



# Effects of semi-constant temperature on embryonic and hatchling phenotypes of six-tubercled Amazon River turtles, *Podocnemis sextuberculata*

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## ABSTRACT

**Purpose:** We evaluated how constant incubation temperatures affect life-history traits pre-hatching and post-hatching of the six-tubercled Amazon River turtle, *Podocnemis sextuberculata*.

**Methods:** We incubated eggs from natural nests at ten semi-constant temperatures between  $22.26 \pm 1.01$  °C and  $37.37 \pm 0.38$  °C (2013) and at six temperatures between  $25.75 \pm 0.22$  °C and  $36.17 \pm 0.15$  °C (2016). In 2013, we raised hatchling for 90 days to evaluate effects of temperature on early hatchling growth. We evaluated maternal effects in 2016.

**Results:** *P. sextuberculata* displays temperature-dependent sex determination and produces males at colder and females at warmer temperatures (TSD Ia). The estimated pivotal temperature was  $33.73 \pm 0.15$  °C and the transitional range of temperatures (TRT)  $1.16 \pm 0.59$  °C. Semi-constant temperatures below 26 °C and above 38 °C were lethal. Intermediate temperatures (32.25 °C and 31.5 °C, respectively) were optimal for hatching success and produced larger hatchlings that grew slower early in life compared to colder or warmer conditions, which produced smaller hatchlings. Warmer incubation temperatures within the optimal range (28 °C–37 °C) accelerated embryonic development. In contrast, comparisons of 30, 60 and 90 days-old suggests that warmer incubation temperatures reduced growth and mass gain rates post-hatching, such that incubation temperature effects on body size at emergence disappeared by 3 months of age.

**Conclusions:** Six-tubercled Amazon River turtles showed the highest pivotal temperature reported for any turtle. The relatively narrow TRT may limit the evolutionary potential of this vulnerable turtle in the face of global warming. Future incubation experiments at a finer scale (33 °C–36 °C) are warranted to refine the sex-ratio reaction norm. Field studies that monitor natural nests are imperative to evaluate conservation measures and the effect of female-biased illegal hunting and climate change. By providing data about the thermal biology of an understudied lineage of non-model species, our study helps fill gaps in our understanding of the evolution of vertebrate sex determination and its potential adaptive value.

## 1. Introduction

Environmental temperature impacts the life histories of ectothermic organisms and is an important ecological driver of individual fitness that mediates adaptive evolution (Booth, 2018; Mitchell et al., 2018). For turtles, external temperature influences the physiology and developmental rate of embryos, hatching success, and hatchling phenotype (reviewed by Packard and Packard 1988; Noble et al., 2018). Among these is the sexual phenotype, to which turtle embryos commit via

temperature-dependent sex determination (TSD), or genotypic sex determination (GSD) with sex chromosomes (Ewert and Nelson, 1991; Valenzuela et al., 2003; Bista and Valenzuela, 2020). Previous reports of co-occurrence of TSD and GSD in the same species of turtle were debunked empirically (Valenzuela et al., 2014; Mu et al., 2015). Temperature-dependent sex determination in reptiles has been found in most turtles, some lizards, all crocodylians and tuatara (Tree of Sex, 2014). However, pleurodire (side-neck turtles) remain understudied with respect to their TSD mechanism compared to their cryptodire

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counterparts.

In most TSD turtles, females are produced at higher incubation temperatures and males produced at lower temperatures (i.e. TSD Ia pattern), and more rarely, males are produced at intermediate temperatures and females above and below those values (TSD II pattern) (Valenzuela and Lance, 2004). The constant incubation temperature that produces a 1:1 sex ratio is known as the pivotal temperature, whereas the range of constant temperatures that produce both sexes is the transitional range temperature (TRT, Mrosovsky and Pieau, 1991).

Temperature-dependent sex determination is likely the ancestral condition in turtles from which GSD evolved at least five times (Valenzuela and Adams, 2011; Pokorná and Kratochvíl, 2016; Sabath et al., 2016), with two potential reversals back to TSD (Valenzuela and Adams, 2011; Litterman et al., 2018), one in the pleurodire lineage of Pelomedusoid turtles (Valenzuela, 2021) to which the turtles of the South American genus *Podocnemis* belong. Of the six species of *Podocnemis*, all but *P. vogli* are reported TSD taxa (Alho et al., 1985; Souza and Vogt, 1994; Pezzuti, 1998; Vogt, 2008; Páez et al., 2009; Carneiro, 2012; Valenzuela and Ceballos, 2012), but only three of them (*P. expansa*, *P. unifilis* and *P. lewyana*) have a well characterized TSD system (Souza and Vogt, 1994; Valenzuela, 2001a; Vogt, 2008; Gallego-García and Páez, 2016). Some *Podocnemis* species display the highest pivotal temperatures known in turtles: 33.23–33.37 °C in *P. lewyana* (Gallego-García and Páez, 2016), 32.6 °C in *P. expansa* (Valenzuela, 2001a), and 31.43 °C in *P. unifilis* (Hulin et al., 2009). Temperature-dependent sex determination in six-tubercled Amazon River turtles, *P. sextuberculata*, was inferred from observations of extreme male-biased sex ratios detected in 40 natural nests (via gonadal inspection of 10 hatchlings per clutch) during a particularly cold year (Pezzuti, 1998), and from sex ratios (calculated from only 5 hatchlings per clutch) and limited temperatures recorded in four natural nests in a second study (Carneiro, 2012). Thus, no formal characterization of the TSD reaction norm has been published for this species under constant incubation conditions, nor has the effect of temperature on other life history traits (Packard and Packard, 1988).

Besides its effect on sex determination in TSD taxa, temperature impacts turtle embryonic physiology, development, and survival, including quantifiable effects on incubation duration, hatching success, and hatchling phenotype, with ultimate fitness consequences (reviewed by Packard and Packard, 1988; Rhen and Lang, 2004). Warmer temperatures accelerate embryonic differentiation, growth and rates of albumen and yolk metabolism (Gutzke and Packard, 1987; Gutzke et al., 1987; Deeming and Ferguson, 1989). Consequently, incubation duration is shortened at warmer temperatures (e.g. Yntema, 1978; Packard et al., 1987; Gutzke and Packard, 1987) and intermediate values produce larger and better performing hatchlings (e.g. Gutzke and Packard, 1987; Packard et al., 1987; Burger, 1991; McKnight and Gutzke, 1993; Bobyn and Brooks, 1994; Allsteadt and Lang, 1995; Du and Ji, 2003). Incubation temperature can have long-term effects on hatchling phenotype (Elphick and Shine, 1998), sometimes mediated by environmental conditions post-hatching (Ceballos et al., 2011; 2014), and can affect habitat choice by juveniles and consequently, their metabolic and growth rates (O'Steen, 1998; Steyermark and Spotila, 2000). Thermal effects on sexual development and other phenotypes are also mediated by maternal effects, that is, non-genetic contributions from females to their offspring that are common in animals with or without parental care (Bernardo, 1996; Moore et al., 2019), and which in turtles include energy and hormonal allocation to the eggs, as well as nest site choice (Topping and Valenzuela, 2021).

Here, we estimate parameters of the thermal reaction norms of sex determination for *P. sextuberculata* turtles for the first time and evaluate the effects of constant temperature on hatching success, incubation duration and hatchling size and growth. Our study helps fill gaps in the broader understanding of the evolution of TSD in turtles, which previous hypotheses have attempted to explain (Charnov and Bull, 1977; Roosenburg, 1996; Ewert and Nelson, 1991; Bull, 1980; Mrosovsky, 1980),

and more recently in *Podocnemis* (Valenzuela, 2021). Our findings also have implications for conservation of the vulnerable *P. sextuberculata*.

## 2. Methods

### 2.1. Study species

*Podocnemis sextuberculata* inhabits the Amazonian regions of Brazil, Bolivia, Colombia and Peru (TTWG, 2021), mainly in white-water rivers, with small populations in clear-water rivers. Individuals migrate up to 85 km annually between feeding areas (flooded forest, backwaters and lakes) and nesting areas (river sand banks that arise during the dry season (Fachín-Terán et al., 2006). Adult females are bigger than males, reaching 34 cm straight carapace length (SCL), but juveniles are not sexually dimorphic. The smallest nesting female reported had a SCL of 26 cm (Fachín-Terán et al., 2004). Females lay 6–25 eggs per clutch, with a mean and standard deviation of  $15.8 \pm 4.99$  (Pezzuti and Vogt, 1999), perhaps up to three times per nesting season (Bernhard, 2001). This turtle suffers poaching of eggs and hunting of adults (Lopes et al., 2012; Pantoja-Lima et al., 2014), which has caused population declines throughout its distribution (Camillo et al., 2012), leading to the species being categorized as “vulnerable” (TTWG, 2021).

