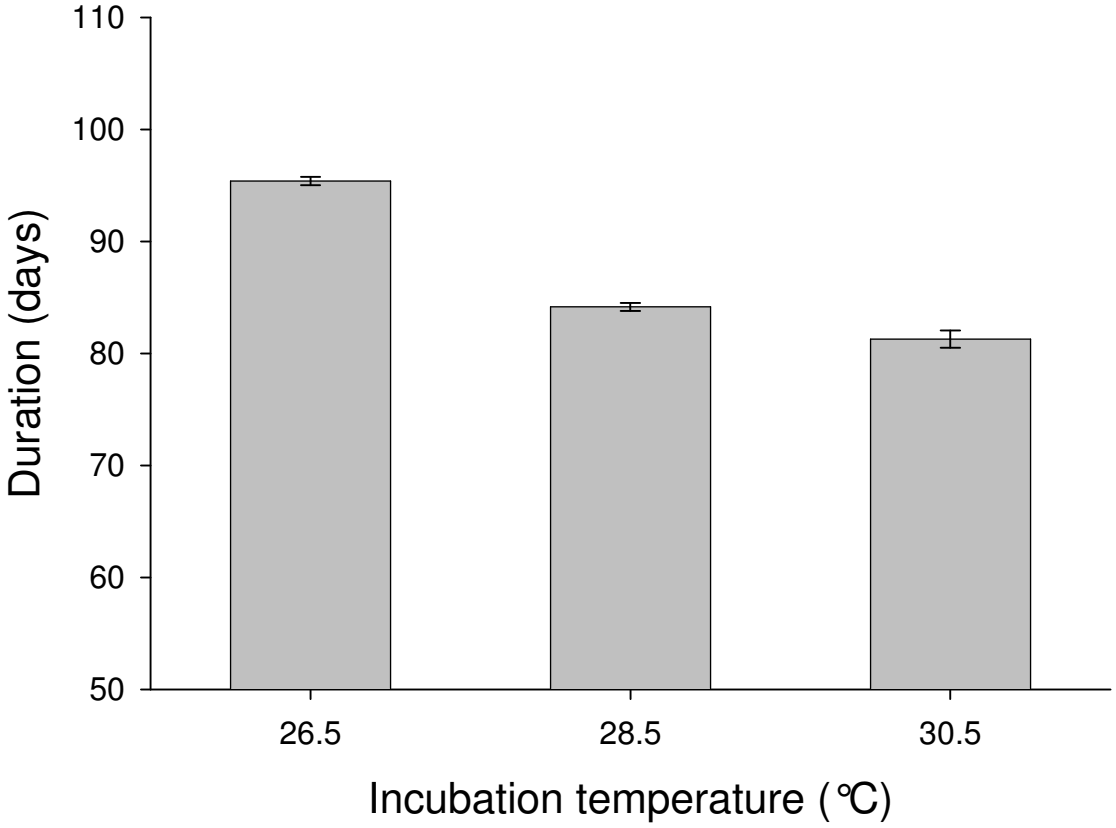

110
100
90
80
70
60
50
Duration (days)
26.5
28.5
30.5
Incubation temperature (℃)

