Zoology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Zoology

journal homepage: www.elsevier.com/locate/zool

Hydric environmental effects on turtle development and sex ratio

Itzel Sifuentes-Romero*, Boris M. Tezak, Sarah L. Milton, Jeanette Wyneken

Department of Biological Sciences, Florida Atlantic University, 777 Glades Rd., Boca Raton, FL 33431-0991, USA

ARTICLE INFO

Keywords:
Trachemys scripta elegans
moisture
temperature
embryo phenotype
incubation
Sox9

ABSTRACT

Experimental and field studies of different turtle species suggest that moisture influences embryonic development and sex ratios, wetter substrates tend to produce more males, and drier substrates produce more females. In this study, we used *Trachemys scripta elegans* to test the effect of moisture on embryonic development and sex ratios. *T. s. elegans* eggs were incubated under different temperature and moisture regimes. We monitored embryonic development until stage 22 (after sex determination) and, for the first time, we estimated sex ratios using a male-specific transcriptional molecular marker, *Sox9*. Among treatments, we found differences in developmental rates, egg mass, and sex ratio. Embryos developed slowly in cooler and wetter sand substrate while water uptake by the eggs was significantly greater on wetter substrates. Developmental differences were due to moisture interacting with temperature where increased water content of the sand resulted in temperatures that were 2–3 °C lower than air temperatures. The coolest and the wettest substrates produced 100% males compared to 42% males from the warmest and driest treatment. Further, we found that embryonic growth appears to be more sensitive to temperature at earlier stages of development and to moisture at later stages. This study shows how moisture may change the incubation conditions inside nests by changing the temperature experienced by eggs, which affects development, growth and sex ratios. The results of this study highlight the importance of including moisture conditions when predicting embryo growth and sex ratios and in developing proxies of embryonic development.

1. Introduction

During development, the incubation environment, combined with the embryo's genetic program, shape the organism's phenotype (Gilbert and Epel, 2015). Several studies show that organisms' phenotypes are under the influence of genetic adaptations to past environmental conditions as well as epigenetic control of phenotypic plasticity to the current environment (Janzen and Krenz, 2004; Morgan and Mackay, 2006). In reptiles, these environmental conditions include temperature and moisture, which affect embryogenesis and the phenotype of the resulting hatchlings (Ackerman, 1997). Nest temperature affects embryonic metabolism (Ligon and Lovern, 2012), hatching success, length of incubation, hatchling body size, hatchling locomotor performance (Elphick and Shine, 1998; Du and Ji, 2003; Booth et al., 2004; Tang et al., 2012); and may indirectly affect turtle growth (Rhen and Lang, 1995; Rhen and Lang, 1999) and behavior (Vervust et al., 2011; Siviter et al., 2017).

Many oviparous reptiles, including crocodilians and the majority of turtles, lack sex chromosomes and have temperature-dependent sex determination (TSD) (Bull, 1987; Pieau et al., 1999; Merchant-Larios, 2001). In species with TSD, differentiation of gonads into ovaries or testes depends on incubation temperature during a critical period of embryonic development known as the thermosensitive period (TSP) (Pieau and Dorizzi, 2004). Following the action of temperature, a downstream network of molecular interactions directs the formation of the ovary or testis. Gene expression analysis found that some genes respond to temperature and show differential expression patterns at male and female promoting temperatures (MPT and FPT respectively) (Shoemaker and Crews, 2009; Rhen and Schroeder, 2010). Among them, *Sox9* has high expression levels toward the end of the TSP at MPT and therefore, is considered a marker of testis differentiation in sea turtles (Torres-Maldonado et al., 2002) and freshwater turtles (Shoemaker et al., 2007; Rhen et al., 2007).