### 2.2. Laboratory experiments

Two incubation experiments using semi-constant temperatures were conducted in 2013 and 2016. Freshly-laid eggs were collected at the Middle Solimoes River within the Mamiraua Sustainable Development Reserve, Central Amazon, Brazil and transported to the laboratory at the Mamiraua Sustainable Development Institute (Tefe, Amazonas State, Brazil). Egg incubation started <24hrs from oviposition. Incubators were made from 120-L Styrofoam boxes divided into two compartments, each with a 60-W lamp connected to a digital thermostat (AddTherm, model: A80). Eggs were buried in moist medium-size vermiculite purchased at a local garden store (in a proportion of 1 g vermiculite:2 g of water following Gallego-García and Paez, 2016) inside plastic containers, along with the thermostat probe. Temperature was recorded hourly with a data logger with ( $\pm 0.5^\circ\text{C}$  accuracy). The thermostat probe and data logger were placed in the middle of the container. To maintain moisture levels, all boxes with vermiculite were incubated for a week, without eggs, at each temperature prior to the onset of the experiment to determine the rate of water loss per treatment by the change of weight of the box. Then, once the eggs were added and the experiment started, water was added at different intervals, between every 2 days (for warmer treatments with the highest rate water loss) to weekly (for colder treatments with lowest rate of water loss).

In 2013, we collected 100 eggs (10 per clutch), and placed one egg per nest at 10 different target temperatures: 22 °C, 23 °C, 28 °C, 29 °C, 30 °C, 31 °C, 32 °C, 33 °C, 36 °C and 37.5 °C. However, the low-tech design of the incubators resulted in thermal regimes that fluctuated with varied amplitude around these target values (Fig. S1) ( $22.86 \pm 1.01$  °C,  $23.22 \pm 0.65$  °C,  $27.89 \pm 0.19$  °C,  $29.14 \pm 0.20$  °C,  $29.99 \pm 0.23$  °C,  $31.11 \pm 0.21$  °C,  $31.7 \pm 1.71$  °C,  $33.18 \pm 0.40$  °C,  $35.95 \pm 0.83$  °C and  $37.37 \pm 0.38$  °C). In 2016, we collected 132 eggs from 11 different nests, and placed two eggs per nest at six different thermal regimes:  $25.75 \pm 0.22$  °C,  $27.94 \pm 0.65$  °C,  $29.83 \pm 0.26$  °C,  $31.95 \pm 0.29$  °C,  $34.01 \pm 0.67$  °C,  $36.17 \pm 0.15$  °C. Because eggs from 22.86 °C to 23.22 °C did not hatch and lacked signs of embryonic development, these temperatures were excluded from all further analyses.

Incubation duration was measured from egg collection to full emergence of hatchlings from the eggshell (emergence). Unhatched eggs were opened at the end of the experiment, and those lacking traces of embryonic development excluded from further analyses, as they were assumed to be infertile or to have suffered embryonic mortality unrelated to temperature (this assumption was based on the fact that statistical tests did not detect any temperature effect on the number of

undeveloped eggs:  $x^2 = 12$ ,  $df = 9$ ,  $p = 0.21$ ). Straight carapace length (SCL), straight carapace width (SCW), plastron length (PL), plastron femoral width (PFW) and head width were measured on day one after emergence using a 150 mm caliper (Mitutoyo®), and hatchling mass was measured using a digital scale (Pesola®, MS500, precision = 0.1 g).

To investigate the influence of incubation temperature on post-hatching growth, in 2013 we kept the hatchlings for eight days in a plastic box containing moist vermiculite and then raised them individually until day 90. We reared them in open plastic boxes (262 × 177 × 147 mm) filled with 40 cm<sup>3</sup> of tap water per millimeter of hatchling SCL (adjusted when hatchlings were measured at 1, 8, 15, 30, 45, 60 and 90 days of age), and provided with a rock as dry surface. Boxes were maintained indoors at 23–31 °C under a ~12 h daylight photoperiod and cleaned every two days. Hatchlings were fed commercial turtle food (Alcon® Reptolife: 34% protein, 10% fiber, 15% mineral, 1.6–3% calcium, 0.7% phosphor) *ad libitum*, starting at 8 days of age. At age 1, 8, 15, 30, 45, 60 and 90 days, we measured and weighed hatchlings.

Hatchlings were euthanized by intravenous (IV) injection of general anesthetics (association of xylazine 2 mg/kg, ketamine 50 mg/kg and acepromazine 0.25 mg/kg), followed by administration of potassium 19.1% chloride (2 mg/kg, IV) after loss of corneal and muscles reflexes, as recommended by Resolution n°1000/2012 of the Brazilian Federal Council of Veterinary Medicine (CFMV, 2012). Gonads were dissected to assess sex by histology, and carcasses were fixed in 10% formalin, stored in 70% ethanol and deposited in the Mamiraua Sustainable Development Institute Reptile Collection in Tefe.

### 2.3. Statistical analysis

We performed all analyses using R v. 3.5.2 (R Core Team, 2018), unless noted otherwise. To estimate pivotal temperature and transitional range temperature we used mean incubation temperature for the entire experiment as the predictor variable (summary statistics for hourly variation of temperature are presented in Fig. S1). We used function “tsd ()” in package “embryogrowth” (Girondot et al., 2018) to estimate TSD parameters: P = pivotal temperature, S = curve representing the relationship between sex ratio and temperature during the transition from masculinizing to feminizing temperatures, and K = an asymmetrical shape parameter (Girondot, 1999; Godfrey et al., 2003). The initial values were P = 32.6, S = -2, K = 0, K1 = 1, K2 = 0. We fitted four models: logistic, Hill, Richards and Double-Richards and used the Akaike Information Criterion (AIC) value to select the best model. Because we had just one temperature that produced males and females, calculating confidence intervals or standard deviations was precluded. Instead, we implemented a modified Metropolis-Hastings Markov chain Monte Carlo (MH-MCMC) algorithm with 100,000 iterations, using function “tsd\_MHmcmc” in package “embryogrowth”.

We tested for the effect of mean temperature on duration, hatching success and hatchling size separately, using multiple regression, including year (2013 or 2016) as a fixed effect. When year was significant (as it was for incubation duration and hatchling size) a linear mixed model was used including year as random effect, using packages “lme4” (Bates et al., 2015) and “emmeans” (Lenth, 2019). For hatching success, a simple logistic regression was used because year was not significant.

To obtain a composite measure of hatchling size, we performed a principal Component Analysis (PCA) using all available linear measurements of size and mass at all ages. PC1 explained 82.8% of the variation in hatchling size and mass and it was used as the composite measure of hatchling size in follow up tests (hereafter referred to as PC1-size). We analyzed the effects of temperature on size for 1 d-old hatchlings, using mean PC1-size per incubator. Egg volume (calculated as the volume of a prolate ellipsoid using egg length and width) was measured only in 2016 and thus was excluded in this analysis. Egg volume was measured because egg size represents a maternal effect that impacts growth in reptiles (Rhen and Lang, 1995; Valenzuela, 2001a, 2001b; Zhu et al., 2006; Ceballos et al., 2011, 2014). For 2016 data, we tested

for the maternal effect of egg size by first performing a linear regression with egg volume and nest as predictor variables and hatchling PC1-size as the response variable. Subsequently, we calculated the mean of the residuals of this regression per incubator and used this value as the response variable to analyze the effects of temperature, while controlling for maternal effects.

To evaluate long-term effects of temperature on hatchling size, we performed a phenotypic trajectory analysis (PTA) using function “trajectory.analysis” in package “geomorph” (Adams and Collyer, 2009; Adams and Otárola-Castillo, 2013). This analysis uses a MANOVA test to evaluate differences in the trajectory of phenotypic traits (hatchling SCL, SCW, PL, FFW, head width and mass) among groups (incubation temperatures) over time (over 1, 8, 15, 30, 45, 60, 90 days). Differences in the length, angle, and shape of the phenotypic trajectories indicate, respectively, differences in the magnitude of change among groups, the overall direction of change from the onset to the end of the experimental period, and variation in the direction of change through more than two time points (Adams and Collyer, 2009). The significance was tested using 100,000 permutations. To further evaluate if the temperature effect on size persisted after three months of age, we extracted PC1 from the trajectory analysis — which explained 95.2% of the variation in hatchling size — for 1 and 90 day-olds hatchlings and performed a regression analysis between temperature and mean PC1 (hereafter referred to as PC1-PTA).

We also evaluated the effect of temperature on SCL growth and mass gain at 30, 60 and 90 days by performing a linear regression using mean temperature as the independent variable and the difference between SCL (growth) and mass (mass gain) at 30, 60 and 90 day-olds and 1-day-old. For all regression analyses, we first tested for a polynomial effect of temperature, since previous studies showed that intermediate temperatures produced more and larger hatchlings than extreme values (Packard and Packard, 1988). If the polynomial effect was not significant, we used a linear model. Bonferroni correction was used to adjust p-values for multiple comparisons.

