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Thyroid hormone modulates offspring sex ratio in a turtle with temperature-dependent sex determination

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The adaptive significance of temperature-dependent sex determination (TSD) has attracted a great deal of research, but the underlying mechanisms by which temperature determines the sex of a developing embryo remain poorly understood. Here, we manipulated the level of a thyroid hormone (TH), triiodothyronine (T_3), during embryonic development (by adding excess T_3 to the eggs of the red-eared slider turtle *Trachemys scripta*, a reptile with TSD), to test two competing hypotheses on the proximate basis for TSD: the *developmental rate hypothesis* versus the *hormone hypothesis*. Exogenous TH accelerated embryonic heart rate (and hence metabolic rate), developmental rate, and rates of early post-hatching growth. More importantly, hyperthyroid conditions depressed expression of *Cyp19a1* (the gene encoding for aromatase) and levels of oestradiol, and induced more male offspring. This result is contrary to the direction of sex-ratio shift predicted by the *developmental rate hypothesis*, but consistent with that predicted by the *hormone hypothesis*. Our results suggest an important role for THs in regulating sex steroid hormones, and therefore, in affecting gonadal sex differentiation in TSD reptiles. Our study has implications for the conservation of TSD reptiles in the context of global change because environmental contaminants may disrupt the activity of THs, and thereby affect offspring sex in TSD reptiles.

1. Introduction

Despite strong genetic similarity, males and females within a population often differ dramatically in a diverse suite of phenotypic traits encompassing behaviour, physiology, and morphology [1–3]. Thus, the gene regulatory pathways that determine whether an individual develops as a male or a female are fundamental to a broad range of processes in ecology and evolution [4–6]. Sex is genotypically determined in mammals, birds, snakes, most amphibians, and many other vertebrates; that is, an offspring's sex depends upon the complement of sex chromosomes that it receives from its parents at the time of conception. By contrast, sex in many ectothermic vertebrates is determined by a remarkable range of other systems that include environmental triggers. For example, nest temperature determines offspring sex in all crocodilians [7], many turtles, and lizards [8,9], and the sole extant rhynchocephalian [10]. Both the ultimate (adaptive) and proximate foundations of temperature-dependent sex determination (TSD) have attracted a great deal of research [11–13], but the underlying mechanisms by which temperature determines the sex of a developing embryo remain poorly understood, and a major challenge for evolutionary biology [14–16].

In this paper, we focus on two competing hypotheses regarding the proximate basis for TSD. The first, which we call the *developmental rate hypothesis*, posits that sex is determined by embryonic development rate rather than

incubation temperature *per se* [17]. In support of this idea, an early study on crocodiles found that offspring sex is more closely related to embryonic development rate during the first half of incubation than it is to incubation temperature [18]. Experimental tests of this hypothesis have been less encouraging. Hatchling sex in turtles is unaffected by manipulations that induce shifts in developmental rate (changes in hydric or oxygen incubation environments [19,20]), nor is sex associated with intraspecific variations in genome size that influence rates of cell division and growth [21].

The second and more broadly accepted hypothesis, which we refer to as the *hormone hypothesis*, posits a central role for levels of sex steroid hormones (androgens and oestrogens), moderated by as-yet-unknown temperature-sensitive genes that direct development into either a male or female trajectory [22]. Support for a central role of sex hormones is provided by experiments that block aromatase (which converts testosterone to oestradiol (E_2)) to influence the outcome of the female/male determining cascade [23,24]; and by the ability of exogenous E_2 to override the effect of temperature on gonadal sex. Applying aromatase inhibitors can generate male offspring at female-producing temperatures, and applying exogenous E_2 can generate female offspring at male-producing temperatures [13,25,26]. Nonetheless, the mechanism by which temperature influences levels of sex steroid hormones, and thus offspring sex, remains unclear.

Other hormones play a critical role both in upregulating or downregulating the level of expression of genes affecting androgens and oestrogens, and in influencing the overall rate of development of the embryo. One such class of hormones, thyroid hormones (THs), regulate metabolic rate during the early growth and development of vertebrates [27–29]. THs control rates of organ differentiation (and thus the pace of embryonic and larval development) in fish, amphibians, reptiles, and birds [30–33]. TH also influences the establishment of gonadal sex in fish, amphibians, and mammals. For example, treatment with goitrogenic compounds or perchlorate (that inhibit TH synthesis) increases the proportion of females among zebrafish (*Daniorerio*) and anuran embryos, whereas application of TH yields male-skewed sex ratios [34–36]. Uniquely, then, TH not only stimulates developmental rate, but also affects gonadal sex. Experiments that modify TH levels thus can directly test contrasting predictions from the *developmental rate hypothesis* and the *hormone hypothesis*.