In addition to temperature, substrate moisture may also influence development, particularly in turtle species with porous, parchment-shelled eggs (Packard, 1991). Higher moisture during incubation causes greater water uptake by the egg and longer incubation, and thus produces larger and heavier hatchlings (Janzen et al., 1995; Tucker et al., 1998; Delmas et al., 2008). Furthermore, moisture may play some role in sex determination. A series of experiments performed with the painted turtle (*Chrysemys picta)* suggested that, under constant incubation temperatures, wetter substrates produced males, while drier

* Corresponding author.
E-mail addresses: isifuentesromero@fau.edu (I. Sifuentes-Romero), btezak@fau.edu (B.M. Tezak), smilton@fau.edu (S.L. Milton), jwyneken@fau.edu (J. Wyneken).

https://doi.org/10.1016/j.zool.2017.11.009
Received 13 September 2017; Received in revised form 18 November 2017; Accepted 26 November 2017

substrates produced both males and females (Gutzke and Paukstis, 1983). In a subsequent study, fluctuating temperatures resulted in nearly equal sex ratios in moist substrates, whereas mostly males were produced in drier substrates (Paukstis et al., 1984). A more recent lab study showed that a daily water application to red-eared slider turtle (*Trachemys scripta)* eggs during the TSP significantly shifted sex ratios to male biases compared to controls (Leblanc and Wibbels, 2009). These differences in the effect of moisture over sex ratios, more than a difference in the response among species, are probably due to different experimental designs coupled with the tight relationship between temperature and moisture that make separating both effects difficult.

The composite effects of moisture and temperature may explain also the weak relationship between natural nest temperature and sex ratios *in situ* (e.g., freshwater species including *Graptemys* spp., Bull, 1985; *C. picta*, Bodensteiner et al., 2015; and a sea turtle *Caretta caretta*, Wyneken and Lolavar, 2015). Particularly, in the loggerhead turtle (*C. caretta)*, average nest temperatures during the TSP were compared to sampled sex ratios; loggerhead nests produced more males than expected based on nest temperatures. This trend was particularly noticeable in years with high rainfall. When the effect of increased moisture by rainfall is considered along with *in situ* nest temperatures, sex ratio trends become more predictable (Wyneken and Lolavar, 2015). This observation and laboratory experiments (Lolavar and Wyneken, 2015; Lolavar and Wyneken, 2017), suggest that moisture influences sex determination and hence sex ratios.

Despite numerous studies on the effects of incubation moisture on hatchling phenotype and performance, little is known about how moisture levels affect the developing embryo *per se*. Here, we tested the effect of moisture on embryonic development and sex ratio using the red-eared slider, which is an emerging animal model that in recent years has gained increasing attention for the study of several developmental processes (Czerwinski et al., 2016; Rowe et al., 2016; Segovia et al., 2016; Treidel et al., 2016). The red-eared slider has a warm female-cool male TSD system, where warmer temperatures ($>$ 31 °C) produce predominantly females and cooler temperatures ($<$ 26 °C) produce predominantly males. In this turtle the embryonic development is divided into 27 stages and the TSP spans from Greenbaum stages (st) 15 to 19 (Greenbaum, 2002) at female promoting temperatures (FPT) and from st 14 to 20 at male promoting temperature (MPT), approximately in the middle third of development (Shoemaker-Daly et al., 2010). The Pivotal Temperature (PT) is the constant temperature (29 °C for *T. scripta*) at which 50:50 sex ratio is expected (Wibbels et al., 1991; Wibbels and Crews, 1995). In this study, red-eared slider eggs were incubated under different temperature and moisture regimes to study the effect of the two environmental factors on developmental rate, egg mass, embryo mass and length, and sex ratio. We show that moisture affects the incubation temperature, influencing developmental rates and sex ratios and greater substrate moisture is associated with the production of larger embryos.