Figure 6

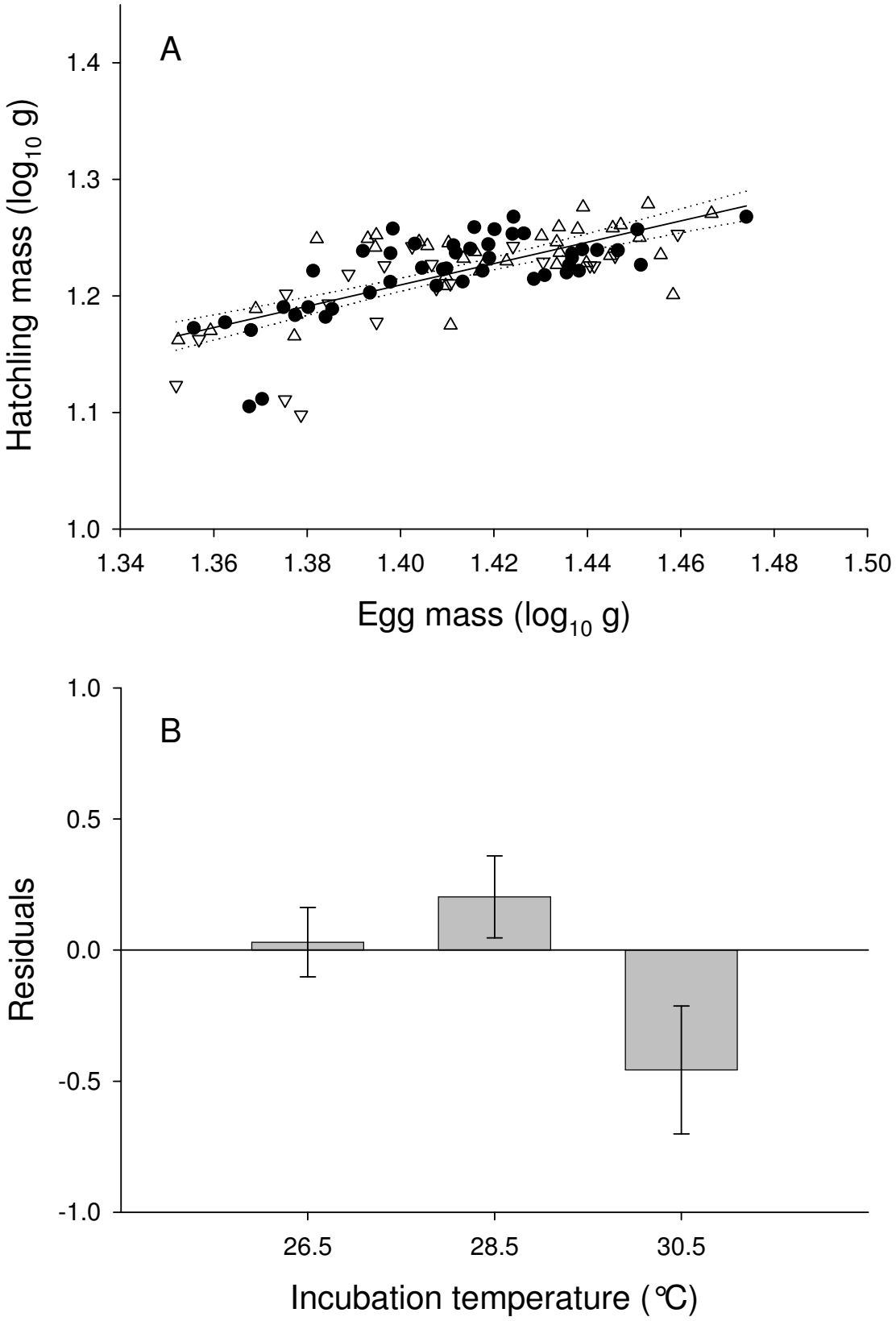

A
Hatchling mass (log_{10} g)
1.4
1.3
1.2
1.1
1.0
1.34
1.36
1.38
1.40
1.42
1.44
1.46
1.48
1.50
Egg mass (log_{10} g)
B
Residuals
1.0
0.5
0.0
-0.5
-1.0
26.5
28.5
30.5
Incubation temperature (°C)

