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Embryonic Temperature Affects Metabolic Compensation and Thyroid Hormones in Hatchling Snapping Turtles

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ABSTRACT

Temperature acclimation of adult vertebrates typically induces changes in metabolic physiology. During early development, such metabolic compensation might have profound consequences, yet acclimation of metabolism is little studied in early life stages. We measured the effect of egg incubation temperature on resting metabolic rate (RMR) and blood thyroid hormone levels of hatchling snapping turtles (*Chelydra serpentina*). Like many reptiles, snapping turtles have temperature-dependent sex determination (TSD), in which embryonic temperature determines sex. Therefore, we designed the experiments to separately measure effects of temperature and of sex on the response variables. We incubated eggs in the laboratory at 21.5°, 24.5°, 27.5°, and 30.5°C, producing both sexes, all males, both sexes, and all females, respectively. Hatchling RMR, when measured at a common temperature (either 25° or 31°C), was negatively correlated with egg temperature in both males and females, such that RMR of turtles from 21.5°C-incubated eggs averaged 160% that of turtles from 30.5°C-incubated eggs. These results indicate that egg temperatures induced positive metabolic compensation in both sexes. Thyroid hormone levels of hatchlings showed similar correlations with egg temperature; thyroxine level of turtles from 21.5°C-incubated eggs averaged 220% that of turtles from 30.5°C-incubated eggs. To examine the possibility that thyroid hormones contribute to positive metabolic compensation, we added triiodothyronine to eggs during mid-incubation. RMR of hatchlings from these treated eggs averaged 131% that of controls, consistent with the previous possibility. Moreover, the effects of embryonic temperature on metabolic physiology, in combination with effects on sex, can result in differences in RMR and thyroid hormone levels between male and female hatchling turtles. Such differences may be important to the ecology and evolution of TSD.

Introduction

The thermal environment influences the body temperature of ectotherms, and so affects nearly every aspect of ectotherm biology (Lillywhite 1987; Bennett 1990; Huey 1991; Rome et al. 1992; Packard and Packard 1994; Johnston and Bennett 1996). Most studies of thermal effects examine adult ectotherms, yet the systemic nature of these effects suggests that temperature could profoundly impact animals during the sensitive early stages of development (Burggren and Just 1992; Johnston et al. 1996). Indeed, studies thus far report striking plasticity during development. In reptiles, embryonic temperature can affect a number of postnatal traits including sex (temperature-dependent sex determination [TSD]; Bull 1983; Janzen and Paukstis 1991; Ewert et al. 1994; Lang and Andrews 1994; Viets et al. 1994), reproductive and thermoregulatory behavior (Gutzke and Crews 1988; Flores et al. 1994; O'Steen 1998), and locomotor performance and survival (Burger 1989; Janzen 1995). Therefore, the physiological bases of plasticity should be broadly important and merit study in embryonic and juvenile reptiles (Packard and Packard 1994).

Physiological plasticity during early development could take several forms. Many studies of adult ectotherms report that long-lasting change in the thermal environment (temperature acclimation or acclimatization) induces positive compensation for the acute effects of temperature, in which adjustments at many levels of biological organization preserve physiologic homeostasis (Precht et al. 1973; Hazel 1989; Bennett 1990; Rome et al. 1992). However, reptiles appear to show several different responses to temperature acclimation. Adult snapping turtles (*Chelydra serpentina*) show inverse metabolic compensation; they enhance the acute affects of temperature by reducing relative rates of oxygen consumption after acclimation to 10°C (Gatten 1978, 1980). This response may serve to reduce energy costs during winter dormancy (Gatten 1978; Hazel 1989; Ultsch 1989). Other reptiles show no compensation, show positive compensation, or show different patterns at different times (Dutton and Fitzpatrick 1975; Ragland et al. 1981; Tsuji 1988*a*, 1988*b*). These diverse patterns show correlations with season,

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climate, and phylogeny, indicating that physiologic responses to temperature are ecologically and evolutionarily labile, and so possibly developmentally labile, among reptiles.

In this study, we test the hypothesis that egg incubation temperature induces positive metabolic compensation in hatchling snapping turtles. Developing turtles might benefit from positive compensation as normal development may require maintaining physiological rates within a limited range. Three previous studies of turtles examined thermal effects on embryonic metabolic rates: one suggests that near perfect compensation occurs during the last half of incubation (Birchard and Reiber 1995), one suggests that partial compensation occurs near the end of incubation (Leshem et al. 1991), and one indicates that no compensation occurs (Booth 1998). Possible reasons for the different results include species differences and unmeasured effects of sex on metabolic rate in snapping turtles (Birchard and Reiber 1995). Snapping turtles have temperature-dependent sex determination, in which intermediate incubation temperatures produce males and high and low temperatures produce females (Yntema 1976). The sexes differentiate just before the developmental phase showing positive compensation (Birchard and Reiber 1995), and thus any influences of sex on metabolic rate could confound influences of temperature on metabolic rate. These previous studies measured the metabolic rates of eggs at their incubation temperatures. We complement these studies by measuring the influence of both egg incubation and acute temperature variation on the metabolic rates of hatchling turtles and by measuring the separate influence of sex on metabolic rate. We also explore a possible endocrine basis for metabolic compensation. Thyroid hormones play a role in mammalian metabolic responses to temperature acclimation (Himms-Hagen 1983; Danforth and Burger 1984; Wang 1989; Tomasi 1991) and can influence reptile metabolic rates (John-Alder 1990; Stamper et al. 1990), but their role in temperature acclimation of reptiles is unknown (Mason 1977; Hulbert and Williams 1988). We measure the effect of incubation temperatures and sex on thyroid hormone levels of hatchling turtles and examine the influence of exogenous thyroid hormones, applied to eggs during mid-incubation, on hatchling metabolic rates.

Material and Methods

Egg Incubation

Eggs were collected in June 1992 (three clutches) and 1993 (five clutches) from <24-h-old nests of *Chelydra serpentina* in Whiteside County, Illinois. Eggs were individually numbered, placed in Styrofoam containers with moistened peat moss, and transported to the University of Chicago. Eggs were weighed to the nearest 0.1 g, and an equal number (range = 4–7) from each clutch was assigned randomly to each of 10 (1992) or 12 (1993) lidded plastic boxes (15 × 33 × 8 cm high) containing moistened vermiculite (-150 kPa ≈ 300 g dry vermiculite : 337 g deionized water; Janzen et al. 1990). Within each box, 20–27 eggs were randomly assigned to a position in a 3 × 9 matrix and half-buried in the substrate 1–2 cm apart.