2. Materials and methods

2.1. Egg incubation

Freshly laid *Trachemys scripta elegans* eggs were purchased from Concordia Turtle Farms (Hammond, LA, USA) and transported to Florida Atlantic University, Boca Raton, Florida. Clutches were divided evenly among four treatments (Tmt); Tmt 1: 29 °C with high moisture (defined as 50% water saturation or $\approx 0.10\ m^3\ m^{-3}$); Tmt 2: 29 °C with moderate moisture (defined as 25% water saturation or $\approx 0.05\ m^3\ m^{-3}$); Tmt 3: 31 °C with high moisture; Tmt 4: 31 °C with moderate moisture.

2.2. Temperature conditions

Groups of 20 eggs were placed in Styrofoam™ boxes (hereafter, nest boxes) containing sterilized sand from a local beach. The nest boxes were then placed in one of two different incubation chambers (hereafter, incubators). Each incubator contained 10 nest boxes (five for each moisture treatment). Temperature of the incubators was set at either 29 °C (PT) or 31 °C (FPT) and was controlled by an Omega.com iSeries Temperature and Process Controller Model CNi32, (Stamford, CT, USA). Humidity in the incubators was maintained at high levels (80-90%) using a fan-assisted mist humidity system (Walgreens Cool Moisture Humidifier Model 890-WGN). Air in incubators and sand temperatures in individual nest boxes were recorded every 15 min using HOBO U22-001 temperature loggers (accuracy ± 0.21 °C and a resolution = 0.02 °C; Onset Computer Corp., Bourne, MA, USA).

2.3. Moisture settings

Water treatments were based on Mcgehee (1990) showing that 25% H_2O saturation of the sand ($\approx 0.05\ m^3\ m^{-3}$) was the optimum level to maximize hatch success and hatchling size in loggerhead turtles. Volumetric sand moisture in each nest box was measured with Decagon EC-5 Soil Moisture probes fitted to HOBO H21-002 Micro Station Data Loggers [Onset Computer Corp., resolution (mean ± S.D) = 0.0007 m^3 H_2O per m^3 sand, accuracy ± 0.031 $m^3\ m^{-3}$ (± 3.1%)] where 25% moisture $\approx 0.05\ m^3\ m^{-3}$ and 50% moisture $\approx 0.10\ m^3\ m^{-3}$. Moisture in the nest boxes was maintained by spraying the surface of the sand with distilled H_2O (Di-H_2O) every day until they reached the treatment's target (≈ 0.05 and $0.10\ m^3\ m^{-3}$). Di-H_2O was kept inside the chambers to maintain it at the same temperature as the incubator air.

2.4. Data collection

Two embryos per treatment, randomly selected from different nest boxes, were sacrificed weekly to verify their developmental stages through st 22 (the stage at which the embryo sex determination is complete) based on Greenbaum's series of embryonic stages for *T. scripta* (Greenbaum, 2002). Developmental trajectories were based on the time required for embryos to reach st 22. To document growth, we harvested 30 eggs per Tmt at st 16 (the start of sex determination) and 20 eggs per Tmt at st 22. The difference in the number of eggs harvested among the stages was due to embryo mortality throughout the experiment. For each harvested egg, the presence and viability of the embryo were verified. Egg mass and embryo mass were recorded only for those eggs with a live embryo. Digital photographs were taken to document the developmental stage. Embryo straight carapace length (SCL) was digitally measured using ImageJ 1.46r (Rasband, 1997–2012).

Sex ratio was estimated based on expression levels of *Sox9*, a male specific gene implicated in testis development. Expression has been shown to be higher in differentiated *T. scripta* testis (Shoemaker et al., 2007). Gonads from two embryos per Tmt at st 16 and from 10-12 embryos per Tmt at st 22, were microdissected and preserved in RNAlater (Ambion, Waltham, MA, USA) for RT-qPCR analysis. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by DNAse I treatment (Promega, Madison, WI, USA). cDNA was reverse-transcribed using random primers and the SuperScript IV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Primers used to assay gene expression were designed from the coding sequence of *T. scripta Sox9* gene (GenBank: EU268286.1) (Forward: 5′-GCC TGG GAA GCA AGA CCT GA-3′; Reverse: 5′-TGA CCG TTG GGT GGG AGG TA-3′) amplifying a fragment of 180 bp. Gene expression was quantified with an Mx3000p qPCR System (Stratagene, San Diego, CA, USA) using SybrGreen® (Invitrogen) as an intercalating dye. Individual samples were analyzed in duplicate. *T. scripta* β-actin was used as an internal control (Forward: 5′-CAC CCA CAC TGT GCC CAT CT-3′; Reverse: 5′-CAC GAT TTC CCT TTC GGC TGT-3′) in order to normalize raw *Sox9* CT data (ΔCT) (Vandesompele et al., 2002). Relative gene expression levels were calculated through the comparative