In this study, we manipulated the level of TH (triiodothyronine, T_3) during embryonic development by adding excess T_3 to the eggs of the red-eared slider turtle (*Trachemys scripta*), a reptile with TSD. The eggs were incubated at three constant temperatures (28°C, 29°C, and 30°C) around the pivotal temperature of 29°C [37] to explore the influence of T_3 on developmental rate, levels of expression of *Cyp19a1* (Cytochrome P450 Family 19 Subfamily A Member 1, encoding for aromatase), oestradiol levels in embryos and offspring sex. Based on the *developmental rate hypothesis*, we expect T_3 treated eggs to produce more females, because faster developmental rates (as generated by higher temperatures, and also by T_3 administration) result in females in this species [37]. Alternatively, if T_3 directly affects oestrogen levels, consistent with the *hormone hypothesis*, we predict lower levels of expression of aromatase and, as a result, lower levels of oestradiol and a male-biased sex ratio in the T_3 treatment (because elevated levels of T_3 and its precursor thyroxine T_4 induce more male offspring [35,36]).

2. Material and methods

(a) Study species

The red-eared slider turtle (*T. scripta*) is widespread globally, due to a flourishing pet trade and generalist ecological niche [38]. *Trachemys scripta* exhibits Type I TSD, whereby 100% females are produced at a constant incubation temperature greater than 30°C and 100% males at less than 26°C, with a pivotal temperature of 29°C [37,39]. Females lay on average 12 eggs that weigh between 9 and 11 g and neonates have been recorded to overwinter in the nest and emerge the following spring (in cool-climate areas: [38,40]).

(b) Egg collection and incubation

In April 2015, we collected freshly laid eggs from a private hatchery in Haikou, Hainan province. Seventy-five clutches of fertilized eggs ($N = 577$) were weighed to the nearest 1 mg on a Mettler balance, and each was individually incubated in an 80 ml jar half-filled with moist vermiculite (water tension −220 kPa) [41]. Each jar was assigned to either a treatment group (T_3 application) or control group (ethanol (EtOH)). Both treatment and control jars were distributed among three incubators (KB240, Binder, Germany) set at 28°C, 29°C, and 30°C. Those temperatures were predicted to result in male-biased sex ratios, even sex ratios, and female-biased sex ratios, respectively [39]. Jars were weighed and rehydrated weekly to account for evaporation and maintain water potential of vermiculite. To counteract potential thermal gradients in the incubator, jars were rotated weekly within chambers. Temperatures within each incubator were monitored using Thermocron iButton temperature loggers (two DS1921 ibuttons in separate jars, MAXIM Integrated Products Ltd., USA), so that we could record the exact thermal environments experienced by the eggs.

(c) Application of thyroid hormone

Eggs in the treatment group were exogenously treated with the TH 3,3′5-triiodo-L-thyronine (T_3; Sigma-Aldrich, USA) dissolved in absolute ethanol (EtOH, Sigma-Aldrich, USA)—4.0 µg T_3 was dissolved in 10.0 µl of EtOH to yield a concentration of 0.4 µg μl^{-1} [42]. Eggs in the control group were treated with EtOH only. The dosage was based on the dosages applied to *Chelydra serpentina* and *Emydura macquarii* embryos at embryonic stages 18–19 [42,43], after estimating and correcting for estimated embryo size (crown to rump length) of *T. scripta* embryo size at the time of application [44,45]. We then conducted a preliminary experiment on T_3 dosage effects to determine the optimal dosage, which was 3 µl of T_3 solution (0.4 µg μl^{-1}) (electronic supplementary material, figure S1).

Every 3 days through the incubation period, we collected three eggs from each incubator and dissected them to determine the stage of embryonic development. When embryos reached stage 15 according to the classification scheme of Greenbaum [45], the beginning of the thermosensitive period of TSD [46], each egg was given a single dose of 3 µl of either the treatment (0.4 µg T_3 μl^{-1} EtOH) or control (EtOH) solutions, applied to the top surface of each egg using a pipette.

(d) Aromatase and oestradiol during the thermosensitive period of temperature-dependent sex determination

After the application of T_3, we dissected three eggs every other day from each treatment to determine the developmental stage of embryos. Embryos at stages 17, 19, and 21 were collected to determine the expression of aromatase and measure levels of oestradiol.