The first two studies required that the effects of incubation temperature be separable from the effects of sex. We met this requirement by using four incubation temperatures to produce each sex from three temperatures. In the study population, *C. serpentina* eggs are viable when incubated at constant temperatures ranging from 21° to 32°C, produce females when incubated at the high or low ends of this range (>28° or <22°C), and produce males at intermediate temperatures (between 23°C and 27°C; S. O'Steen and F. Janzen, unpublished data). We placed egg boxes in constant-temperature (±0.5°C) incubators at 21.5°, 24.5°, 27.5°, and 30.5°C, to produce both sexes, all males, both sexes, and all females, respectively. Incubator temperatures were recorded continuously and remained stable throughout the experiments. In 1992, two egg boxes were placed at 21.5°, two at 24.5°, two at 30.5°C, and four at 27.5°C. In 1993, two boxes were placed at each of 24.5° and 30.5°C and four boxes at each of 21.5° and 27.5°C. All experiments used equal numbers of hatchlings from each egg box within an egg incubation temperature.

The boxes were repositioned in the incubators once daily to minimize potential differences in the environments within boxes and between boxes resulting from thermal gradients (Bull et al. 1982) The possible effect of egg box on hatchling traits was examined by including a factor for egg box, nested within incubation temperature, in the analysis of covariance (ANCOVA) for each trait (see "Statistical Analyses"). Egg box had no effect on any trait except 1992 hatchling mass, and so egg box was excluded from all final analyses except those for hatchling mass. To maintain stable hydric environments in the boxes, substrates were rehydrated once weekly by adding deionized water sufficient to return each box, including contents, to its initial mass. Temperatures and hydric conditions used in this experiment are within the range of those measured in natural *C. serpentina* nests (Packard et al. 1985). Hatching success was greater than 95% in all boxes except those in the triiodothyronine (T3) application study (see "Results").

Hatchling Care

On pipping, each egg was isolated in the incubation box by a plastic divider that identified the hatchling. Within 24 h of hatching, turtles were rinsed of vermiculite and adhering membranes, blotted dry, and weighed to the nearest 0.1 g. Turtles were given an identifying tag consisting of a labeled 1-cm square of thin plastic, secured with dental floss tied through two needle holes in the marginal scutes of the carapace. Turtles were then returned to their incubators and housed in the incubation boxes on moistened vermiculite. Turtles were not fed during any of the following experiments. A 12L : 12D cycle (lights on at 0700

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hours) was initiated in each incubator when the first hatchling emerged and was maintained throughout these experiments.

Resting Metabolic Rate

Resting metabolic rate (RMR) was measured as the rate of oxygen consumption of hatchling turtles resting at night. We consider the measurement resting rather than standard metabolic rate, because hatchling turtles typically contain yolk reserves and cannot be considered postabsorptive. RMR of all 1992 hatchlings was measured at both 25° and 31°C (timing protocol below), whereas all 1993 hatchlings were measured at 25°C. We selected these assay temperatures for several reasons. We wished to measure all turtles at common temperatures, because in nature hatchlings move from nests into common environments (nearby water), and one goal of this project was to examine relative energy requirements of hatchlings in such environments (see companion study, O'Steen 1998). We selected 25° and 31°C for the 1992 study as these temperatures were within the range of frequently selected body temperatures of snapping turtles in both laboratory and field studies (Ernst et al. 1994), approximated two of our egg incubation temperatures, and provided a measure of acute effects of temperature on RMR. We selected 25°C for the 1993 study as the effects of egg temperature on RMR did not differ between assay temperatures in 1992, and as turtles showed less activity at 25° than at 31°C (allowing activity-free measurements to be obtained in less time), and as 25°C was nearer the mean selected nighttime body temperature (22.7°C) of snapping turtles in the field (Brown et al. 1990). Finally, the severe logistic restrictions of measuring many animals within days of hatching precluded measuring RMR at additional temperatures.

We measured RMR by using flow-through respirometry. Turtles were assayed in 70-mL metabolic chambers containing a clean paper towel moistened with 4–6 mL of deionized water, which prevented any desiccation during the assays (verified by comparing weights of turtles before and after the assay). Air was drawn from outside the building and pumped through Drierite and Ascarite and then through the chambers at a rate of 48 mL/min. Air leaving the chambers passed through Drierite and Ascarite and then through the analysis cell of an S-3A Applied Electrochemistry oxygen analyzer (Ametek, Pittsburgh) interfaced with a computer. Data were recorded and analyzed by using a Datacan data acquisitions system (Sable Systems, Las Vegas, Nev.). The metabolic chambers were housed in a darkened incubator that maintained chamber temperatures within 0.5°C of target.

On the assay day, turtles were weighed to the nearest 0.1 g, placed in the chambers between 1700 and 1900 hours, and left undisturbed for 2 h before oxygen concentration recordings were initiated. Oxygen consumption of each turtle was recorded for two to three 20-min intervals separated by 1–2 h. The recordings were monitored for indications of turtle movement. Any recordings indicating movement were discontinued and repeated 1–2 h later. Oxygen consumption of some turtles was discontinuous, indicating that these turtles took single breaths followed by 1–5 min of apnea. If no recording of continuous oxygen consumption (stable for at least 5 min) could be obtained for a given individual, we calculated RMR for that animal by using the minimum average consumption integrated over three complete discontinuous breathing cycles. For recordings of continuous breathing, we calculated RMR from the minimum consumption over a 5-min interval. *G*-tests (Sokal and Rohlf 1981) indicated that discontinuous breathing did not differ in frequency among incubation groups, and ANCOVA indicated that neither discontinuous breathing nor time of day affected RMR (see "Statistical Analyses": continuous vs. discontinuous breathing was tested as a categorical factor and time of day as a covariate in the primary ANCOVAs; as neither factor was significant, they both were excluded from further analyses). RMR was calculated by using Withers's (1977) equation (4a), in units of milliliters of oxygen consumed per hour (mL O_2/h). The influence of mass on RMR was examined and controlled for in the ANCOVAs. In addition, turtle mass data are provided in the tables; thus RMR can be readily converted to units per gram if desired.