ΔCT method according to Livak and Schmittgen (2001). PCR conditions were as follows: one cycle at 95 °C 10 min, 40 cycles at 95 °C 30 s, and 60 °C 60 s. PCR specificity was confirmed through melting curve analysis.

2.5. Statistical analyses

All data were tested for normality (Kolmogorov-Smirnov) and for homogeneity (Bartlett's test) before statistical analysis. Temperature data of the air and the sand were compared using a two samples T-Test. One-way ANOVA followed by an all-pairwise multiple comparison analysis (Tukey Test) was performed to identify statistical differences among treatments ($\alpha < 0.05$) for egg mass and embryo mass and length. For *Sox9* expression levels, ΔCT values were converted to the linear form using the $2^{-\Delta CT}$ equation (Livak and Schmittgen, 2001). One-way ANOVA coupled to an all pairwise multiple comparison analysis (Holm-Sidak test) was performed to identify any statistical differences in gene expression between st 22 embryos of the four treatments and st 16 embryos as the control group, since it is known that *Sox9* expression is lower in undifferentiated embryos compared to fully differentiated embryos ($\alpha < 0.05$). Statistical procedures were based on (Zar, 1999) and SigmaStat software (ver 4.0).

3. Results

3.1. Temperature outside and inside the nest

In all four treatments, the sand temperature was significantly cooler than the air temperature (Table 1) and was affected by sand moisture in the nest boxes. Differences between air and sand temperature (mean ± S.D) were 3.14 °C ± 0.02 in high moisture Tmts and 2.04 °C ± 0.10 in the moderate moisture treatments. Although sand temperatures differed from air temperatures, we were able to maintain the two target moisture treatments throughout the experiment with expected daily fluctuations due to water evaporation (Table 1). This means that temperatures were primarily at MPTs (26 and 27 °C) and at temperatures closer to the PT (28° and 29 °C). While moisture significantly cooled the sand below air temperatures, the effect was not great enough to override the differences due to air temperatures. Thus sand temperatures were distinct and characterized: cooler (26 °C) with high moisture < cooler (27 °C) with moderate moisture < warmer (28 °C) with high moisture < warmer (29 °C) with moderate moisture. Therefore the treatments are hereafter termed: 26-H (Tmt 1), 27-M (Tmt 2), 28-H (Tmt 3) and 29-M (Tmt 4) for the remainder of this paper.

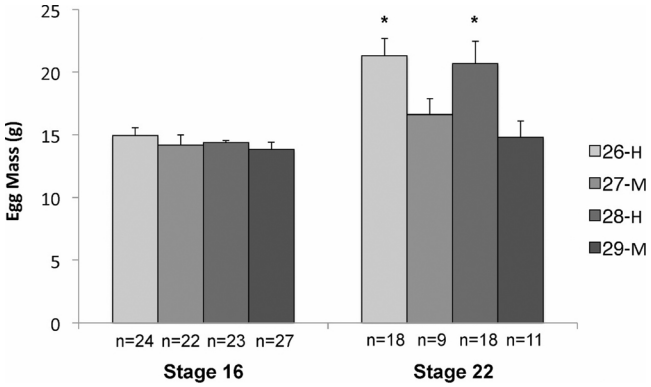

Fig. 1. Difference in mean egg mass (g) among the four treatments at stage 16 and stage 22 (during and after the TSP, respectively). * Denotes statistically significant differences by ANOVA (Table 2) and post hoc Tukey Test (Table 3, $\alpha < 0.05$); error bars represent 95% confidence intervals. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture.