## 3. Results

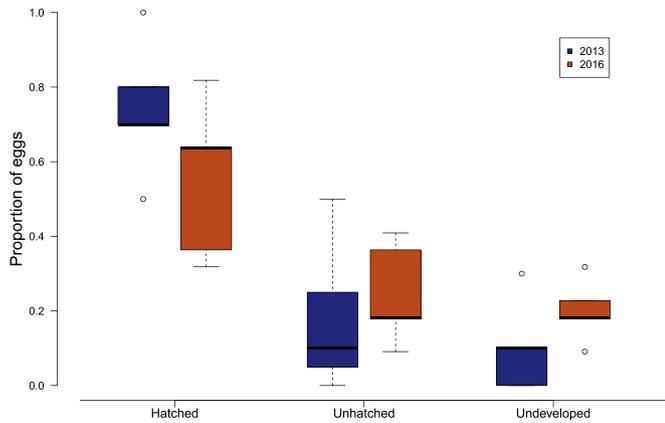
Mean incubation temperatures influenced sex ratio, incubation duration, hatching success, hatchling size, and post-hatching growth and mass gain of *Podocnemis sextuberculata*. Of the 212 eggs we incubated, 115 hatched (54.3%), 59 contained dead embryos in different stages of development (27.8%), and 38 lacked obvious embryonic development (17.9%). We tested for a year effect by comparing values for incubators with similar temperatures (i.e. excluding 37.37 °C in 2013 and 25.75 °C in 2016), and although hatching success appeared lower and both proportion of dead embryos and undeveloped eggs was higher in 2016 (Fig. 1), differences between years were not significant (Mann-Whitney U test:  $p > 0.05$ ).

### 3.1. Pattern of TSD

We used two approaches to estimate the TSD pattern, and both support the existence of a TSD Ia in this turtle species (i.e. males produced at low temperatures and females at high temperatures), although the specific details of the reaction norm varied between models. First, the GLM model estimated the pivotal value of 33.91 °C, and a 5% transitional range of temperatures (TRT) of 0.37 °C (Fig. 2A). Although the best fit model (lowest AIC) was logistic, the goodness of fit for all tested models was 1.0 because only one temperature produced both sexes (34.01 °C in 2016). Second, the MH-MCMC with 100,000 iterations estimated a mean pivotal temperature of  $33.73 \pm 0.15$  °C and a TRT of  $1.16 \pm 0.59$  °C (Fig. 2B).

### 3.2. Thermal effects on embryonic and post-hatching development

Mean temperature explained 92% of the variation in incubation



**Fig 1.** Proportion of unhatched (dead embryos), undeveloped and hatched eggs for both years (2013 and 2016). Boxplots and whiskers represent the five percentiles: 0, 0.25, median (black line) 0.75 and 1. Open circles represent outliers. The data presented are just for incubators with a mean temperature range similar in both experiments (ranging from 27.9°C to 36°C).

duration, whereas temperature and year (2013 or 2016) together explained 99% of the variation (Table 1). Mean incubation duration declined with increasing temperature but began to increase slightly at the highest temperature treatments (Fig. 3A).

Mean incubation temperature strongly affected hatching success, explaining 85% of its variation (Table 1). The incubator with a 25.75 °C mean produced no hatchlings, while the incubator with a 37.37 °C mean yielded just 2 hatchlings from 7 eggs (3 were classified as undeveloped), one of which died 11 days after emergence. The model fitted to our data predicts a hatching success below 10% for 26.25 °C and 38.5 °C, and the highest hatching rate at 32.25 °C (Fig. 3B). The optimum temperature and tolerance limits varied slightly (but not significantly) between years.

Mean incubation temperature alone explained 40% of the variation in hatchling size, and 69% when combined with year (Table 1). The mixed model fitted to our data shows that temperature has a bell-shaped effect on hatchling size, and that 31.5 °C (a masculinizing temperature – Fig. 2A) enhanced hatchling size the most (Fig. 3C). But it should be noted that egg volume is highly correlated with hatchling size and both are influenced by female size (Camillo et al., unpublished data). Since each incubator received one (in 2013) or two (in 2016) eggs from each female, this explains the high standard deviation observed in the mean hatchling size for each incubator.

As previously noted, we measured eggs only in 2016. For this year, we analyzed the combined effect of egg volume and nest identity (which reflect maternal effects) on hatchling PC1-size, and then analyzed the effect of mean temperature on the residuals of the first regression (Table 1). Egg volume and nest identity explained 88% of the variation in hatchling PC1-size. When fitting a regression between temperature and the regression residuals of PC1-size versus egg volume and nest combined, temperature had a significant, polynomial effect, like that

observed in the mixed model and for the 2013 data (Fig. 3C), explaining 98% of the variation in mean residuals (Fig. 3D).

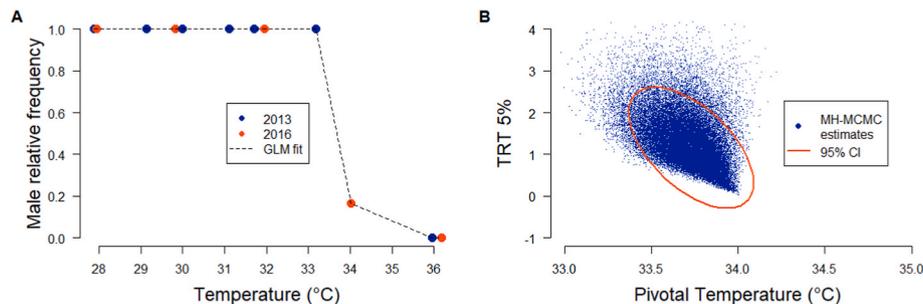
Survival was high for hatchlings raised for 90 days in 2013, with just two hatchlings dying, one at 11 days of age (from the highest temperature, 37.37 °C), and one at 7 days (from 29.14 °C). Temperature had no effect on hatchling growth trajectory overall, however, pairwise comparisons revealed differences in trajectory orientation (angle) between the lowest (27.89 °C), and highest (37.37 °C), temperatures when compared to all others (Fig. 4). It should be noted that only one of the two individuals that hatched from 37.37 °C survived, such that variance cannot be estimated for this treatment. However, comparisons of means are possible for sequential trajectories, such as the growth trajectories reported here. Shape differences were also detected between the lowest temperature trajectory and all but the highest temperatures, revealing distinct patterns of growth over more than 2 time points.

Mean temperature affected hatchling size (PC1-PTA) at emergence (1-day old), with 32.75 °C producing the largest hatchlings on average (Fig. 5A, Table 1), but these differences disappear after 90 days ( $p = 0.13$  after Bonferroni correction). The smallest hatchling was produced at 37.37 °C, and remained the smallest throughout the study. Analyses of the effects of temperature on SCL growth and mass gain confirm this pattern of effects of temperature on long-term hatchling size and on size at emergence. Hatchlings from the lower temperatures grew faster in SCL during the first two months, and gained more mass by their second and third month of life than hatchlings from intermediate and higher temperatures (Fig. 5B and C, Table 1). Standard deviations of mean SCL, mean mass, mean PC1-size, mean growth and mean mass gain for each temperature increased with age, such that differences in the mean values for these variables among treatments (Figs. 4 and 5) became non-significant by three months of age.

#### 4. Discussion

An expanded understanding of the remarkable evolution of sex determination in vertebrates (Bachtrog et al., 2014) is emerging from a growing number of studies in non-model organisms, which is challenging classical paradigms (Kratohvil et al., 2021). Yet, information about understudied taxa is much needed to fill existing knowledge gaps (Stöck et al., 2021). Little is known about the thermal biology of the vulnerable six-tubercled turtle, *Podocnemis sextuberculata*, in relation to embryonic development, sex determination, and early growth post-hatching, and our study helps fill this gap.

While limited field data from natural nests of *P. sextuberculata* and the prevalence of TSD in all other *Podocnemis* species examined indicated that incubation temperature was the likely driver of sex determination in six-tubercled turtles (Alho et al., 1985; Souza and Vogt, 1994; Pezzuti, 1998; Vogt, 2008; Páez et al., 2009; Carneiro, 2012), our results provide the first empirical confirmation of TSD in *P. sextuberculata*. Our data provide the first characterization of the TSD reaction norm in this species, and the first estimates of its pivotal (Tpiv) and transitional range temperatures (TRT). Our Tpiv estimates for this



**Fig 2.** Temperature sex determination in Six-tubercled Amazon River turtles. A) Relative male frequency recorded in 2013 and 2016 and fitted line from the logistic model. B) Pivotal temperatures and transitional range temperatures for MH-MCMC (100,000 iterations).

**Table 1**

ANOVA results for the tested models: influence of mean incubation temperature on hatching success, mean incubation duration, mean hatchling size PC1, hatching size over a 90 days period, and hatchling mean growth and mass gain at 30, 60 and 90 day-olds. Exp: experiment (2013 and 2016), Nd(Df): null deviance (degrees of freedom), Rd(Df): residual deviance (degrees of freedom).