In 1992, we measured oxygen consumption of hatchling turtles from two clutches; the number measured per clutch was balanced within and among incubation treatment groups. Within each group, half of the turtles were first assayed at 25°C and the other half at 31°C. Two nights later, oxygen consumption was remeasured at the second temperature. Turtle age at the first oxygen assay ranged from 2 to 13 d old; demand for the oxygen analyzer from multiple studies precluded measuring turtles of a narrower age range. Turtle age influenced RMR and is included in the main analysis. To reduce the influence of age in 1993, we measured oxygen consumption at 25°C of 3- and 4-d-old turtles only (mean age = 3.3 d), and among these turtles age did not influence RMR. In 1993 we used turtles from three clutches, balanced within and among incubation groups. Sample sizes are listed in the tables.

Hatchling Thyroid Hormone Levels

We measured blood thyroxine levels of hatchlings 8–14 d old, from each of the three clutches incubated in 1992. Thus, turtles from two clutches were siblings of the turtles in the RMR study. We used 22–26 turtles per clutch, balanced within and among incubation groups. The hatchlings provided sufficient plasma to measure only one of the two central thyroid hormones. Thyroxine (T4) was selected over triiodothyronine (T3) because T4 is the primary thyroid hormone circulating in the blood and assay methods are well documented. Turtles were removed from the egg incubators at 1400 hours and transferred to a 4°C refrigerator. Turtles were removed from the refrigerator 1–5 h later and killed by decapitation in accordance with animal care

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regulations, and blood was collected from the central trunk artery in heparinized tubes. This collection method may introduce small quantities of other body fluids into the sample. However, as the same method was used for all turtles, this possibility should not influence measured T4 differences among incubation groups. The blood samples were stored at 4°C for 24 h and centrifuged to separate plasma from red cells. The plasma was stored at −20°C. T4 levels were determined by radioimmunoassay, by using the method of Denver and Licht (1988). All plasma samples were measured during a single large assay, in which each sample was measured in a triplicate dilution series. These plasma dilutions produced T4 curves that paralleled those of T4 standards. Turtle age and time at 4°C before blood collection were included as covariates in the primary ANCOVAs of plasma T4 level (see "Statistical Analyses"); neither covariate was significant and both were excluded from further tests.

Thyroid Hormone Application to Eggs

This study measured the effect on hatchling RMR of exogenous thyroid hormone added to incubating eggs, as a preliminary test of the hypothesis that thyroid hormones contribute to positive metabolic compensation. Because this was the first study of the kind, we limited our goal to measuring the effects of hormone applications at two egg incubation temperatures and did not try to separate the influences of temperature and sex. To eliminate the possible influence of sex within temperatures, we used eggs from the two single-sex egg temperatures, 24.5°C (all males) and 30.5°C (all females), from the 1993 incubations. We also limited the study to measuring the effects of one hormone, triiodothyronine (T3). We chose T3 to maximize the probability of influencing hatchling RMR, as T3 is considered the active form of thyroid hormone in most animals (T4 is the primary circulating form of the hormone, and T3 results primarily from intracellular deiodination of T4; DiStefano et al. 1982*a*, 1982*b*).

We applied T3 to the shell of eggs at mid-incubation, during developmental stages 18–19. The thyroid gland differentiates before this period and responds to antithyroidal agents during this period, suggesting that the gland is functional (Dimond 1954; Ewert 1985). Stages 18–19 are within the sex-determining phase of development (Yntema 1976, 1979) and just before the phase that displays positive metabolic compensation (Birchard and Reiber 1995). We used published relationships to predict the dates that the embryos should reach stage 18 (Yntema 1968, 1978). To verify that the embryos were at the correct stage, on several days we opened two eggs from each of two egg boxes per temperature and staged and preserved the embryos. The 30.5°C embryos reached stage 18 and received first treatment on day 20 of incubation; the 24.5°C embryos reached stage 18 and were first treated on day 33. T3 (2.0 μg T3 dissolved in 5.0 μL EtOH) or control (5.0 μL EtOH) solutions were applied to the top of the exposed eggshells. T3 and ethanol were obtained from Sigma Chemical Company. Each egg box and clutch received 60% T3 and 40% control treatments. Within this framework, eggs were assigned at random to treatment groups. Applications were repeated after 24 and 48 h to the 30.5°C eggs and after 36 and 72 h to the 24.5°C eggs. The difference in application timing was designed to approximately equalize timing relative to the different developmental rates at the two temperatures. The eggs were treated normally for the remainder of incubation. We recorded incubation time (days from start of incubation to egg pipping), hatching success, hatchling sex, mass, and RMR at 25°C of 3- and 4-d-old turtles. We also took blood samples of hatchlings for hormone analysis, but these samples were most unfortunately destroyed in a freezer accident. Therefore, our study does not address the effect of the exogenous hormones on circulating levels. Table 3 presents the study sample sizes.

Sexing of Turtles

The sex of all turtles from 21.5° and 27.5°C incubation treatments, all turtles from the two thyroid experiments, and any turtles that died during the experiments was determined by visual inspection of the gonads of dissected animals (Yntema 1976; Janzen et al. 1990). Turtles from the thyroid studies were dissected 1–2 wk posthatching, turtles from 21.5° and 27.5°C at approximately 1 yr of age. The turtles were killed with an overdose of anesthetic (0.8 mL of 1 : 1 distilled H_2O : Nembutal = sodium pentobarbitol) injected into the pericardial cavity, then dissected and examined by S. O'Steen. After this sexing procedure, the turtles were preserved with only their original identity tags, which do not identify sex or incubation treatment. Each author independently determined the sex of the preserved turtles on a later date. Agreement among the three sexing analyses was 100%. Turtles from 24.5° and 30.5°C that were not in a thyroid study and were not dissected were assumed to be 100% males and females, respectively, for several reasons. First, all studies of snapping turtle eggs incubated at these and similar temperatures have found 95% to 100% single sexes to result (reviewed in Paukstis and Janzen 1990). Previous studies using the same equipment and population of snapping turtles as this study found 25 of 25 turtles incubated at 30°C to be female and 36 of 36 turtles incubated at 26°C to be male (Janzen 1995). Finally, in this and a concurrent study (O'Steen 1998), a total of 45 turtles from 24.5°C and 36 turtles from 30.5°C were dissected and determined to be all males and all females, respectively.

Statistical Analyses

Data were primarily examined with analyses of covariance (ANCOVA). ANCOVAs were generated on a Macintosh com-

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puter with SuperANOVA (Abacus Concepts, Berkeley) by using Type III sums of squares, which examine the effect of each factor after removing the effects of other factors in the model. The primary ANCOVA model contained the following factors: egg temperature, hatchling sex, egg temperature by sex interaction, clutch, and a covariate of either egg or hatchling mass. Variations of this primary model required for different dependent traits are presented below. Each analysis was initially conducted using all factors. We then tested for the possible influence of insignificant factors by removing such factors from the model one at a time. In all cases except the repeated-measures model, removal of these factors in any combination had no effect on the significance of the remaining factors. Therefore, with the one exception, we present only the results of the complete models. After completing the ANCOVAs, we further examined the effects of egg temperature with post hoc orthogonal polynomial tests, to determine the separate significance of linear, quadratic, and cubic influences of temperature.