3.2. Egg mass

Fig. 1 summarizes egg mass at st 16 and st 22 for the four treatments. At st 16, water uptake by the eggs (reflected in egg mass but not embryo size, see below) showed a lesser influence of moisture or temperature; therefore we found no difference in egg mass among the four treatments (Table 2). At st 22, H_2O uptake by the eggs differed among treatments. Eggs maintained on wetter substrates (26-H and 28-H) absorbed significantly more H_2O than the eggs incubated in drier substrates (27-M and 29-M; Table 3) regardless of the incubation temperature.

3.3. Developmental trajectory

Since moisture differences resulted in sand temperature differences even when air temperatures in the incubators were the same, the embryos developed at different rates. Embryos from the different treatments were approximately one stage apart from each other throughout development (Fig. 2). Embryos from 29-M (warmer, moderate moisture) developed the fastest, reaching st 22 in only 6 weeks while embryos from 26-H (cooler, high moisture) were the slowest, taking 10 weeks to reach st 22.

3.4. Embryo mass and length

Along with embryos reaching stage 22 at different rates, embryonic growth rates also differed under the different treatment regimens. At st 16, both mass (Fig. 3) and straight carapace length (SCL, Fig. 4) differed

Table 1
Incubation conditions (moisture and temperature) in each one of the treatments throughout the experiment. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture. Air T: mean ± s.d. of the air temperature in the incubators. Sand T: mean ± s.d. of the sand temperature in the nest boxes. Significant differences are in bold. T-test: t, (df, degrees of fredom); P = value. Moisture Min: mean ± s.d. of the daily minimum moisture in the nest boxes. Moisture Max: mean ± s.d. of the daily maximum moisture in the nest boxes. Moisture Average: mean ± s.d. of the moisture maintained in the nest boxes throughout the experiment.

Treatment	Air T (°C)	Sand T (°C)	T-test results	Moisture (m^3/m^3)		
				Min	Max	Average
26-H	29.10 ± 0.29	25.84 ± 0.61	t = 59.375 (84) **P = < 0.001**	0.088 ± 0.018	0.117 ± 0.009	0.103 ± 0.021
27-M	29.10 ± 0.29	27.04 ± 0.73	t = 29.665 (84) **P = < 0.001**	0.046 ± 0.015	0.059 ± 0.009	0.053 ± 0.014
28-H	31.01 ± 0.20	27.88 ± 0.50	t = 81.002 (84) **P = < 0.001**	0.083 ± 0.022	0.120 ± 0.011	0.100 ± 0.025
29-M	31.01 ± 0.20	28.85 ± 0.64	t = 45.422 (84) **P = < 0.001**	0.048 ± 0.017	0.062 ± 0.010	0.055 ± 0.016

Table 2
ANOVA analysis for egg mass, embryo mass and size, and *Sox9* expression comparing the four different treatments in two developmental stages (st). 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture. Significant differences are in bold. df, degrees of freedom; SS, sum of squares; MS, mean sum of squares.

	Source	df	SS	MS	F-ratio	P-value
Egg mass st 16	Between groups	3	15.883	5.294	1.723	0.168
	Residual	92	282.68	3.073		
	Total	95	298.563			
Egg mass st 22	Between groups	3	392.367	130.789	13.836	< **0.001**
	Residual	52	491.542	9.453		
	Total	55	883.909			
Embryo mass st 16	Between groups	3	0.384	0.128	40.3830	< **0.001**
	Residual	92	0.292	0.00317		
	Total	95	0.676			
Embryo mass st 22	Between groups	3	1.692	0.564	3.287	**0.028**
	Residual	52	8.924	0.172		
	Total	53	10.616			
Embryo size st 16	Between groups	3	2.978	0.993	12.748	< **0.001**
	Residual	92	7.165	0.078		
	Total	95	10.14			
Embryo size st 22	Between groups	3	10.712	3.573	3.317	**0.027**
	Residual	52	56.015	1.077		
	Total	55	66.735			
***Sox9* Expression**	Between groups	4	0.0417	0.0104	17.2316	< **0.001**
	Residual	50	0.0303	0.0006		
	Total	54	0.072			