Response variable	Predictor variable(s)	Analysis	Statistics	p-value	R <sup>2</sup>
<b>TESTS OF 2013 + 2016 DATA</b>					
Hatching success	MeanT + MeanT <sup>2</sup>	GLM	Nd(13) = 70.94 Rd(11) = 15.19	0.03	0.85 <sup>d</sup>
Incubation duration	MeanT + MeanT <sup>2</sup> + Year	LMER	F <sub>2,11</sub> = 549.33	<0.001 <sup>b</sup>	0.92/ 0.99 <sup>c</sup>
PC1-size at emergence (day 0)	MeanT + MeanT <sup>2</sup> + Year	LMER	F <sub>2,11</sub> = 7.82	0.001 <sup>b</sup>	0.40/ 0.69 <sup>c</sup>
<b>TESTS OF 2016 DATA ALONE</b>					
PC1-size	Egg volume + nest ID	LM	F <sub>20,40</sub> = 23.75	<0.001	0.88
Residuals of PC1-size by (egg volume + nest ID) regression	MeanT + MeanT <sup>2</sup>	LM	F <sub>2,2</sub> = 131.5	0.008	0.98
<b>TESTS OF 2013 DATA ALONE</b>					
Growth trajectories	MeanT	PTA	F <sub>7,44</sub> = 1.43	0.17	
PC1-PTA to Day 1	MeanT + MeanT <sup>2</sup>	LM	F <sub>2,5</sub> = 31.6	0.01 <sup>d</sup>	0.9
PC1-PTA to Day 90	MeanT + MeanT <sup>2</sup>	LM	F <sub>2,5</sub> = 10.59	0.13 <sup>d</sup>	
SCL Growth to Day 30	MeanT + MeanT <sup>2</sup>	LM	F <sub>2,5</sub> = 80.24	0.001 <sup>d</sup>	0.96
SCL Growth to Day 60	MeanT	LM	F <sub>1,6</sub> = 19.77	0.03 <sup>d</sup>	0.73
SCL Growth to Day 90	MeanT	LM	F <sub>1,6</sub> = 6.66	0.33 <sup>d</sup>	
Mass Gain to Day 30	MeanT	LM	F <sub>1,6</sub> = 8.61	0.21 <sup>d</sup>	
Mass Gain to Day 60	MeanT	LM	F <sub>1,6</sub> = 16.32	0.05 <sup>d</sup>	0.69
Mass Gain to Day 90	MeanT + MeanT <sup>2</sup>	LM	F <sub>1,6</sub> = 31.33	0.01 <sup>d</sup>	0.81

<sup>a</sup> Adjusted, estimated using package “rsq” (Zhang 2018).

<sup>b</sup> Estimated from a normal distribution of t-values.

<sup>c</sup> Estimated using package “piecewiseSEM”: Marginal R<sup>2</sup> (proportion of variance explained by the fixed effect factor alone)/Conditional R<sup>2</sup> (proportion of variance explained by both fixed and random effects (Lefcheck, 2015)).

<sup>d</sup> Adjusted for multiple comparisons using Bonferroni correction method.

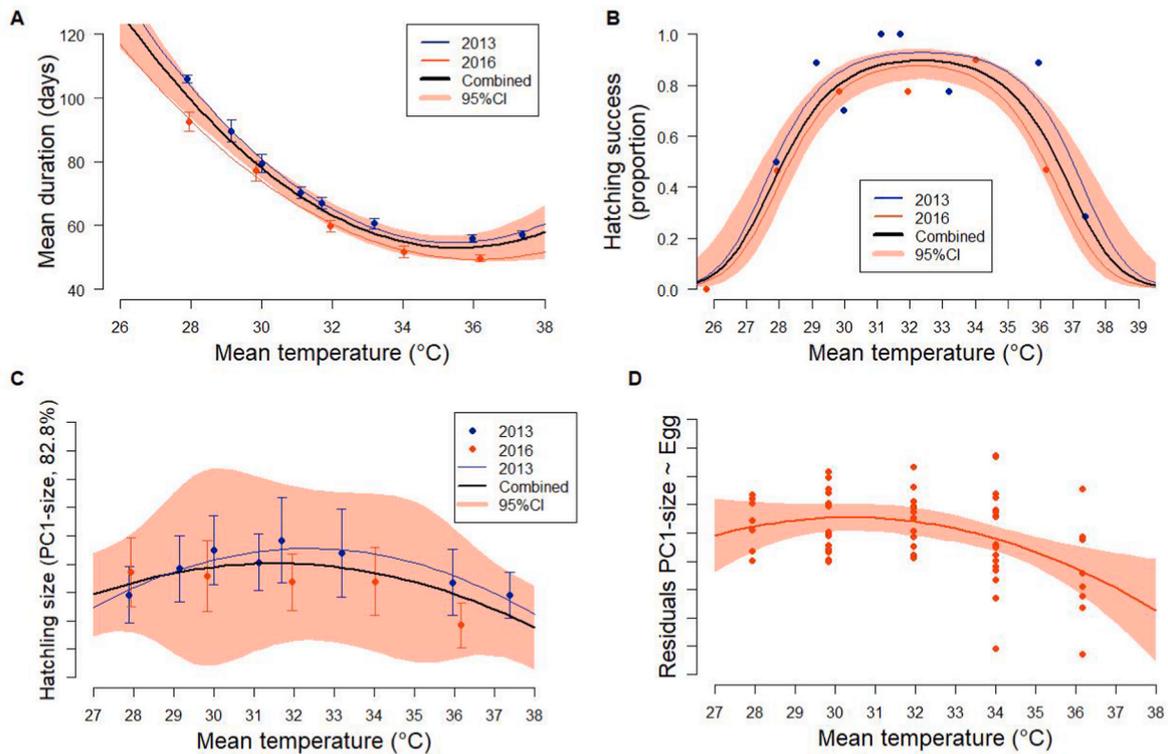
population (33.73 ± 0.15 °C or 33.91 °C) for *P. sextuberculata* represent the highest T<sub>piv</sub> ever documented for any turtle species, followed by two other Podocnemididae: *P. expansa*'s 32.6 °C (Valenzuela, 2001a) and *P. lewyana*'s 33.23–33.37 °C (Gallego-García and Páez, 2016). Further, *P. sextuberculata*'s TRT from the study population (1.16 ± 0.59 °C) is fairly narrow — 8th narrowest out of 32 species reported (Hulin et al., 2009) and the narrowest among *Podocnemis* species reported (*P. expansa* = 4.86 °C; *P. unifilis* = 6.84 °C). The narrowest TRT (0.08 °C) overall for chelonians was reported in leatherback sea turtles (*Dermochelys coriacea*) from French Guiana and Suriname, and the second (0.36 °C) in the pancake tortoise (*Malacochersus tornieri*) (Hulin et al., 2009).

In our study area, six-tubercled turtles build relatively shallow nests (20.1 ± 3.3 cm) on open sandbars which may experience highly variable temperatures from 22.6 °C to 44.2°, although mean temperatures rarely exceeded 33 °C (C.S. Camillo, unpublished data). Thus, high pivotal temperatures may be adaptive to ensure the production of both sexes. However, the benefit of such high pivotal temperature under climate change may be reduced by the narrow TRT observed here, which reduces the proportion of nests that produce mixed sex ratios, where genetic variation or maternal effects that permit adaptation of the TSD

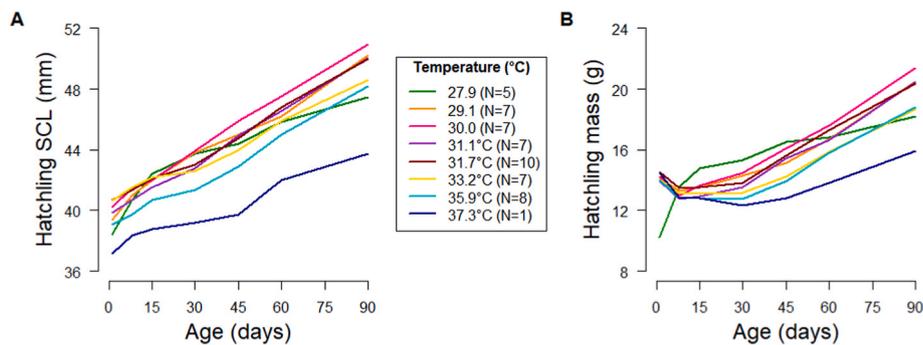
system are more likely expressed (Hulin et al., 2009). Thus, *P. sextuberculata* may be at higher risk of extinction in the face of global warming than given solely by its high pivotal temperature. Natural sex ratios in *P. sextuberculata* are affected by year-to-year variation in ambient temperatures and can be highly biased. Indeed, Pezzuti (1998) recorded a sex ratio of 0.97 males (N = 40 nests) in the Mamiraua Reserve in 1996, while Carneiro (2012) recorded 0.34 males in 2007, and 0.06 males in 2008 and 2010 in the Xingu River (N = 10, 9, and 8 nests respectively), although the sampling of only 5 hatchlings per clutch in the later study renders these estimates less reliable.