The 1992 RMR analyses were performed in several steps. First, two separate ANCOVAs were constructed for the 25° and the 31°C RMR assays. These ANCOVAs included a covariate of turtle age on the assay date (see "Material and Methods") in addition to the primary model. The two analyses gave qualitatively identical results, and the results for the 25°C data are presented. Then, to determine the effect of assay temperature on RMR, the 25° and 31°C data were combined in a repeated-measures ANCOVA, composed of the above model plus the repeated factor, assay temperature. This analysis reproduced the results of the separate ANCOVAs and showed no significant effect of assay temperature ($P = 0.12$). However, this last result deserved further examination, because there was a large measured difference in RMR at the two assay temperatures, and because the repeated-measures ANCOVA contained terms for the interaction of each factor with assay temperature, and all of these terms were insignificant. Removal of these interaction terms, and/or removal of nonsignificant main factors (Table 2) from the repeated-measure model, resulted in highly significant assay temperature effects. Therefore, we consider that assay temperature did influence RMR and report results of the repeated-measures ANCOVA with nonsignificant factors removed.

The 1993 RMR analysis required only the primary ANCOVA model. The analysis of hatchling mass required adding one factor to the primary model, egg box nested within egg temperature (see "Egg Incubation"). Analysis of the blood plasma T4 data required several steps because mass data were unavailable for turtles from one of the three clutches used in the T4 study. These turtles were excluded from the initial analysis, and the remaining data were examined with and without the mass covariate. The presence of the covariate did not change the significance of the other effects. The results were also unaffected by adding data from the third clutch while excluding the mass covariate. Therefore, mass was excluded and data from all three clutches were included in the final analysis.

The T3 application study required a different ANCOVA. In the T3 study, egg temperature and hatchling sex were equivalent variables, because only the two single-sex egg temperatures were used. Thus, in the ANCOVAs, these variables were treated as one factor, egg temperature–sex, with two states, 24.5°C-male and 30.5°C-female. The remaining factors were T3 treatment group (T3, control), the interaction of T3 group and egg temperature–sex, clutch, and a mass covariate. Separate ANCOVAs were conducted for incubation time, for hatchling mass (covariate equals egg mass), and for RMR (covariate equals hatchling mass). *G* statistics determined the effect of T3 treatment on hatching success (Sokal and Rohlf 1981).

Results

Egg Incubation Temperature Effects on Hatchling Mass and Sex Ratio

Egg temperature influenced the mass of hatchling turtles (ANCOVA, $P < 0.003$), such that hatchlings from intermediate temperatures weighed more than hatchlings from high and low temperatures (Table 1). Orthogonal polynomial analyses show this quadratic effect of egg temperature in both 1992 and 1993 ($P = 0.0006$ and 0.026, respectively) and show a linear effect in 1993 ($P = 0.012$). The ANCOVAs show clutch ($P < 0.02$) and egg mass effects in both years (heavier eggs produce heavier hatchlings, $P < 0.0001$), egg box effects in 1992 ($P = 0.016$), and no sex or sex by temperature interaction effects ($P > 0.10$). Egg temperature influenced sex ratio as expected; eggs at 21.5° and 27.5°C produced both sexes, whereas sexed turtles from 24.5° and 30.5°C eggs were all males and all females, respectively (Table 1). These results are qualitatively identical to those for the complete group of hatchlings resulting from the 1992 and 1993 egg incubations (O'Steen 1995, 1998).

Egg Incubation Temperature and Sex Effects on Hatchling RMR

Egg temperature markedly influenced the RMR of hatchling turtles. When RMR was measured at a common temperature (either 25° or 31°C), then hatchling RMR showed a strong negative correlation with egg temperature, such that the average RMR of hatchlings from 30.5°C eggs was only 64% that of hatchlings from 21.5°C (Fig. 1; Tables 1, 2). This effect occurred in both years (Table 1) and was strongly linear ($P < 0.005$), with no quadratic or cubic components ($P > 0.20$, orthogonal polynomial tests). Assay temperature also influenced RMR; RMR assayed at 31°C was higher than RMR at 25°C in turtles from all egg temperatures (repeated-measures ANOVA, $P = 0.0001$; Table 1). The Q_{10} of this acute effect did not differ among turtles from the different egg temperatures (repeated-measures ANOVA, egg incubation temperature × RMR assay tempera-

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Table 1: Mass, RMRs, and blood thyroxine (T4) levels of hatchling snapping turtles from four egg incubation temperatures

	Egg Incubation Temperature			
	21.5°C	24.5°C	27.5°C	30.5°C
Hatchling mass (g):				
1992:				
Females	10.7 ± .4 (4)	…	11.3 ± .1 (24)	10.0 ± .2 (20)
Males	9.9 ± .2 (16)	11.4 ± .2 (23)	11.1 ± .2 (20)	…
1993:				
Females	9.0 ± .2 (12)	…	9.7 ± .1 (9)	9.6 ± .2 (12)
Males	9.3 ± .1 (9)	9.8 ± .1 (12)	9.9 ± .1 (12)	…
RMR (mL O_2/h):				
1992:				
Assayed at 31°C:				
Females	1.82 ± .09 (3)	…	1.45 ± .02 (13)	1.22 ± .08 (12)
Males	1.74 ± .05 (8)	1.50 ± .05 (12)	1.50 ± .06 (11)	…
Assayed at 25°C:				
Females	1.15 ± .04 (3)	…	.90 ± .02 (13)	.71 ± .04 (12)
Males	1.09 ± .02 (8)	.98 ± .05 (12)	.86 ± .04 (11)	…
1993:				
Assayed at 25°C:				
Females	1.09 ± .08 (12)	…	.71 ± .04 (9)	.72 ± .06 (12)
Males	1.17 ± .08 (9)	.92 ± .06 (12)	.83 ± .06 (12)	…
Blood T4 (ng/mL):				
1992:				
Females	4.8 ± 1.2 (2)	…	2.3 ± .3 (19)	1.2 ± .4 (12)
Males	2.3 ± .5 (10)	1.6 ± .2 (16)	1.4 ± .4 (11)	…

Note. The 1992 mass data include the RMR turtles and their clutchmates among the T4 turtles. The two assay temperatures for 1992 RMR reflect repeated measures of the same turtles. The 1992 RMR values are standardized for hatchling age, and the T4 values are standardized for clutch (see "Results"). Values are mean ± 1 SE (*N*).

ture interaction term, $P = 0.11$) and averaged 2.32. Interestingly, Table 2 indicates that sex influenced RMR in 1993, with RMR being slightly higher in males than in females (Table 1). However, there was no sex effect in 1992, and the effect of egg temperature on RMR was similar in both sexes in both years (Table 2). These results suggest that the primary differences in RMR among hatchling turtles, in a common environment, are caused by their egg temperatures and not by their sex. Nonetheless, because egg temperature determines sex, males on average experienced lower egg temperatures than females, and then as hatchlings displayed higher average RMR than females, in both years of the study (Table 1; overall mean difference in 25°C RMR = 0.11 mL O_2/h, $P = 0.006$, *t*-test).