For aromatase, we collected a total of 90 embryos (five embryos at each stage from each treatment). The Adrenal Kidney Gonad Complex (AKG) of each embryo was separated immediately on dissection and frozen in liquid nitrogen. RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. The quality and quantity of each total RNA sample was assessed through spectrophotometer reading at 260 nm and 280 nm. The RNA sample was then treated with DNase I to eliminate genomic DNA contamination and single-stranded complementary DNA (cDNA) was reverse-transcribed using an RT reagent kit with genomic DNA (gDNA) eraser (Takara Bio Inc., CA, USA). After reverse transcription, we mixed the cDNA of five embryos at each stage from each treatment together to obtain enough cDNA for polymerase chain reaction (PCR). Relative levels of *Cyp19a1* expression were quantified using SYBR Green (Hercules, CA, USA) following the manufacturer's protocol and Roche LightCycler 480 real-time PCR cycler. Each reaction was performed in triplicate and normalized to constitutive expression of peptidylprolyl isomerase (PPI). Primers used to assay gene expression were designed according to Ramsey *et al*. [47]. The qPCR cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 30 s, 59°C for 20 s, 72°C for 20 s, and then melt curve from 55 to 95°C. Relative gene expression was calculated from the equation $2^{-\triangle\triangle CT}$.

For measuring levels of oestradiol, we collected 125 embryos (six to eight embryos at each stage from each treatment). Each embryo was homogenized with a glass homogenizer in ice cold phosphate buffer saline (PBS). The suspensions were then centrifuged at 13 000 r.p.m. for 10 min. The volume of each supernatant was only large enough for us to assay oestradiol levels with radioimmunoassay. Even so, 11 embryos were too small for us to detect the oestradiol level accurately, and data on these individuals were excluded from subsequent analyses.

(e) Embryonic heart rate

We measured heart rates of 15 eggs from each treatment 24 h after T_3 application, in order to infer the metabolic rate of embryos [48]. For this purpose, eggs were individually removed from the incubation container and immediately placed in a Buddy digital egg monitor system (Avian Biotech, UK) in complete darkness for a maximum of 2 min [48,49]. Eggs were not disturbed during the recording period. If any embryonic movement was detected, we waited for movement to cease and heart rate to stabilize before recording [49].

(f) Incubation duration and hatching success

Towards the end of incubation, eggs were inspected twice daily for signs of pipping (initial break of egg with caruncle) [50]. The duration of incubation was measured for each egg as the number of days from oviposition to pipping. Hatching success was recorded as the number of neonates that hatched as a percentage of all eggs assigned to the respective treatment.

(g) Hatchling traits

Directly after the eggs hatched, we measured maximum carapace length, width, and height, and mass of each hatchling. The young turtles were each individually housed in plastic containers (150 × 100 × 60 mm) for the next three months. The containers were set in a temperature-controlled room at 28 ± 0.5°C, with a 12 light (L) : 12 dark (D) photoperiod. Sufficient commercial food was provided for hatchlings every day. Hatchling size and growth rate were recorded twice (at one and three months of age) to measure the effects of TH and incubation temperature on post-hatching development. Hatchlings were sexed at three months of age, when the sex of the turtles can be reliably identified by their secondary sexual characteristics [51].

(h) Data analysis

We used the Kolmogorov–Smirnov test and Levene's test to evaluate the normality of distributions and homogeneity of variances for all data. χ^2-tests were used to explore the effects of incubation temperature and T_3 on hatching success and hatchling sex ratio. Factorial ANOVA was used to detect the effect of temperature and T_3 on embryonic heart rate and incubation duration, as well as the effect of temperature, T_3, and developmental stage on the expression of aromatase and levels of oestradiol. Two-way ANCOVA was used to analyse the effects of temperature and T_3 on hatchling sizes (with initial egg mass as the covariate) and growth rates (with hatchling carapace length as the covariate). Post hoc Tukey–Kramer honest significant difference (HSD) tests were carried out to determine the locations of among-treatment differences.

3. Results

Expression of aromatase in embryos increased with incubation temperature ($F_{2,36} = 72.1$, $p < 0.00001$) and as the embryos developed ($F_{2,36} = 135.7$, $p < 0.00001$), but was depressed by T_3 application ($F_{1,36} = 23.0$, $p < 0.0001$; figure 1). Oestradiol levels in embryos increased as the embryos developed ($F_{2,96} = 36.2$, $p < 0.00001$), were not affected by incubation temperature ($F_{2,96} = 2.9$, $p = 0.061$), but were depressed by T_3 application ($F_{1,96} = 27.1$, $p < 0.0001$; figure 2).