Figure 7

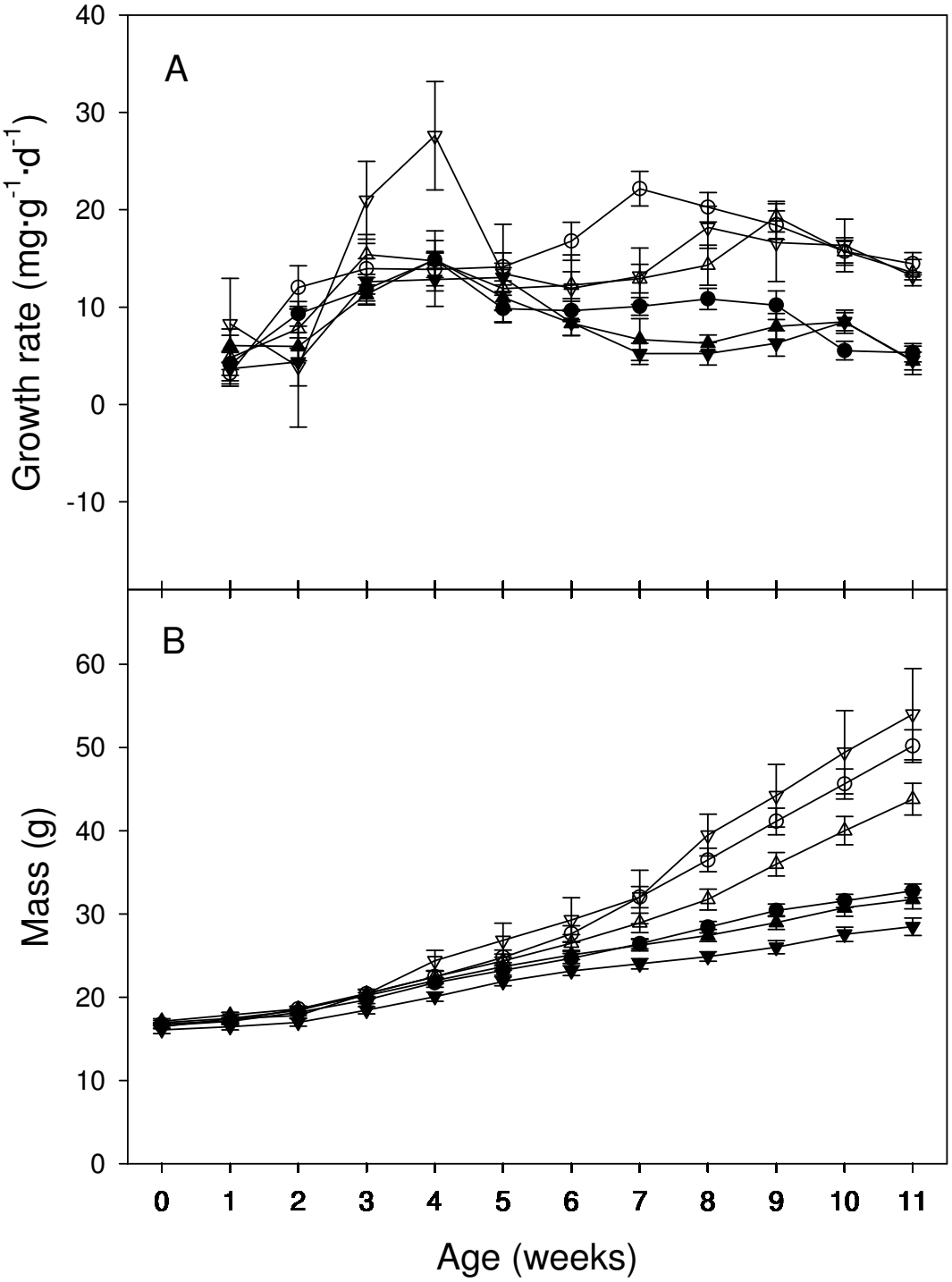