Temperature extremes can cause abnormal development and increased embryo mortality in reptiles and there is typically an intermediate optimum which produces higher hatching success (reviewed by Packard and Packard, 1988, but see also Burger, 1991; Du and Ji, 2003; Les et al., 2009; Valenzuela et al., 2019). Consistently, our data support the notion of the existence in *P. sextuberculata* of a viable range of temperatures (that induce a developmental rate >0) spanning from >26 °C to <38 °C, and a narrower optimal range of temperatures spanning >28 °C and <37 °C. Indeed, intermediate temperatures produced more and larger hatchlings in our experiment, in agreement with results from multiple reptiles (Gutzke and Packard, 1987; Packard et al., 1987; Burger, 1991; McKnight and Gutzke, 1993; Bobyn and Brooks, 1994; Allsteadt and Lang, 1995; Du and Ji, 2003; Páez et al., 2009; Ceballos et al., 2014), but opposite to *P. expansa* (Valenzuela, 2001b), indicating evolutionary divergence among these congeners. The shortening of the incubation period with increasing temperatures has also been widely documented (e.g., Yntema, 1978; Packard et al., 1987; Gutzke and Packard, 1987; Burger, 1991; Páez et al., 2009). Warmer temperatures accelerate reptilian embryonic differentiation, growth rates, and the pace at which albumen and yolk are consumed, such that larger hatchlings are produced at warmer temperatures (Ferguson and Joanen, 1983; Gutzke and Packard, 1987; Gutzke et al., 1987; Deeming and Ferguson, 1989; Allsteadt and Lang, 1995). But importantly, studies that report a linear relationship between temperature and hatching success or hatchling size probably failed to test extreme temperatures for those species (Packard et al., 1987; Brooks et al., 1991; Bobyn and Brooks, 1994; Páez and Bock, 2004). In fact, most of those studies also found that hatchlings from intermediate temperatures grew faster than those from extreme temperatures (e.g. Gutzke and Packard, 1987; Gutzke et al., 1987; McKnight and Gutzke, 1993), explained by the fact that hatchlings from lower temperatures would have less residual yolk than those from warmer temperatures, due to the longer incubation duration that requires more yolk to be metabolized before hatching (Gutzke and Packard, 1987). We found that hatchlings from the intermediate temperatures used in our experiments hatched larger and grew less in the first 30 days of life than hatchlings from extreme temperatures (which were smaller at hatching). Our findings contradict the pattern seen in the congeneric *P. expansa*, where intermediate temperatures produced smaller hatchlings than extreme temperatures, but they coincide in that smaller individuals at hatching grew more than larger ones, in a compensatory growth pattern (Valenzuela, 2001b). At the highest temperatures we used though, near the upper tolerance limit for *P. sextuberculata*, hatchlings emerged smaller and grew less than hatchlings from intermediate and lower temperatures, suggesting that metabolism was so accelerated that embryos used yolk without efficiently converting it to tissue (Packard and Packard, 1988). O'Steen (1998) found that warmer incubation temperatures slowed down growth rates of snapping turtles (*Chelydra serpentina*), similar to our results, except that such a negative relationship was caused by behavioral differences in selection of subsequent thermal environments by hatchlings. Similar effects remain unevaluated in *P. sextuberculata*.

Importantly, some of the temperature effects on hatchling size we observed in early life of *P. sextuberculata* were transient, such that when hatchlings reached 90 days of age any size differences among treatments were not significant. Similar compensatory responses were observed in *P. expansa* (Valenzuela, 2001b). Results from other studies analyzing



**Fig 3.** Relationship between mean incubation temperature and A) mean incubation duration until emergence (note that only 2 eggs hatched from 37.37°C — see text for details); B) hatching success; C) size of 1-day old hatchlings; and D) the residuals of the regression between egg volume and hatchling size, for *Podocnemis sextuberculata* turtles. A linear model was fitted for each year separately (2013 and 2016) and for both years combined. Dots represent means and bars  $\pm 1$ SD for each incubator.



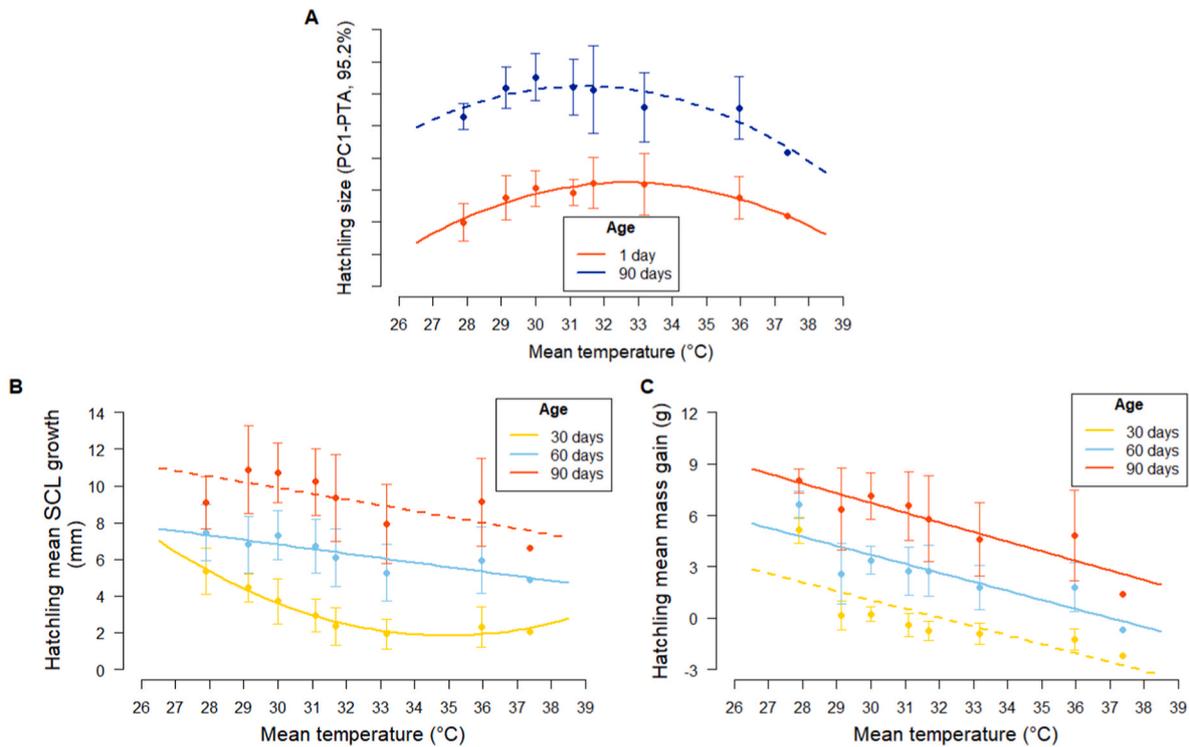
**Fig 4.** A) SCL growth and B) mass gain curves over 90 days after emergence, for hatchling of Six-tubercled Amazon River turtle incubated at eight different temperatures, in 2013. A) mean SCL growth and B) mean mass gain.

long-term effects of temperature on hatchling traits in reptiles are equivocal. Some data from lizards are congruent with the results presented here (e.g., Elphick and Shine, 1998), while others reveal a long-term effect of incubation temperature on hatchling phenotype in *Chelydra serpentina* (e.g. Brooks et al., 1991; Bobyn and Brooks, 1994; McKnight and Gutzke, 1993), a turtle with significant and sexually-dimorphic plasticity in post-hatching growth (Ceballos and Valenzuela, 2011).

Our analysis of the effect of egg size (a maternal effect measured only in 2016) revealed that egg size explained 65% of variation in hatchling size, and that the effect of temperature on hatchling size for this year was significant only when egg size was accounted for in the analysis. Females can affect hatchling size by influencing egg size and amount and composition of yolk reserves (Congdon, 1989). Additionally, the deposition of yolk steroids can alter sex ratios in TSD turtles (Bowden et al., 2000). Indeed, when accounted for, maternal effects had a greater effect

on hatchling phenotypes than any environmental variable analyzed in other reptiles (e.g. Brooks et al., 1991; Allsteadt and Lang, 1995; Bobyn and Brooks, 1994; O'Steen, 1998; Rhen and Lang, 1998; Tucker et al., 1998; Páez et al., 2009, 2015). We observed an increase in the standard deviation of hatchling SCL growth and mass gain for each incubator through time in both years, but whether these enhanced individual differences with age reflect genetic variation or maternal effects could not be disentangled in our study.

We observed differences between the experiments in 2013 and 2016, regarding incubation duration, hatching success, and the relationship between temperature and hatchling size. While the exact causes are unknown, such year effect could be due to differences in water potential, fungal infections, or the time span between collecting eggs and placing them in the incubators. We note that even in studies that maintained incubation conditions constant among years, high embryonic mortality is sometimes observed in certain years from unknown causes



**Fig 5.** Long-term effects of mean incubation temperature on *Podocnemis sextuberculata* hatchling growth and mass gain. Relationship between mean incubation temperature and A) hatchling PC1-PTA, which represents 95.2% of the variation in size and mass, at 1 and 90 day-olds, B) hatchling mean SCL growth and C) mass gain over 30, 60 and 90 days after hatching. Points represent means and bars  $\pm$  1SD, solid lines are significant regressions after Bonferroni correction ( $p \leq 0.05$ ) and dashed lines are nonsignificant regressions.

(Valenzuela et al., 2019).