Incubation temperature accelerated embryogenesis: it increased embryonic heart rates and decreased incubation durations (table 1; figure 3). Exogenous TH (T_3) also increased embryonic heart rates (by 4–8%: figure 3*a*), and shortened incubation duration of turtle eggs (by 4–7%: figure 3*b*, table 1). The effect of T_3 on incubation duration depended on incubation temperature (i.e. significant interaction between T_3 treatment and incubation temperature: table 1). The effect of T_3 was greater at lower incubation temperatures (figure 3).

Hatching success of turtle eggs was high (approx. 90%), and was not significantly affected by either T_3 (28°C: $x^2 = 0.33$, d.f. = 1, $p = 0.57$; 29°C: $x^2 = 0.78$, d.f. = 1, $p = 0.38$; 30°C: $x^2 = 0.12$, d.f. = 1, $p = 0.73$) or incubation temperature (T_3 treatment: $x^2 = 1.388$, d.f. = 2, $p = 0.50$; control: $x^2 = 0.084$, d.f. = 2, $p = 0.96$; figure 4).

The sex ratio of hatchlings was affected by exogenous T_3 when eggs were incubated at 28°C ($x^2 = 6.35$, d.f. = 1, $p = 0.01$) and 29°C ($x^2 = 6.76$, d.f. = 1, $p < 0.01$), but not at 30°C ($x^2 = 2.65$, d.f. = 1, $p = 0.10$). Generally, T_3 caused a male-biased sex ratio relative to the control group (figure 5). Hatchling sex was also affected by incubation temperature (T_3 treatment: $x^2 = 94.8$, d.f. = 2, $p < 0.001$; control: $x^2 = 65.64$, d.f. = 2, $p < 0.001$). Hatchling sex ratios were male-biased at 28°C and 29°C, but female-biased at 30°C (figure 5).

T_3 treatment, incubation temperatures, and the interaction between these two factors also affected hatchling size (carapace length, width, and height) and mass (table 1). Hatchlings from the T_3 treatment were smaller than their counterparts from the control group. In the T_3 treatment, hatchlings from 28°C were smaller than those from 29°C and 30°C, whereas hatchling size was not significantly affected by incubation temperature in the control group (electronic supplementary material, figure S2).

During the first month of life, a hatchling's growth was affected by both the T_3 treatment and incubation temperature under which it had developed as an embryo (table 1). Hatchlings from the T_3 treatment grew more rapidly than did those from the control groups and hatchlings from 28°C grew more