Table 3
Tukey-Test analyses to identify sources of statistical difference in egg mass and embryo mass and size. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture. Significant differences are in bold.

	Multiple contrasts	Difference	P-value
Egg mass st 22	26-H vs. 29-M	0.484	< **0.001**
	26-H vs. 27-M	0.350	**0.003**
	26-H vs. 28-H	0.190	0.926
	28-H vs. 29-M	0.294	< **0.001**
	28-H vs. 27-M	0.161	**0.011**
	27-M vs. 29-M	0.133	0.569
Embryo mass st 16	27-M vs. 28-H	0.168	< **0.001**
	27-M vs. 29-M	0.124	< **0.001**
	27-M vs. 26-H	0.051	0.140
	26-H vs. 28-H	0.116	< **0.001**
	26-H vs. 29-M	0.073	< **0.001**
	29-M vs 28-H	0.043	2.047
Embryo mass st 22	26-H vs. 27-M	0.484	**0.030**
	26-H vs. 29-M	0.350	0.134
	26-H vs. 28-H	0.190	0.522
	28-H vs. 27-M	0.294	0.314
	28-H vs. 29-M	0.161	0.742
	29-M vs. 27-M	0.133	0.890
Embryo size st 16	27-M vs. 28-H	0.436	< **0.001**
	27-M vs. 29-M	0.345	< **0.001**
	27-M vs. 26-H	0.088	0.684
	26-H vs. 28-H	0.348	< **0.001**
	26-H vs. 29-M	0.257	**0.0076**
	29-M vs. 28-H	0.090	0.652
Embryo size st 22	26-H vs. 27-M	1.153	**0.043**
	26-H vs. 29-M	0.671	0.340
	26-H vs. 28-H	0.050	0.899
	28-H vs. 27-M	1.103	0.057
	28-H vs. 29-M	0.622	0.411
	29-M vs. 27-M	0.482	0.708

among treatments (Table 2). Embryos incubating at cooler temperatures (≈26 and 27 °C) were significantly bigger and heavier than those incubating at warmer temperatures (≈28 and 29 °C) but did not differ with moisture regime (Table 3). Interestingly, at st 22, high moisture treatments embryos (26-H and 28-H) tended to be bigger and heavier than embryos incubated at moderate moisture treatments regardless of incubation temperatures. However, only embryos at the ≈26 °C and high moisture treatment (26-H) were significantly different from the rest of the treatments (Table 3).

3.5. Sex ratio: Sox9 expression

We found that undifferentiated st 16 embryos of the four treatments were expressing low levels of *Sox9* (Fig. 5), while differentiated st 22 embryos of 26-H, 27-M, and 28-H expressed significantly higher levels of *Sox9*, suggesting that in those three treatments 100% of the embryos were males (Tables 2 and 4). However, 29-M was not statistically different from st 16 embryos (Tables 2 and 4) due to the low levels of *Sox9* found in some of the embryos analyzed (7 out of 12), suggesting a mix of male and female embryos.