In 1992, turtle age was negatively correlated with RMR ("Material and Methods"; Table 2) and for this reason only 3- and 4-d-old turtles were measured in 1993. To allow comparison of 1992 and 1993 data in Table 1, we used the model coefficient for the age effect (generated by the ANCOVA in Table 2) to standardize the 1992 data to the mean age of 1993 turtles (3.3 d old). Yolk retained by hatchling turtles, a variable not measured here, might also affect RMR. Yolk mass can account for 1% to 22% of total mass of hatchling snapping turtles (Wilhoft 1983; Packard et al. 1987) and be positively correlated with egg temperature (Packard et al. 1987; Packard and Packard 1994). Yolk might be metabolically inert relative to hatchling tissue (Burggren and Just 1992) or might increase RMR due to some form of specific dynamic action (increased metabolic rate resulting from digestion; reviews in Secor and Diamond 1997; Peterson et al. 1998); the effect of yolk on metabolic rate of hatchling reptiles merits further study. However, these possible effects should not explain our results. In a separate experiment S. O'Steen measured yolk in seven 30.5°C and nine 24.5°C hatchlings from the 1993 incubations. Yolk mass averaged 7% and 2%, respectively, of total wet mass, a difference insufficient to account for egg temperature effects on RMR.

Egg Incubation Temperature and Sex Effects on Hatchling Blood T4 levels

The level of T4 in blood plasma of hatchlings was negatively correlated with egg temperature, as was RMR (Fig. 1; Tables 1,

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Table 2: ANCOVA of resting metabolic rates (RMR) and blood thyroxine (T4) levels of hatchling turtles

Dependent Variable and Source of Variance	df	*F*	*P*
RMR (mL O_2/h at 25°C):			
1992:			
Egg incubation temperature	3	17.0	.0001
Sex	1	.9	.3540
Egg temperature × sex	1	.0	.8674
Clutch	1	.3	.5949
Turtle mass	1	.3	.5905
Turtle age	1	20.0	.0001
Residual	50		
1993:			
Egg incubation temperature	3	9.4	.0001
Sex	1	6.6	.0120
Egg temperature × sex	1	1.1	.2991
Clutch	4	3.0	.0264
Turtle mass	1	.9	.3422
Residual	55		
Blood T4 (ng/mL):			
Egg incubation temperature	3	5.4	.0023
Sex	1	8.2	.0057
Egg temperature × sex	1	2.2	.1393
Clutch	2	7.5	.0012
Residual	62		

2). The relationship was such that T4 of turtles from 30.5°C eggs averaged only 44% that of turtles from 21.5°C (Table 1). Orthogonal polynomial tests indicated that this relationship was strongly linear ($P = 0.0008$), with no quadratic or cubic components ($P > 0.32$). The ANOVA interaction term indicated that the temperature effect was the same in males and females (Table 2). However, sex had a main effect on T4 (Table 2), with females having higher levels of T4 than males (Fig. 1; Table 1). This result is tentative because of the low sample size of 21.5°C-females. Removing their data from the ANOVA left the egg temperature effect intact ($P = 0.01$) but rendered the sex effect insignificant ($P = 0.22$). Nonetheless, we found no anomalies in the data or methods that would predicate discounting the data for these females.

T4 levels differed strongly among the three clutches in the study (Table 2). To determine whether T4 response to egg temperature also differed among clutches, we added the clutch by egg temperature interaction to the ANOVA. The result showed that all clutches responded similarly to egg temperature ($P = 0.94$). Nonetheless, the strong main effect of clutch on T4 rendered the sex and egg temperature effects difficult to detect visually in the raw data, because each clutch had a relatively small number of eggs at each egg temperature ("Material and Methods"), which, by chance, resulted in very different sex ratios among clutches at the mixed sex egg temperatures. Therefore, to provide accurate comparisons for Figure 1 and Table 1, we used the model coefficients for the clutch effects (generated by the ANOVA in Table 2) to standardize the T4 data to the intermediate clutch. All statistics result from the ANOVA of the raw data (Table 2).

Effects of Egg T3 Treatments

Applying T3 to incubating eggs strongly increased the RMR of the hatchling turtles (Table 3). Among the 24.5°C-males, the RMR of T3 treatment turtles was 144% that of the controls. The RMR of these T3 turtles was also greater than that of the highest RMR group from the first study, the turtles from 21.5°C eggs (cf. Tables 1 and 3). Among the 30.5°C-females, RMR of T3 turtles was 118% that of the controls, a smaller response to T3 than that of the 24.5°C-males (Table 3, ANCOVA interaction term). T3 treatment also influenced egg incubation time