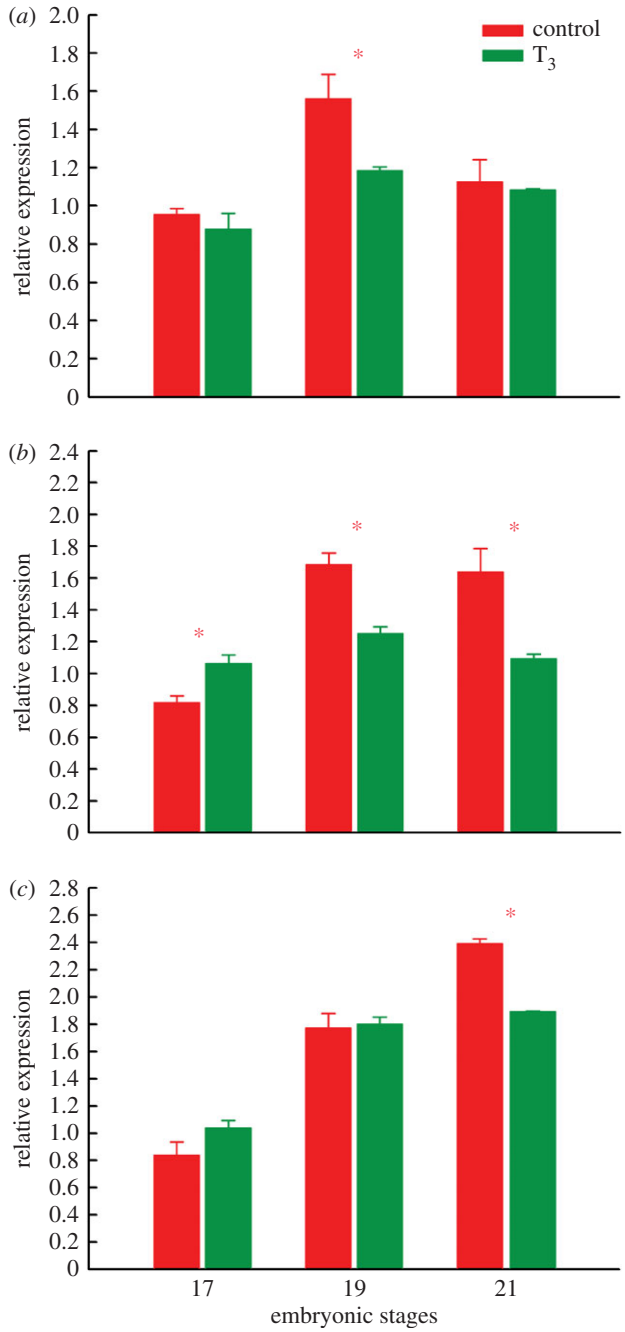

Figure 1. Effects of triiodothyronine (T_3) treatment on expression of aromatase in different developmental stages of *Trachemys scripta* embryos incubated at (*a*) 28°C, (*b*) 29°C, and (*c*) 30°C. Data are expressed as mean ± standard error, and the sample sizes are all three. The asterisk indicates a significant difference between control and T_3 application. (Online version in colour.)

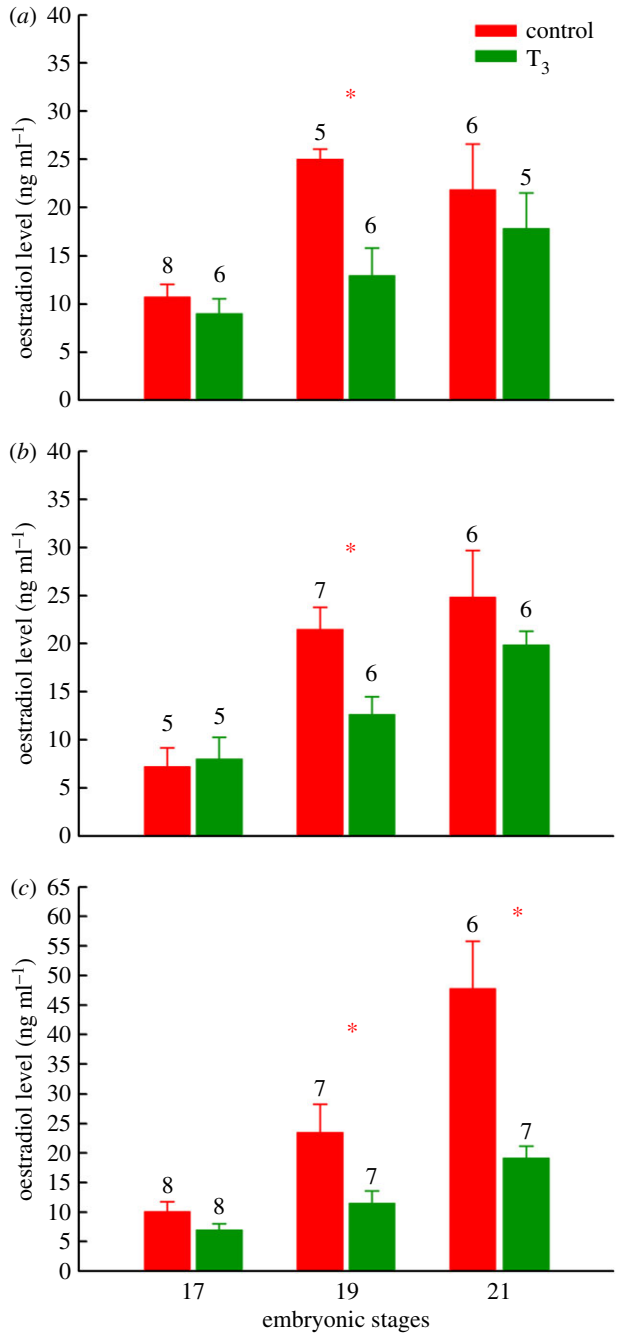

Figure 2. Effects of triiodothyronine (T_3) treatment on oestradiol levels in different developmental stages of *Trachemys scripta* embryos incubated at (*a*) 28°C, (*b*) 29°C, and (*c*) 30°C. Data are expressed as mean ± standard error, and sample sizes are shown above the error bars. The asterisk indicates a significant difference between control and T_3 application. (Online version in colour.)

rapidly than did those from 29°C and 30°C (electronic supplementary material, figure S3). By contrast, rates of growth by hatchlings during the second and third months of life were affected by incubation temperature but not by T_3 treatment (table 1). Hatchlings from 28°C grew more rapidly than did those from 29°C and 30°C (electronic supplementary material, figure S4).