Thermal reaction norms (tolerance limits, optimum temperature range, pivotal temperatures and transitional range temperature) are heritable traits that can evolve among populations and species (Bull et al., 1982; Mrosovsky and Pieau, 1991; Ewert et al., 1994, 2005; Rhen and Lang, 1998; Páez et al., 2009, 2015; Gallego-García and Páez, 2016; Gómez-Saldarriaga et al., 2016). The estimates we presented here for *P. sextuberculata* provide a baseline to guide the design of future experiments to refine estimates of pivotal temperature and TRT under constant temperature, and the reaction norms in natural nests, which can vary from those observed in the lab (Mrosovsky, 1994) and remain unknown in this species. Intriguingly, temperatures that produce larger hatchlings and induced faster growth by the third month of life in *P. sextuberculata* produce males (the smaller sex in this species). At first glance, these observations appear to counter to expectations from the Charnov and Bull (1977) model for adaptive evolution of sex determination, if size at emergence or growth rates during the first trimester of life were assumed to be linked to life-time differential fitness. But because differences disappeared by the third month of life, further research is needed to test this model conclusively, with longer-term data on differential growth rates, age and size at maturity between males and females, which may be the variables under selection in this species. In contrast, the enhanced size and growth of males relative to females observed here, at least in early life, are consistent with previous findings, that *P. sextuberculata* evolved a less pronounced female-larger sexual size dimorphism than present in their ancestor, via the stronger reduction of female body size than of male body size compared to ancestral estimates for this lineage (Ceballos et al., 2013).

Six-tubercled turtles are a vulnerable species, threatened by growing human consumption and illegal trade. Human harvest is asymmetrical with greater pressure on females than males because they are larger, may be gravid (eggs are also highly valued), and are easy to capture when nesting on land. Future studies that assess population dynamics

directly for this species must use analytical methods to account for the difficulty that river levels affect capture rates, and consequently, the estimates of sex ratios and age structure of populations. However, studies conducted so far point to male-biased sex ratios in two populations in the Brazilian Amazon (Fachín-Terán et al., 2003; Perrone, 2012; Araújo, 2017), but it is uncertain whether this is a consequence of greater hunting pressure on females, of biased offspring sex ratios, of differential sampling rates, or a combination of these factors. Finally, climate change poses another threat to the survival of *P. sextuberculata*, since its narrow TRT suggest that the species is less likely to adapt to new thermal conditions (Hulin et al., 2009). Further research on thermal reaction norms and long-term effects of temperatures on hatchling phenotype and fitness, combined with genetic population studies as done for other congeners (Bock et al., 2001; Valenzuela, 2001c; Pearce et al., 2006; Escalona et al., 2009; Vargas-Ramirez et al., 2012), are needed to inform management decisions.

## 5. Conclusions

Ours is the first study of the effect of semi-constant incubation temperature on hatchling phenotypes of *P. sextuberculata*, including the formal characterization of its pattern of TSD. The pivotal temperature reported here ( $33.73 \pm 0.15$  °C) is the highest ever documented for a turtle species and the transitional range temperature of  $1.16 \pm 0.59$  °C is relatively narrow, which may limit the evolutionary potential of this vulnerable turtle in the face of global warming. Constant temperatures below 26 °C and above 38 °C are lethal to this species. Intermediate temperatures are optimal for hatching success (32.25 °C and 31.5 °C, respectively) and produce larger hatchlings that grow slower during the first month of life than the smaller hatchlings produced under colder or warmer conditions (although size and mass differences disappeared by 3 months of age).

Future lab experiments should more closely examine temperatures

between 33 °C and 36 °C to refine the reaction norm described here. Field studies that monitor natural nests are essential to understand how male and female *P. sextuberculata* develop in the wild, and to help evaluate conservation measures that consider female-biased illegal hunting pressure and global warming threats.

### Ethics approval and permits

This research was carried out in accordance with codes of ethics for animal experiments and was approved by the animal ethics committee at the Mamiraua Institute (CEP #002/2014) and at the University of Florida (IACUC #201609490). The Chico Mendes Institute for Biodiversity Conservation provided permits for data and sample collection (SISBIO #19898, #28805, #47078, #54614), while the Brazilian Institute of the Environment and Renewable Natural Resources/CITES and the US Fish and Wildlife Services (#2017995120) provided permits for sample export/import to the USA (#16BR022350/DF).

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### CRediT authorship contribution statement

**Cassia S. Camillo:** Conceptualization, Methodology, Data curation, Visualization, Investigation, Formal analysis, Writing – original draft, Funding acquisition. **Nicole Valenzuela:** Validation, Writing – review & editing. **Steve A. Johnson:** Supervision, Funding acquisition, Writing – review & editing.

### Declaration of competing interest

None.

### Data availability

Data has been made available in Dryad: <https://doi.org/10.5061/dryad.stjq2c63>

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtherbio.2022.103292>.

### References

- Adams, D.C., Collyer, M.L., 2009. A general framework for the analysis of phenotypic trajectories in evolutionary studies. *Evolution* 63, 1143–1154.
- Adams, D.C., Otárola-Castillo, E., 2013. Geomorph: an R package for the collection and analysis of geometric morphometric shape data. *Methods Ecol. Evol.* 4, 393–399.
- Alho, C.J.R., Danni, T.M.S., Padua, L.F.M., 1985. Temperature-dependent sex determination in *Podocnemis expansa* (Testudinata: Pelomedusidae). *Biotropica* 17, 75–78.
- Allsteadt, J., Lang, J.W., 1995. Incubation temperature affects body size and energy reserves of hatchling American Alligators (*Alligator mississippiensis*). *Physiol. Zool.* 68, 76–97.
- Araújo, C.G., 2017. Estrutura populacional de *Podocnemis sextuberculata* Cornalia, 1849 (Testudines: Podocnemididae) na Reserva de Desenvolvimento Sustentável Mamirauá, Amazonas, Brasil. Instituto Nacional de Pesquisas da Amazônia, Manaus, Brasil. M.S. thesis.
- Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T.-L., et al., 2014. Sex determination: why so many ways of doing it? *PLoS Biol.* 12, e1001899. <https://doi.org/10.1371/journal.pbio.1001899>.
- Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Software* 67, 1–48. <https://doi.org/10.18637/jss.v067.i01>.
- Bernardo, J., 1996. Maternal effects in animal ecology. *Am. Zool.* 36, 83–105.
- Bernhard, R., 2001. Biologia reprodutiva de *Podocnemis sextuberculata* (Testudines, Pelomedusidae) na Reserva de Desenvolvimento Sustentável Mamirauá, Amazonas, Brasil. Instituto Nacional de Pesquisas da Amazônia, Manaus, Brasil. M.S. thesis.
- Bista, B., Valenzuela, N., 2020. Turtle insights into the evolution of the reptilian karyotype and the genomic architecture of sex determination. *Genes* 11, 416.
- Bobyn, M.L., Brooks, R.J., 1994. Interclutch and interpopulation variation in the effects of incubation conditions on sex, survival and growth of hatchlings turtles (*Chelydra serpentina*). *J. Zool.* 233, 233–257.
- Bock, B.C., Páez, V.P., White, M.M., 2001. Genetic population structure of two threatened South American River Turtle species, *Podocnemis expansa* and *Podocnemis unifilis*. *Chelonian Conserv. Biol.* 4, 47–52.
- Booth, D.T., 2018. Incubation temperature induced phenotypic plasticity in oviparous reptiles: where to next? *J. Exp. Zool.* 329, 343–350. <https://doi.org/10.1002/jez.2195>.
- Bowden, R.M., Ewert, M.A., Nelson, C.E., 2000. Environmental sex determination in a reptile varies seasonally and with yolk hormones. *Proc. Biol. Sci.* 267, 1745–1749.
- Brooks, R.J., Bobyn, M.L., Galbraith, D.A., Layfield, J.A., Nancekivell, E.G., 1991. Maternal and environmental influences on growth and survival of embryonic and hatching Snapping Turtles (*Chelydra serpentina*). *Can. J. Zool.* 69, 2667–2676.
- Bull, J.J., 1980. Sex determination in reptiles. *Q. Rev. Biol.* 55, 3–21.
- Bull, J.J., Vogt, R.C., Bulmer, M.G., 1982. Heritability of sex ratio in turtles with environmental sex determination. *Evolution* 36, 333–341.
- Burger, J., 1991. Effects of incubation temperature on behavior of hatchling Pine snakes: implications for reptilian distribution. *Behav. Ecol. Sociobiol.* 28, 297–303.
- Camillo, C.S., Santos, O.M., Sousa, I.S., Queiroz, H.L., 2012. Community-based freshwater turtle conservation in middle Solimões River, AM, Brazil. *Uakari* 8, 33–44.
- Carneiro, C.C., 2012. Influência do meio ambiente de nidificação sobre a taxa de eclosão, a duração da incubação e a determinação sexual em *Podocnemis* (Reptilia, Podocnemididae) no tabuleiro do Embaubal, Rio Xingu, Pará. Universidade Federal do Pará, Belem, Brazil. M.S. thesis.
- Ceballos, C.P., Valenzuela, N., 2011. The role of sex-specific plasticity in shaping sexual dimorphism in a long-lived vertebrate, the snapping turtle *Chelydra serpentina*. *Evol. Biol.* 38, 163–181.
- Ceballos, C.P., Adams, D.C., Iverson, J.B., Valenzuela, N., 2013. Phylogenetic patterns of sexual size dimorphism in turtles and their implications for Rensch’s rule. *Evol. Biol.* 40, 194–208.
- Ceballos, C.P., Hernández, O.E., Valenzuela, N., 2014. Divergent sex-specific plasticity in long-lived vertebrates with contrasting sexual dimorphism. *Evol. Biol.* 41, 81–98.
- CFMV. Conselho Federal de Medicina Veterinária, 2012. Resolução no1000, de 11 de maio de 2012. CFMV. [http://www.cfmv.org.br/portal/legislacao/resolucoes/resolucao\\_1000.pdf](http://www.cfmv.org.br/portal/legislacao/resolucoes/resolucao_1000.pdf).
- Charnov, E.L., Bull, J.J., 1977. When is sex environmentally determined? *Nature* 266, 828–830.
- Congdon, J.D., 1989. Proximate and evolutionary constraints on energy relations of reptiles. *Physiol. Zool.* 62, 356–373.
- R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- Deeming, D.C., Ferguson, M.W.J., 1989. Effects of incubation temperature on growth and development of embryos of *Alligator mississippiensis*. *J. Comp. Physiol. B* 159, 183–193.
- Du, W.G., Ji, X., 2003. The effects of incubation thermal environments on size, locomotor performance and early growth of hatchling soft-shelled turtles, *Pelodiscus sinensis*. *J. Therm. Biol.* 28, 279–286.
- Elphick, M.J., Shine, R., 1998. Longterm effect of incubation temperatures on the morphology and locomotor performance of hatchling lizards. *Biol. J. Linn. Soc.* 63, 429–447.