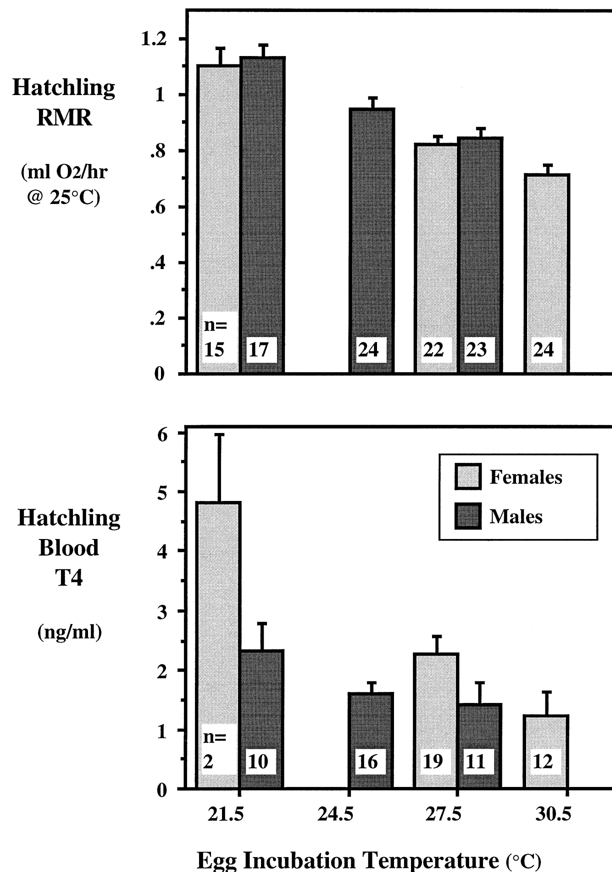

Figure 1. RMR measured at 25°C and blood thyroxine (T4) levels of hatchling turtles. In male and female hatchlings, both RMR and blood T4 are strongly negatively correlated with egg incubation temperature. The RMR graph presents combined data from 1992 and 1993. Table 2 presents statistical analyses. Error bars represent standard errors.

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Table 3: Effects of treating eggs with triiodothyronine (T3) during mid-incubation

	Egg Temperature-Sex	
Variable and Treatment	24.5°C-Male	30.5°C-Female
Hatchling RMR (mL O_2/h at 25°C):[a]		
T3	1.34 ± .07 (13)	.99 ± .08 (6)
Control	.93 ± .04 (8)	.84 ± .06 (7)
Incubation time (d):[b]		
T3	68.7 ± .6 (21)	54.6 ± 1.4 (7)
Control	72.5 ± .6 (15)	57.9 ± .5 (14)
Hatchling mass (g):[c]		
T3	8.5 ± .3 (21)	8.2 ± .4 (7)
Control	9.1 ± .3 (16)	8.9 ± .4 (14)
Hatching success (%):		
T3	81 (22 of 27)	26 (7 of 27)
Control	94 (16 of 17)	74 (14 of 19)

Note. T3 treatment increased hatchling resting metabolic rates (RMR), while decreasing egg incubation time, hatching success, and hatchling mass. The ANCOVA model is described in "Material and Methods." *G* statistics indicate that T3 treatment did not affect hatching success within the 24.5°C-males ($G^2 = 1.6$, $P = 0.21$) but strongly affected hatching success within the 30.5°C-females ($G^2 = 10.6$, $P = 0.001$). In addition, hatching success of the T3 24.5°C-males was greater than that of T3 30.5°C-females ($G^2 = 17.8$, $P < 0.0001$) and that hatching success did not differ among control eggs ($G^2 = 2.9$, $P = 0.09$). Trait values are mean ± 1 SE (*N*).

[a] ANCOVA: treatment $P = 0.0001$; egg temperature-sex $P = 0.013$; interaction $P = 0.009$.

[b] ANCOVA: treatment $P = 0.0001$; egg temperature-sex $P = 0.0001$; interaction $P = 0.83$.

[c] ANCOVA: treatment $P = 0.0003$; egg temperature-sex $P = 0.96$; interaction $P = 0.29$.

and hatchling mass, decreasing both variables in both egg temperature–sex groups (Table 3). T3 treatment decreased hatching success of 30.5°C eggs but not of 24.5°C eggs (Table 3). The only variable not affected by T3 was hatchling sex ratio. All turtles sexed from the 24.5°C eggs were male (T3 group $n = 14$; EtOH group $n = 9$) and from the 30.5°C eggs were female (T3 group $n = 8$; EtOH group $n = 7$).

Discussion

Egg Incubation Temperature and Hatchling RMR

When measured at a common temperature, RMR of hatchling snapping turtles is strongly negatively correlated with egg incubation temperature (Fig. 1). This result supports the possibility that egg incubation temperature induces positive metabolic compensation in the hatchling turtles. Notably, this effect of egg temperature is quite different from the quadratic effect of egg temperature on hatchling mass (Table 1); thus mass cannot explain RMR patterns. In addition, RMR patterns do not differ between male and female hatchlings (Fig. 1; Tables 1, 2), and thus sex cannot explain RMR patterns, nor can RMR patterns explain TSD.

The data on acute affects of temperature provide additional evidence of positive compensation. RMR is sensitive to assay temperature (Table 1), thus without positive compensation, these acute temperature effects could create differences in RMR among turtles developing at different egg temperatures. Compensation would be complete if RMR was the same in all hatchlings measured at their egg temperatures. We can test this possibility across two egg temperatures. In 1992, we measured RMR of hatchlings from 24.5°C eggs at 25°C and measured hatchlings from 30.5°C eggs at 31°C (Table 1). The RMR of the latter group was higher ($P = 0.0001$, *t*-test), but this difference was less than half that created by the acute effects of temperature (Table 1). Thus, positive compensation occurs but is not complete between these incubation temperatures. It was beyond the scope of this study to determine the precise degree of compensation, but this could be accomplished by examining RMR at additional incubation temperatures and determining the contributions of different body components to RMR (e.g., yolk mass; see "Results").

Three previous studies of turtles examined temperature effects on metabolic rate during early development (Leshem et al. 1991; Birchard and Reiber 1995; Booth 1998). These studies measured oxygen consumption of eggs at their incubation temperatures, and so can address the development of compensation before hatching. In snapping turtles, oxygen consumption of eggs incubating at 24°C (thus, males) and 30°C (females) was similar in eggs at similar stages of development (on the basis of embryonic mass), during the latter half of incubation (Birchard and Reiber 1995). This result is consistent with near-perfect positive compensation during this period of incubation.

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Our data additionally suggest that sex did not influence these metabolic patterns. In contrast, eggs of the Nile soft-shelled turtle *Trionyx triunguis* displayed minimal compensation until nearly the end of incubation (Leshem et al. 1991), and the Brisbane river turtle *Emydura signata* displayed no compensation throughout incubation (Booth 1998). Whereas these three studies did not report acute temperature effects, and so cannot define exact levels of compensation, they nonetheless suggest that patterns of metabolic compensation differ among turtles. Notably, the snapping turtle, with the strongest pattern of positive compensation during development, is the only species of the three mentioned that displays TSD. Prevailing theory suggests that TSD should tend to evolve (or be retained) in species where incubation temperature strongly influences traits other than sex (such as metabolic physiology), and these traits are differentially important to the sexes (Charnov and Bull 1977; Bull 1983; Deeming and Ferguson 1988; Ewert and Nelson 1991; Janzen and Paukstis 1991). It should prove interesting to determine if TSD species generally show stronger patterns of metabolic compensation than non-TSD species (preliminary data suggest five of six additional species fit this pattern; Ewert 1985), and the fitness consequences of compensation (also see below).