4. Discussion

Exogenous TH (T_3) accelerated embryonic heart rate (and hence metabolic rate), developmental rate, and rates of early post-hatching growth. More importantly, the administration of T_3 during embryonic development depressed expression of aromatase and levels of oestradiol, and induced more male offspring in *T. scripta*. This result is contrary to the direction of sex-ratio shift predicted by the *developmental rate hypothesis*, but consistent with that predicted by the *hormone hypothesis*.

THs play critical roles in metabolism, differentiation, development, and growth [31,32,52–54]. Hyperthyroid conditions enhance oxygen consumption and metabolic rate, and thereby affect growth and development [54]. Consistent with such stimulatory effects, our study indicated that application of exogenous T_3 increased the heart rate (and hence metabolic rate) of embryonic turtles. In addition, exogenous

Table 1. Two-way ANOVA or ANCOVA results for embryonic heart rate, incubation length, hatchling sizes, and hatchling growth in *Trachemys scripta*. Growth 1, growth during the first month; growth 2, growth during the subsequent two months. The significant differences are shown in bold.

variable	T_3 treatment	incubation temperature	interaction
heart rate	**$F_{1,84} = 55.9$, $p < 0.00001$**	**$F_{2,84} = 79.9$, $p < 0.00001$**	$F_{2,84} = 2.2$, $p = 0.12$
incubation length	**$F_{1,272} = 147.8$, $p < 0.00001$**	**$F_{2,272} = 361.7$, $p < 0.00001$**	**$F_{2,272} = 6.6$, $p < 0.01$**
carapace length (CL)	**$F_{1,271} = 23.9$, $p < 0.00001$**	**$F_{2,271} = 3.9$, $p = 0.02$**	$F_{2,271} = 1.5$, $p = 0.24$
carapace width (CW)	**$F_{1,271} = 14.4$, $p < 0.001$**	**$F_{2,271} = 15.2$, $p < 0.00001$**	$F_{2,271} = 2.6$, $p = 0.07$
carapace height (CH)	**$F_{1,271} = 17.2$, $p < 0.0001$**	**$F_{2,271} = 35.8$, $p < 0.00001$**	**$F_{2,271} = 11.5$, $p < 0.001$**
body mass (BM)	**$F_{1,271} = 26.3$, $p < 0.0001$**	**$F_{2,271} = 4.0$, $p = 0.02$**	**$F_{2,271} = 7.6$, $p < 0.001$**
growth 1 in CL	**$F_{1,268} = 16.2$, $p < 0.0001$**	$F_{2,268} = 1.5$, $p = 0.22$	**$F_{2,268} = 3.5$, $p = 0.03$**
growth 1 in CW	**$F_{1,268} = 6.6$, $p = 0.01$**	**$F_{2,268} = 5.0$, $p < 0.01$**	**$F_{2,268} = 10.8$, $p < 0.0001$**
growth 1 in CH	$F_{1,268} = 0.34$, $p = 0.56$	**$F_{2,268} = 48.3$, $p < 0.0001$**	**$F_{2,268} = 10.8$, $p < 0.0001$**
growth 1 in BM	**$F_{1,268} = 9.1$, $p < 0.01$**	**$F_{2,268} = 28.3$, $p < 0.0001$**	$F_{2,268} = 0.38$, $p = 0.68$
growth 2 in CL	$F_{1,268} = 1.2$, $p = 0.27$	**$F_{2,268} = 7.1$, $p = 0.001$**	$F_{2,268} = 2.1$, $p = 0.12$
growth 2 in CW	$F_{1,268} = 0.9$, $p = 0.34$	**$F_{2,268} = 4.9$, $p < 0.01$**	$F_{2,268} = 1.7$, $p = 0.19$
growth 2 in CH	$F_{1,268} = 3.4$, $p = 0.07$	**$F_{2,268} = 12.8$, $p < 0.0001$**	**$F_{2,268} = 3.3$, $p = 0.04$**
growth 2 in BM	$F_{1,268} = 1.4$, $p = 0.24$	**$F_{2,268} = 8.9$, $p < 0.001$**	$F_{2,268} = 2.2$, $p = 0.11$

T_3 reduced the incubation period of turtle eggs (figure 3). The reduced incubation period may be owing to faster developmental rate, or because the final hatchling size is smaller (and thus, can be achieved more rapidly). In *E. macquarii*, exogenous application of T_3 reduced the incubation period of neonates with no cost to development, suggesting that embryonic development was complete [43]. In zebrafish, an experimental reduction in levels of endogenous TH decreased body lengths of developing embryos, an effect that was not reversed by exogenous application of T_4, the precursor to T_3 [55]. If the same phenomenon occurs in turtle embryos, our application of exogenous T_3 may have induced a corresponding reduction in the embryo's production of its own T_4 thyroxin, in turn leading to reduced hatchling size. Young turtles from the T_3-treated eggs grew faster than the controls, indicating a carry-over effect of embryonic T_3 treatment. However, this effect was relatively brief (only seen in the first month after hatching).