- Escalona, T., Engstrom, T.N., Hernandez, O.E., Bock, B.C., Vogt, R.C., Valenzuela, N., 2009. Population genetics of the endangered South American freshwater turtle, *Podocnemis unifilis*, inferred from microsatellite DNA data. *Conserv. Genet.* 10, 1683–1696.
- Ewert, M.A., Nelson, C.E., 1991. Sex determination in turtles: diverse patterns and some possible adaptive values. *Copeia* 50–69, 1991.
- Ewert, M.A., Jackson, D.R., Nelson, C.E., 1994. Temperature-dependent sex determination in turtles. *J. Exp. Zool.* 270, 3–15.
- Ewert, M.A., Lang, J.W., Nelson, C.E., 2005. Geographic variation in the pattern of temperature-dependent sex determination in the American Snapping Turtle (*Chelydra serpentina*). *J. Zool.* 265, 81–95.
- Fachin-Terán, A., Vogt, R.C., Thorbjarnarson, J.B., 2003. Estrutura populacional, razão sexual e abundância de *Podocnemis sextuberculata* (Testudines, Podocnemididae) na Reserva de Desenvolvimento Sustentável Mamirauá, Amazonas, Brasil. *Phyllomedusa* 2, 43–63.
- Fachin-Terán, A., Vogt, R.C., Thorbjarnarson, J.B., 2004. Patterns of use and hunting of turtles in the Mamirauá Sustainable development reserve, Amazonas, Brazil. In: Silvius, K.M., Bodmer, R.E., Fragoso, J.M.V. (Eds.), *People in Nature: Wildlife Conservation in South and Central America*. Columbia University Press, New York, pp. 362–377.
- Fachin-Terán, A., Vogt, R.C., Thorbjarnarson, J.B., 2006. Seasonal movements of *Podocnemis sextuberculata* (Testudines: Podocnemididae) in the Mamirauá Sustainable development reserve, Amazonas, Brazil. *Chelonian Conserv. Biol.* 5, 18–24.
- Ferguson, M., Joanen, T., 1983. Temperature-dependent sex determination in *Alligator mississippiensis*. *J. Zool.* 200, 143–177.
- Gallego-García, N., Páez, V.P., 2016. Geographic variation in sex determination patterns in the river turtle *Podocnemis lewyana*: implications for global warming. *J. Herpetol.* 50, 256–262.
- Girondot, M., 1999. Statistical description of temperature-dependent sex determination using maximum likelihood. *Evol. Ecol. Res.* 1, 479–486.
- Girondot, M., Monsinjon, J., Guillon, J.M., 2018. Delimitation of the embryonic thermosensitive period for sex determination using an embryo growth model reveals a potential bias for sex ratio prediction in turtles. *J. Therm. Biol.* 73, 32–40.
- Godfrey, M.H., Delmas, V., Girondot, M., 2003. Assessment of patterns of temperature-dependent sex determination using maximum likelihood model selection. *Ecoscience* 10, 265–272.
- Gómez-Saldarriaga, C., Valenzuela, N., Ceballos, C.P., 2016. Effects of incubation temperature on sex determination in the endangered Magdalena River turtle, *Podocnemis lewyana*. *Chelonian Conserv. Biol.* 15, 43–53.
- Gutzke, W.H.N., Packard, G.C., 1987. Influence of the hydric and thermal environments on eggs and hatchlings of Bull Snakes *Pituophis melanoleucus*. *Thermal Zool.* 60, 9–17.
- Gutzke, W.H.N., Packard, G.C., Packard, M.J., Boardman, T.J., 1987. Influence of the hydric and thermal environments on eggs and hatchlings of Painted Turtles (*Chrysemys picta*). *Herpetologica* 43, 393–404.
- Hulin, V., Delmas, V., Girondot, M., Godfrey, M.H., Guillon, J.M., 2009. Temperature-dependent sex determination and global change: are some species at greater risk? *Oecologia* 160, 493–506.
- Kratochvíl, L., Stöck, M., Rovatsos, M., Bullejos, M., Herpin, A., Jeffries, D.L., Peichel, C. L., Perrin, N., Valenzuela, N., Pokorná, M.J., 2021. Expanding the classical paradigm: what we have learnt from vertebrates about sex chromosome evolution. *Phil. Trans. Biol. Sci.* 376 <https://doi.org/10.1098/rstb.2020.0097>, 2020009720200097.
- Lefcheck, J.S., 2015. piecewiseSEM: Piecewise structural equation modeling in R for ecology, evolution, and systematics. *Methods Ecol. Evol.* 7, 573–579.
- Lenth, R., 2019. Emmeans: Estimated Marginal Means, Aka Least-Squares Means. R package version 1.3.2. <https://CRAN.R-project.org/package=emmeans>.
- Les, H.L., Paitz, R.T., Bowden, R.M., 2009. Living at extremes: development at the edges of viable temperature under constant and fluctuating conditions. *Physiol. Biochem. Zool.* 82, 105–112.
- Literman, R., Burrett, A., Bista, B., Valenzuela, N., 2018. Putative independent evolutionary reversals from genotypic to temperature-dependent sex determination are associated with accelerated evolution of sex-determining genes in turtles. *J. Mol. Evol.* 86, 11–26.
- Lopes, G.P., Valsecchi, J., Vieira, T.M., Amaral, P.V., Costa, E.W.M., 2012. Hunting and hunters in lowland communities in the region of the middle Solimões, Amazonas, Brazil. *Uakari* 8, 7–18.
- McKnight, C.M., Gutzke, W.H.N., 1993. Effects of the embryonic environment and of hatchling housing conditions on growth of young Snapping Turtles (*Chelydra serpentina*). *Copeia* 475–482, 1993.
- Mitchell, T.S., Janzen, F.J., Warner, D.A., 2018. Quantifying the effects of embryonic phenotypic plasticity on adult phenotypes in reptiles: a review of current knowledge and major gaps. *J. Exp. Zool.* 329, 203–214. <https://doi.org/10.1002/jez.2187>.
- Moore, M.P., Whiteman, H.H., Martin, R.A., 2019. A mother's legacy: the strength of maternal effects in animal populations. *Ecol. Lett.* 22, 1620–1628.
- Mrosovsky, N., 1980. Thermal biology of sea turtles. *Am. Zool.* 20, 531–547.
- Mrosovsky, N., 1994. Sex ratios of sea turtles. *J. Exp. Zool.* 270, 16–27.
- Mrosovsky, N., Pieau, C., 1991. Transitional range of temperature, pivotal temperatures and thermosensitive stages for sex determination in reptiles. *Amphibia-Reptilia* 12, 169–179.
- Mu, Y., Zhao, B., Tang, W., Sun, B., Zeng, Z., Valenzuela, N., Du, W., 2015. Temperature-dependent sex determination ruled out in the Chinese soft-shelled turtle (*Pelodiscus sinensis*) via molecular cytogenetics and incubation experiments across populations. *Sex. Dev.* 9, 111–117.
- Noble, D.W.A., Stenhouse, V., Schwanz, L.E., 2018. Developmental temperatures and phenotypic plasticity in reptiles: a systematic review and meta-analysis. *Biol. Rev.* 93, 72–97. <https://doi.org/10.1111/brv.12333>.
- O'Steen, S., 1998. Embryonic temperature influences juvenile temperature choice and growth rate in snapping turtles *Chelydra serpentina*. *J. Exp. Biol.* 201, 439–449.
- Packard, G.C., Packard, M.J., 1988. The physiological ecology of reptilian eggs and embryos. In: Gans, C., Huey, R.B. (Eds.), *Biology of the Reptilia*, vol. 16, pp. 523–605. *Ecology B*. Alan R. Liss, New York.
- Packard, G.C., Packard, M.J., Miller, K., Boardman, T.J., 1987. Influence of moisture, temperature, and substrate on Snapping Turtle eggs and embryos. *Ecology* 68, 983–993.
- Páez, V.P., Bock, B.C., 2004. Pre- and post-hatching factors affecting juvenile growth rates in the yellow-spotted river turtle (*Podocnemis unifilis*). *Actual. Biol.* 26, 137–151.
- Páez, V.P., Correa, J.C., Cano, A.M., Bock, B.C., 2009. A Comparison of maternal and temperature effects on sex, size, and growth of hatchlings of the Magdalena River Turtle (*Podocnemis lewyana*) incubated under field and controlled laboratory conditions. *Copeia* 698–704, 2009.
- Páez, V.P., Echeverri-G, L., Bock, B.C., Bowden, R.M., Hinestroza, L.M., 2015. Preovulatory maternal effects on intra- and interpopulation variation in sex ratios and phenotypic characteristics of Magdalena River Turtles (*Podocnemis lewyana*). *Herpetologica* 71, 196–202.
- Pantoja-Lima, J., Aride, P.H.R., de Oliveira, A.T., Félix-Silva, D., Pezzuti, J.C.B., Rebêlo, G.H., 2014. Chain of commercialization of *Podocnemis* spp. turtles (Testudines: Podocnemididae) in the Purus River, Amazon basin, Brazil: current status and perspectives. *J. Ethnobiol. Ethnomed.* 10, 8.
- Pearse, D.E., Arndt, A.D., Valenzuela, N., Miller, B.A., Cantarelli, V., Sites, J.W.J., 2006. Estimating population structure under non-equilibrium conditions in a conservation context: continent-wide population genetics of the giant Amazon river turtle *Podocnemis expansa* (Chelonia; Podocnemidae). *Mol. Ecol.* 15, 985–1006.
- Perrone, E.L., 2012. Estrutura populacional de *Podocnemis sextuberculata* Cornalia, 1849 (Testudines: Podocnemididae) na Reserva Biológica do Rio Trombetas, Pará, Brasil. Instituto Nacional de Pesquisas da Amazônia, Manaus, Brasil. M.S. thesis.
- Pezzuti, J.C.B., 1998. Reprodução da iaçá, *Podocnemis sextuberculata* (Testudines, Pelomedusidae), na Reserva de Desenvolvimento Sustentável Mamirauá, Amazonas, Brasil. Instituto Nacional de Pesquisas da Amazônia/Universidade Federal do Amazonas. M.S. thesis.
- Pezzuti, J.C.B., Vogt, R.C., 1999. Nesting ecology of *Podocnemis sextuberculata* (Testudines, Pelomedusidae) in the Japurá river, Amazonas, Brazil. *Chelonian Conserv. Biol.* 3, 419–424.
- Pokorna, M.J., Kratochvíl, L., 2016. What was the ancestral sex-determining mechanism in amniote vertebrates? *Biol. Rev.* 91, 1–13.
- Rhen, T., Lang, J.W., 1995. Phenotypic plasticity for growth in the common snapping turtle—effects of incubation, temperature, clutch and their interaction. *Am. Nat.* 146, 726–747.
- Rhen, T., Lang, J.W., 1998. Among-family variation for environmental sex determination in reptiles. *Evolution* 52, 1514–1520.
- Rhen, T., Lang, J.W., 2004. Phenotypic effects of incubation temperature in reptiles. In: Valenzuela, N., Lance, V.A. (Eds.), *Temperature Dependent Sex Determination in Vertebrates*. Smithsonian Books, Washington, DC, pp. 90–98.
- Roosenburg, W.M., 1996. Maternal condition and nest site choice: an alternative for the maintenance of environmental sex determination? *Am. Zool.* 36, 157–168.
- Sabath, N., Itescu, Y., Feldman, A., Meiri, S., Mayrose, I., Valenzuela, N., 2016. Sex determination and the birth and death of species. *Ecol. Evol.* 6, 5207–5220.
- Souza, R.R., Vogt, R.C., 1994. Incubation temperature influences sex and hatchling size in the neotropical turtle *Podocnemis unifilis*. *J. Herpetol.* 28, 453–464.
- Steyermark, A.C., Spotila, J.R., 2000. Effects of maternal identity and incubation temperature on snapping turtles (*Chelydra serpentina*) metabolism. *Physiol. Biochem. Zool.* 73, 298–306.
- Stöck, M., Kratochvíl, L., Kuhl, H., Rovatsos, M., Evans, B.J., Suh, A., Valenzuela, N., Veyrunes, F., Zhou, Q., Gamble, T., Capel, B., Schartl, M., Guiguen, Y., 2021. A brief review of vertebrate sex evolution with a pledge for integrative research: towards 'sexomics'. *Phil. Trans. Biol. Sci.* 376 <https://doi.org/10.1098/rstb.2020.0426>, 3762020042620200426.
- Topping, N.E., Valenzuela, N., 2021. Turtle Nest-Site choice, anthropogenic Challenges, and evolutionary potential for adaptation. *Front. Ecol. Evol.* 9, 808621.
- Tree of Sex Consortium, 2014. Tree of Sex: a database of sexual systems. *Sci. Data* 1. <https://doi.org/10.1038/sdata.2014.1015>.
- TTWG - Turtle Taxonomy Working Group, Rhodin, A.G.J., Iverson, J.B., Bour, R., Fritz, U., Georges, A., Shaffer, H.B., van Dijk, P.P.J., 2021. Turtles of the world: annotated checklist and atlas of taxonomy, synonymy, distribution, and conservation status. In: Rhodin, A.G.J., Iverson, J.B., van Dijk, P.P., Stanford, C.B., Goode, E.V., Buhlmann, K.A., Mittermeier, R.A. (Eds.), *Conservation Biology of Freshwater Turtles and Tortoises: A Compilation Project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group*, vol. 8. Chelonian Research Monographs, pp. 1–472. <https://doi.org/10.3854/crm.8.checklist.atlas.v9.2021>.
- Tucker, J.K., Filoramo, N.L., Paukstis, G.L., Janzen, F.J., 1998. Response of Red-Eared Slider, *Trachemys scripta elegans*, eggs to slightly differing water potentials. *J. Herpetol.* 32, 124–128.
- Valenzuela, N., 2001a. Constant, shift, and natural temperature effects on sex determination in *Podocnemis expansa* Turtles. *Ecology* 82, 3010–3024.
- Valenzuela, N., 2001b. Maternal effects on life history traits in the Amazonian giant river turtle *Podocnemis expansa*. *J. Herpetol.* 35, 368–378.
- Valenzuela, N., 2001c. Genetic differentiation among nesting beaches in the highly migratory giant river turtle (*Podocnemis expansa*) from Colombia. *Herpetologica* 57, 48–57.