Positive metabolic compensation during development contrasts with temperature acclimation effects reported in adult *Chelydra serpentina.* Adult snapping turtles exhibit inverse compensation, reducing relative rates of oxygen consumption after acclimation to 10°C (Gatten 1978, 1980). Our results show that *C. serpentina* is capable of both positive and inverse responses to temperature. The direction of response may depend on season (Tsuji 1988*b*) or developmental stage. For example, soon after hatching, snapping turtles from northern populations must hibernate through a long winter (Ultsch 1989; Ernst et al. 1994). These hatchlings might benefit by shifting from positive to inverse metabolic responses to temperature before hibernation. Alternatively, acclimation response may depend entirely on the absolute temperatures experienced. After hatching, turtles tolerate body temperatures ranging from 1°–7°C below freezing up to 31°C and exhibit normal activity between 21°C and 31°C, the egg viability range (Ernst et al. 1994; Birchard and Packard 1997). Once hatched, all snapping turtles may exhibit positive compensation in response to 20°–30°C temperatures and inverse compensation in response to 10°C and below.

Egg Incubation Temperature and Hatchling Thyroid Hormone Levels

Blood thyroxine (T4) levels of hatchling turtles show a strong negative correlation with egg incubation temperature (Fig. 1). This response parallels that of RMR, suggesting either that thyroid hormones play a functional role in positive metabolic compensation or that T4 and RMR share a temperature sensitive regulatory pathway. In many other vertebrates, cold acclimation can increase thyroid activity (Dauncey 1990; Tomasi 1991), and thyroid hormones can increase RMR and influence cellular and biochemical factors, such as the fluidity of plasma membranes, that counter the acute effects of cold (Danforth and Burger 1984; Hoch 1988; Oppenheimer et al. 1991; Cossins 1994). However, very few studies examine the relationship between temperature acclimation and thyroid activity in turtles. Thyroidal uptake of radioiodine was much lower in adult *Pseudemys floridana* and *Terrapene carolina* turtles housed at ≤10°C than at 21°C (Shellabarger et al. 1956), and was lower in adult *Chelodina longicollis* turtles housed at 21° than at 31°C (Hulbert and Williams 1988). Yet blood T4 and T3 levels did not differ with temperature in these *C. longicollis* (Hulbert and Williams 1988), and T4 levels similarly did not differ between 7°C-acclimated and 28°C-acclimated adult *Chrysemys picta* turtles (Mason 1977).

Thus, in contrast to our findings in hatchlings, adult turtles appear to show no correlation between thyroid hormone levels and acclimation temperature and a positive correlation between activity of the thyroid gland and acclimation temperature. These studies of adults did not examine metabolic responses to temperature, and so the relationship between these thyroidal responses and metabolic compensation is unclear. Different findings in adults and hatchlings might reflect different metabolic responses to temperature or different expression of factors that can mediate thyroid activity (e.g., thyroid binding proteins), or thyroid hormones might play a different or no role in metabolic compensation in adults. Clearly, considerably more data is needed to elucidate relationships between thyroid hormones and temperature in turtles. Relationships between thyroid hormones and metabolic rate are somewhat better studied and are discussed further below.

Effects of Exogenous Thyroid Hormones

T3 treatments of eggs creates several responses that counter the acute effects of low temperature, consistent with the hypothesis that thyroid hormones contribute functionally to positive metabolic compensation. Acute reductions in temperature decrease hatchling RMR (Table 1), whereas applying T3 to eggs increases hatchling RMR (Table 3). Similarly, reduced temperature decreased metabolic rate in adult *C. longicollis*, whereas T4 injections increased and thyroidectomy decreased metabolic rate (Hulbert and Williams 1988). In this study, lower incubation temperatures increase incubation time (Table 1; Yntema 1978), and T3 decreases incubation time (Table 3). This result nicely complements an earlier study in which *C. serpentina* embryos responded to thiourea (a goitrogen or antithyroidal) treatment with slowed developmental rates and increased incubation times (Dimond 1954). Our study also found that T3 treatment decreases hatchling mass (Table 3), which otherwise increases with declining egg temperature down to 24°C (Table 1; reviews

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in Rhen and Lang 1995; O'Steen 1998). Together these studies support the hypothesis that thyroid hormones influence metabolic rates and can contribute to metabolic compensation in turtles.

The effect of T3 on RMR is notably consistent with the positive correlation between hatchling RMR and T4 levels that occurred in response to egg incubation temperatures (Fig. 1). In most vertebrates, T4 level provides at least a general predictor of T3 level and activity (see caveats, next paragraph). T4 is the primary thyroid hormone secreted by the thyroid gland and circulating in the blood, whereas T3 is considered the active form of thyroid hormone, produced primarily by deiodination of T4 in the liver and target cells (DiStefano et al. 1982*b*; Hadley 1996). In reptiles, blood levels of the two hormones show similar seasonal cycles and some direct, positive correlations (reviews in John-Alder 1984; O'Steen 1995). The pattern of T4 levels found here (Fig. 1) suggests that T3 will show a similar pattern and may be causally related to RMR. Showing a causal relationship will require determining the physiologic range of thyroid hormone levels in developing turtles and the effects of this variation on RMR.

Importantly, we were unable to measure the effect of egg T3 treatments on blood thyroid hormone levels in this study, and so do not know if the responses to T3 represent physiologic or pharmacologic effects. Previous studies of the relationship between thyroid hormones and RMR in reptiles indicate that detecting and showing a physiologic relationship will be difficult. Pharmacologic manipulations of thyroid hormone levels affect RMR in lizards (John-Alder 1983) and turtles (Hulbert and Williams 1988; Stamper et al. 1990), but physiologic manipulations do not do so consistently (John-Alder 1990; O'Steen 1995). In unmanipulated reptile populations, RMR can be positively correlated (Kar and Chandola 1985; Kar and Chandola-Saklani 1985), negatively correlated (Sellers et al. 1982), or not correlated with blood thyroid hormone levels (John-Alder 1984; O'Steen 1995). Several factors may contribute to the complexity of these results. First, total blood plasma levels of hormones will not always indicate hormone activity. Factors including circulating levels of thyroid binding proteins (Licht et al. 1990, 1991; Glennemeier and Licht 1993), cellular expression of thyroid receptors, and activity of enzymes that convert T4 to T3 (Samuels et al. 1989; Dauncey 1990; Shepard and Eberhardt 1993) may all mediate interactions among hormone levels in the blood, hormone actions, and RMR. In addition, thyroid hormones are broad acting, and RMR reflects the total of all resting metabolic processes of an organism. Thus both hormone levels and RMR vary in response to many physiologic and ecologic factors that may mask direct interactions between them. Despite such complexities, this study found two positive correlations between thyroid hormones and RMR. As nearly all previous studies have used adult animals, our findings suggest that developmental systems should form a critical complement to adult systems for explicating the relationship of thyroid and metabolic traits in reptiles.