Although the close relationship between hatchling sex ratio and developmental rate in crocodiles supports the developmental rate hypothesis [17,18], manipulative studies on turtles have failed to support the idea [19,20,56]. First, a change in developmental rate (induced by incubation conditions independent of temperature) did not modify the sex ratio of hatchlings [19,20]. Second, Georges *et al.* [56] manipulated the sex ratio of a turtle from 100% male to 100% female by modifying diel schedules of thermal variation while holding overall development rate (incubation duration) constant. Third, changes in developmental rate due to hormones (this study) or thermal conditions [57,58] have been associated with biased sex ratios, but in the opposite direction to that predicted by the developmental rate hypothesis.

Our study provides the first evidence that TH affects the offspring sex ratio in TSD reptiles, as it is known to do in some teleosts and amphibians [34–36]. Generally, hyperthyroid conditions during early development produce more male offspring in vertebrates, whereas hypothyroid conditions produce more female offspring [36,55]. For example, perchlorate, a TH synthesis inhibitor, skewed the sex ratio of zebrafish towards female, but yielded male-skewed sex ratios when co-treated with exogenous TH [36]. Our results suggest that TH affects sex-determining pathways in TSD reptiles (figures 1,2,5). However, we caution that our work is based only on a single species, and studies in fish have

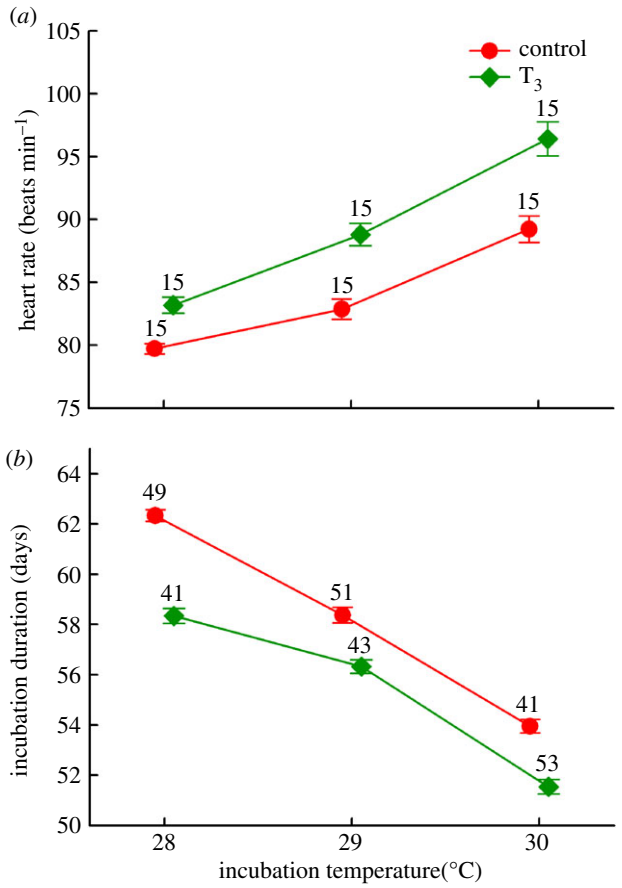

Figure 3. Effects of triiodothyronine (T_3) treatment on embryonic heart rate (*a*) and incubation duration (*b*) in *Trachemys scripta* eggs incubated at different temperatures. Data are expressed as mean ± standard error. Numbers above the error bar are sample sizes. (Online version in colour.)

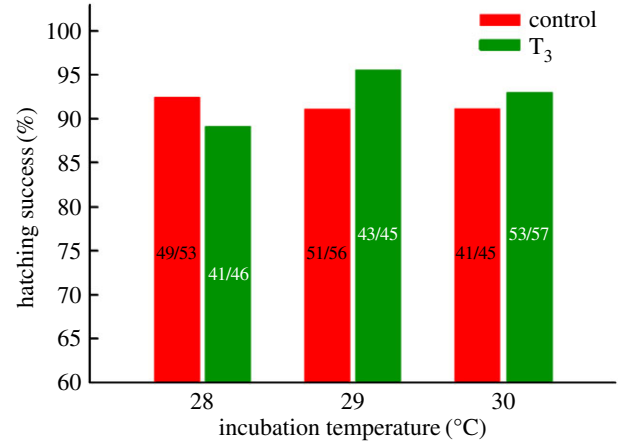

Figure 4. Hatching success of *Trachemys scripta* eggs treated with triiodothyronine (T_3) and incubated at different temperatures. Numbers in the bars are sample sizes. (Online version in colour.)

reported contradictory results. For example, perchlorate did not affect the gonadal sex phenotype of zebrafish [59], or induce male-skewed sex ratios in three-spined sticklebacks (*Gasterosteus aculeatus*) [60]. The broader question of how TH affects sex-determining mechanisms merits further studies in a wide range of vertebrate species.