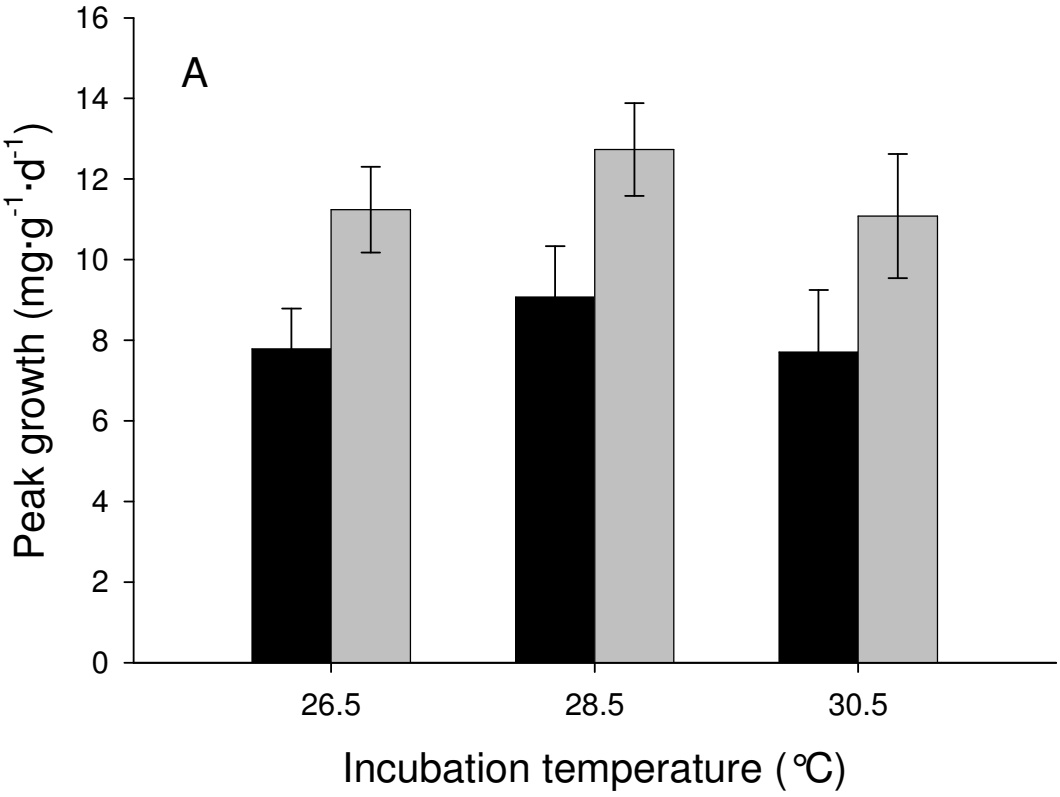