Possible Functions of Positive Metabolic Compensation

Positive metabolic compensation may serve to maintain physiologic processes within a range that promotes normal development. This possibility predicts that extreme metabolic rates will decrease fitness of embryonic turtles. Data from the T3 study fit this prediction. T3 treatment did not affect hatching success of 24.5°C eggs but greatly reduced the hatching of 30.5°C eggs (Table 3), which apparently also experienced unusually high RMRs. The Q_{10} measured between 25°C and 31°C in the first study (see "Results") provides a prediction of RMR at egg temperature for the T3 study hatchlings. Predicted RMR at egg temperature was highest in the T3-treated 30.5°C female hatchlings (Fig. 2), the group with highest egg mortality (Table 3), whereas the predicted RMR at egg temperature of T3-treated 24.5°C-males was below that of the 30.5°C controls (Fig. 2). Mortality data of unmanipulated eggs show similar patterns. In snapping turtles, hatching success decreases above 30.5°C (F. Janzen, unpublished data; Yntema 1978), and in *T. triunguis* hatching success decreases at 33°C (Leshem et al. 1991). In both species, these temperatures also produce the highest mea-

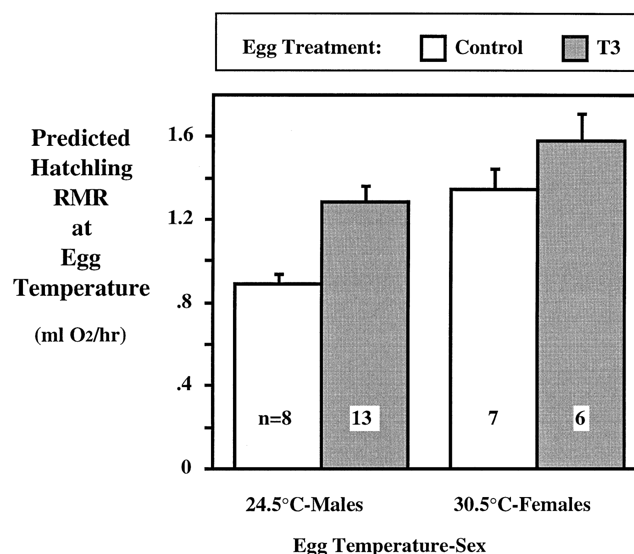

Figure 2. Predicted RMR at egg incubation temperature of hatchlings from the T3 study. Predicted RMR is highest in hatchlings from the T3-treated, 30.5°C eggs, which also experienced the highest mortality (Table 3). Overall, predicted RMR is higher in hatchlings from T3-treated than from control eggs and higher in 30.5°C female hatchlings than in 24.5°C-males (ANCOVA: T3 treatment, $P = 0.001$; egg temperature–sex, $P = 0.0007$; interaction, $P = 0.15$). Predicted RMR was calculated by transforming RMR measured at 25°C (Table 3) with the mean Q_{10} for RMR (2.32) measured between 25° and 31°C (see "Results"). Error bars represent standard errors.

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sured rates of oxygen consumption at incubation temperature, despite positive compensation (this study; Leshem et al. 1991).

Positive compensation may also function to promote hatching at an ecologically appropriate time. For example, in northern populations of snapping turtles, eggs need to hatch while the climate is warm enough for hatchlings to leave the nest and locate overwintering sites; hatchlings that overwinter in the nest rarely survive (reviewed in Ultsch 1989; Packard and Packard 1994). The high RMR and T4 levels of 21.5°C turtles (Fig. 1) may reflect processes that decrease incubation time (Table 3) and thus promote hatchling survival. In addition, positive compensation increases hatching synchrony, which may function to reduce predation risk and energetic costs of hatching (see review in Booth and Thompson 1991).

Importance of Metabolic Traits to TSD

The strong effect of egg incubation temperature on hatchling turtle physiology has interesting implications for the ecologic and evolutionary significance of TSD, which is as yet little understood. As stated previously, TSD should be favored by selection if egg temperature influences traits that are differentially important to males and females (Charnov and Bull 1977; Bull 1983; Deeming and Ferguson 1988; Ewert and Nelson 1991; Janzen and Paukstis 1991). RMR and thyroid hormone levels appear to fit these criteria. Reptiles frequently show sex differences in activity rates, habitat use, and body size (Brown and Brooks 1993), and these traits can be influenced by thyroid activity and metabolic capacity. For example, thyroid hormone levels of reptiles are correlated with activity level and capacity, temperature tolerance, growth rates, and reproductive behavior (Lynn 1970; Bona-Gallo et al. 1980; Sellers et al. 1982; John-Alder 1983, 1984; Stamper et al. 1990; Denver and Licht 1991; John-Alder and Joos 1991; Gerwien and John-Alder 1992; O'Steen 1995). One caveat to this suggestion is that thyroid levels and RMR are both highly plastic traits in adult animals, and thus egg incubation temperature effects may not be long lasting. However, egg temperature has some markedly enduring consequences in TSD reptiles. Egg temperature affects the temperature preferences of juvenile snapping turtles for at least 36 wk after hatching (O'Steen 1998), and in other TSD species affects steroid hormone levels and reproductive behaviors of adults (Gutzke and Crews 1988; Flores et al. 1994; Tousignant and Crews 1995) and has long-lasting effects on growth (Joanen et al. 1987; Brooks et al. 1991; Bobyn and Brooks 1994; Rhen and Lang 1995; Tousignant and Crews 1995; Roosenburg and Kelley 1996; O'Steen 1998). In addition, in *Pseudemys scripta* turtles, a TSD species, adult females have higher plasma T4 levels than males (Licht et al. 1990), suggesting that the similar relationship found here (Fig. 1) is not anomalous and may persist into adulthood. Together these facts suggest that physiologic responses to egg temperature could play a role in the evolution of TSD, and the phylogenetic patterns and fitness consequences of these responses merit study.

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