The underlying mechanisms by which TH affects sex determination in TSD reptiles may be associated with interactions between TH and the sex steroid hormones (androgen and oestrogen) that appear to play a critical role in determining

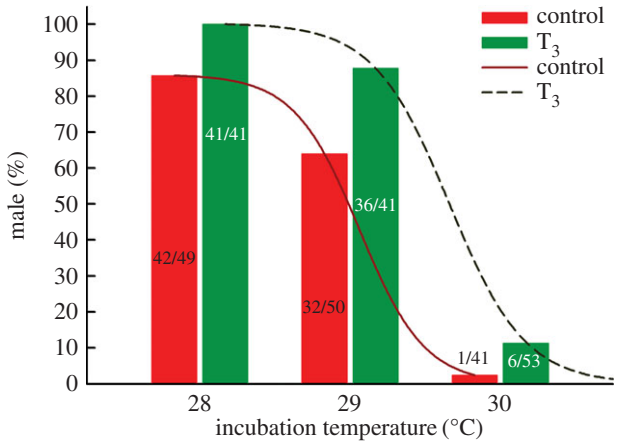

Figure 5. Effects of triiodothyronine (T_3) treatment on the sex ratio of *Trachemys scripta* hatchlings from different incubation temperatures. Numbers in the bars are number of males produced out of sample size. (Online version in colour.)

gonadal sex of TSD reptiles [55,61]. TH might affect sex-determining pathways in several ways. First, TH may affect the activity of gonadal aromatase [27,36]. Aromatase is a key factor in the sex determination of vertebrates; experimental inhibition of aromatase activity reduces oestrogen production and, therefore, generates male-biased sex ratios in species with both genotypic and TSD [22,62,63]. Likewise, hyperthyroid conditions may reduce aromatase activity and thus generate male-biased gonadal sex ratios [36]. Our results on the expression of aromatase support the hypothesis that hyperthyroid conditions can depress aromatase activity (figure 1), which may block the conversion of testosterone to oestradiol and, therefore, promote the male regulatory cascade at the expense of the female cascade [23,24]. Second, TH may upregulate androgen receptors and downregulate oestrogen receptors [61,64,65], again promoting the male regulatory cascade. Unfortunately, we were not able to test this hypothesis in this study. Future studies on how TH affects the regulation of these receptors would deepen our understanding of the physiological mechanism by which hyperthyroid conditions produce more male offspring in reptiles.

Sex steroid hormones play critical roles in determining gonadal sex in TSD reptiles, and thus have been the focus of most studies on physiological pathways underlying TSD in these animals [66]. However, we know little about interactions between sex steroid hormones and other important hormones in determining the sexual phenotype of TSD reptiles. Our results suggest an important role for TH in regulating sex steroid hormones, and therefore, in affecting gonadal sex differentiation in TSD reptiles. For example, application of exogenous T_3 inhibited the activity of aromatase and consequently decreased the level of oestradiol in *T. scripta* (figure 2), thereby inducing male offspring at female-producing temperatures (see also [13,25]). Our study not only highlights the importance of studying interactions among different hormones when we attempt to elucidate physiological mechanisms underlying the TSD in reptiles, but also has implications for the conservation of TSD reptiles under the context of global change. For example, environmental contaminants like perchlorate may disrupt the activity of TH, and thereby affect the sex of TSD reptiles.

Ethics. This research was performed in accordance with the NIH *Guide for the Principles of Animal Care*. The Animal Ethics Committee

at the Institute of Zoology, Chinese Academy of Sciences approved our protocol and study (Permit No: IOZ14001).

Data accessibility. Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.hk1vv [67].

Authors' contributions. B.-J.S., R.S., and W.-G.D. designed the study; B.-J.S., T.L., Y.M., and J.-K.M. conducted the experiments. B.-J.S. and W.-G.D. analysed data; B.-J.S., A.G., R.S., and W.-G.D. wrote the manuscript.

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