- Valenzuela, N., 2021. *Podocnemis expansa* turtles hint to a unifying explanation for the evolution of temperature-dependent sex determination in long-lived and short-lived vertebrates. *Sex. Dev.* 15, 23–37.
- Valenzuela, N., Adams, D.C., 2011. Chromosome number and sex determination co-evolve in turtles. *Evolution* 65, 1808–1813.
- Valenzuela, N., Ceballos, C.P., 2012. Evolución y mecanismos de determinación sexual en tortugas. In: Paez, V.P., Morales-Betancourt, M.A., Lasso, C.L., Castaño-Mora, O. V., Bock, B.C. (Eds.), *Biología y Conservación de las Tortugas Continentales de Colombia*. Instituto Humboldt, Bogotá, Colombia, pp. 115–124.
- Valenzuela, N., Lance, V., 2004. *Sex Determination in Vertebrates*. Smithsonian Institution Press, Washington, DC.
- Valenzuela, N., Adams, D.C., Janzen, F.J., 2003. Pattern does not equal process: exactly when is sex environmentally determined? *Am. Nat.* 161, 676–683.
- Valenzuela, N., Badenhurst, D., Montiel, E.E., Literman, R., 2014. Molecular cytogenetic search for cryptic sex chromosomes in painted turtles *Chrysemys picta*. *Cytogenet. Genome Res.* 144, 39–46.
- Valenzuela, N., Literman, R., Neuwald, J.L., Mizoguchi, B.A., Iverson, J.B., Riley, J.L., Litzgus, J.D., 2019. Extreme thermal fluctuations from climate change unexpectedly accelerate demographic collapse of vertebrates with temperature-dependent sex determination. *Sci. Rep.* <https://doi.org/10.1038/s41598-019-40597-4>.
- Vargas-Ramirez, M., Stuckas, H., Castano-Mora, O.V., Fritz, U., 2012. Extremely low genetic diversity and weak population differentiation in the endangered Colombian river turtle *Podocnemis lewyana* (Testudines: Podocnemididae). *Conserv. Genet.* 13, 65–77.
- Vogt, R.C., 2008. *Amazon Turtles*. Peru, Lima.
- Yntema, C.L., 1978. Incubation times for eggs of the turtle *Chelydra serpentina* (Testudines: Chelydridae) at various temperatures. *Herpetologica* 34, 274–277.
- Zhang, D., 2018. *rsq: R-Squared and Related Measures*. R package version 1.1. <https://CRAN.R-project.org/package=rsq>.
- Zhu, X.P., Wei, C.Q., Zhao, W.H., Du, H.J., Chen, Y.L., Gui, J.F., 2006. Effects of incubation temperatures on embryonic development in the Asian yellow pond turtle. *Aquaculture* 259, 243–248.