A
B
Growth rate (mg·g-1·d-1)
Mass (g)
Age (weeks)
40
30
20
10
0
-10
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11

Figure 8


A
Peak growth (mg·g-1·d-1)
0
2
4
6
8
10
12
14
16
26.5
28.5
30.5
Incubation temperature (°C)

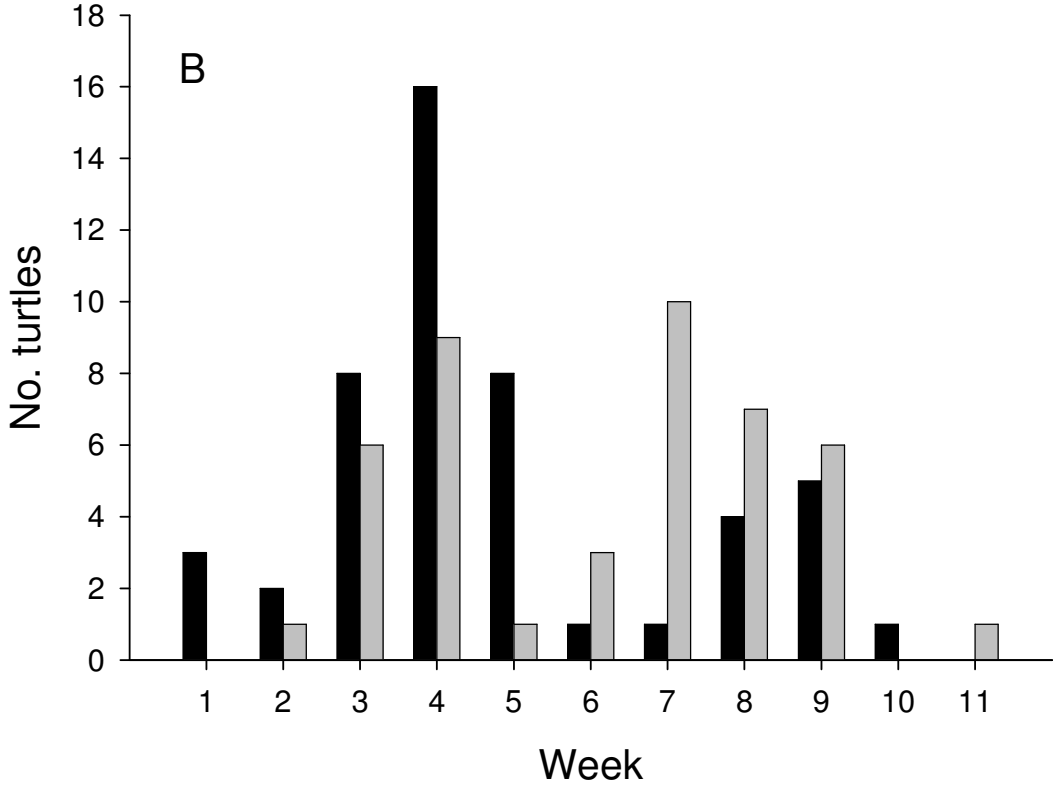

B
No. turtles
0
2
4
6
8
10
12
14
16
18
1
2
3
4
5
6
7
8
9
10
11
Week

Figure 9

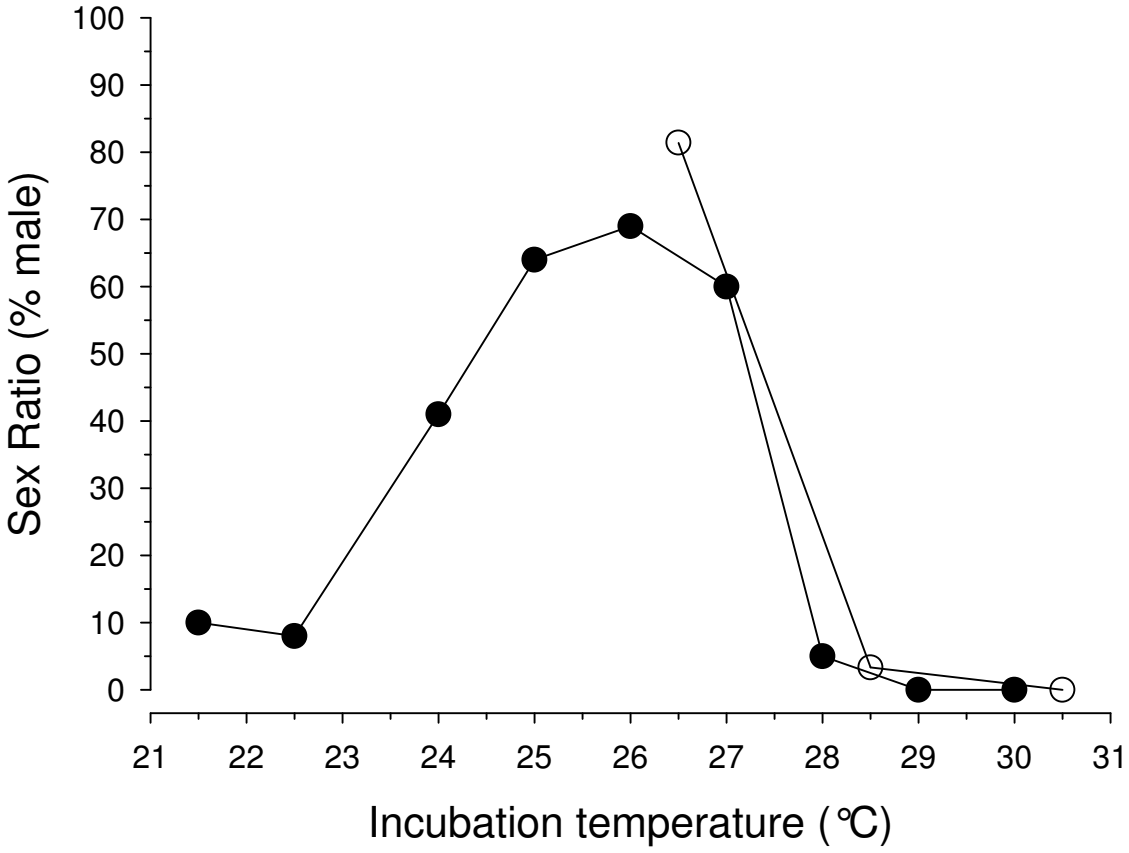

Sex Ratio (% male)
100
90
80
70
60
50
40
30
20
10
0
21
22
23
24
25
26
27
28
29
30
31
Incubation temperature (°C)

Figure 10

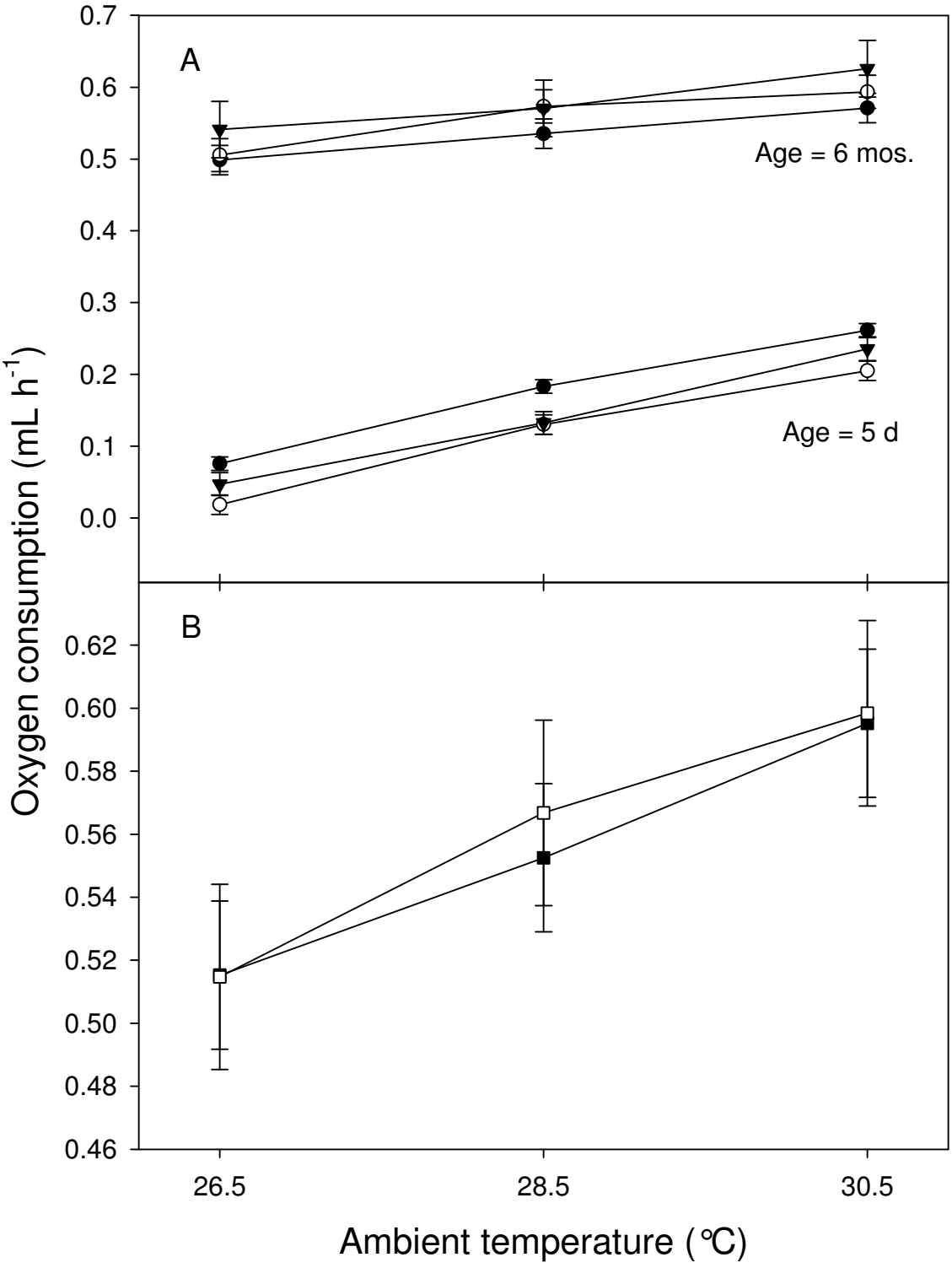
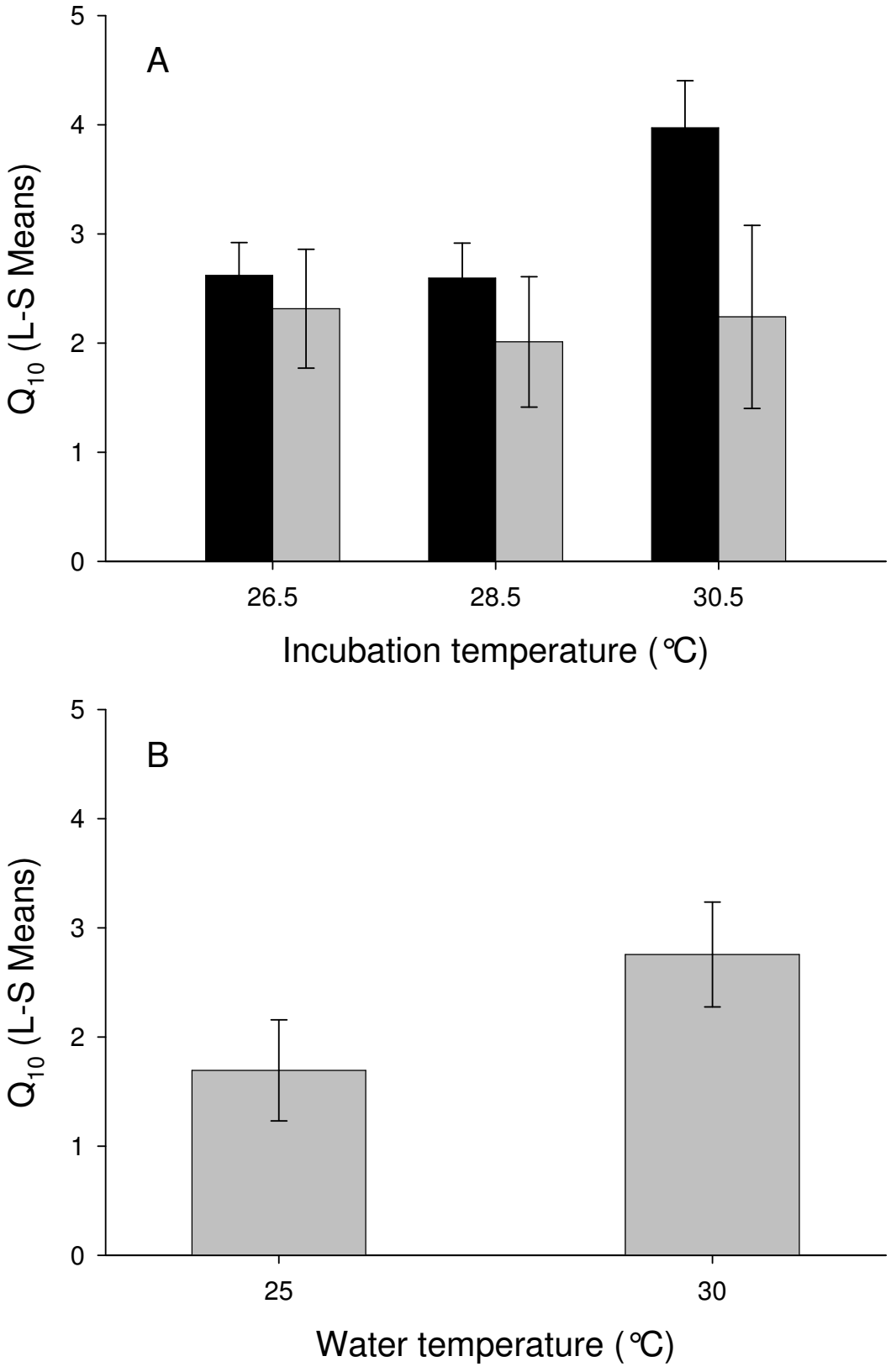

A
B
Age = 6 mos.
Age = 5 d
Oxygen consumption (mL h-1)
Ambient temperature (°C)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.62
0.60
0.58
0.56
0.54
0.52
0.50
0.48
0.46
26.5
28.5
30.5

Figure 11


A
Q_{10} (L-S Means)
0
1
2
3
4
5
26.5
28.5
30.5
Incubation temperature (°C)
B
Q_{10} (L-S Means)
0
1
2
3
4
5
25
30
Water temperature (°C)

VITA

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