4. Discussion

During incubation, the turtle embryo grows inside the nest from a few cells to a fully formed and independent organism at hatching. For proper development, embryos require an appropriate range of temperature, moisture, salinity, and respiratory gases (reviewed by Ackerman, 1997). In this study, we focused our attention on how moisture conditions inside the nest affect the development of *T. s. elegans* embryos. We found that the temperature inside the nest is strongly influenced by the water content of the sand; the sand temperatures

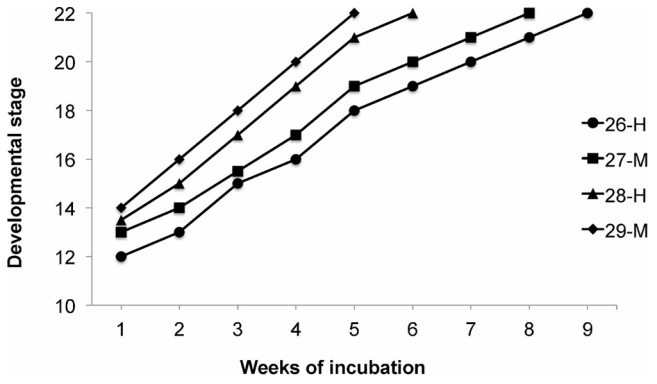

Fig. 2. Developmental trajectories of *Trachemys scripta elegans* embryos showing differences in the speed at which they developed as a function of incubation conditions. Developmental trajectories were determined by stage two embryos per treatment, weekly. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture.

were approximately 2–3 °C cooler than the air temperatures within each incubator (29 °C and 31 °C) (Table 1). This drop in sand temperature likely occurred due to evaporative cooling. Our results show that moisture changes the microclimate experienced by the eggs inside the nest and that this can significantly affect their development.

Effects of moisture on nest temperatures have been reported for sea turtles nesting in Suriname (Godfrey et al., 1996), in Florida (Foley et al., 2000; Lolavar and Wyneken, 2015) and in Costa Rica (Hill et al., 2015); and also in painted turtle *Chrysemys picta* nesting areas (Bodensteiner et al., 2015). None have focused upon why and how H_2O affects embryonic phenotype. The tight biophysical relationship

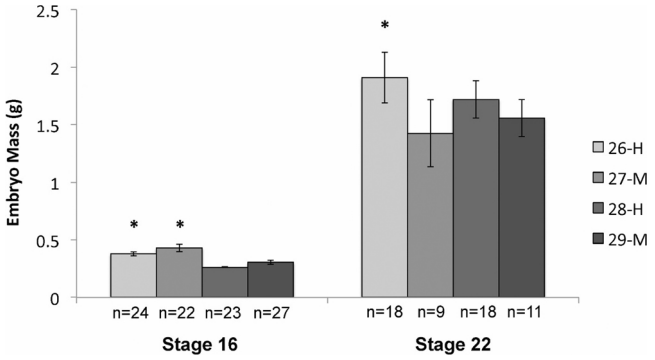

Fig. 3. Difference in mean embryo mass among the four treatments at stage 16 and stage 22 (during and after the TSP, respectively). * Denotes statistically significant differences by ANOVA (Table 2) and post hoc Tukey Test (Table 3, $\alpha < 0.05$); error bars represent 95% confidence intervals. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture.

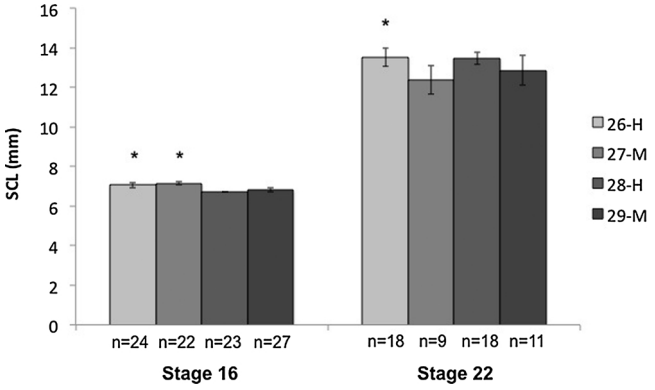

Fig. 4. Difference in mean embryo length (SCL, straight carapace length) among the four treatments at stage 16 and stage 22 (during and after the TSP, respectively). * Denotes statistically significant differences by ANOVA (Table 2) and post hoc Tukey Test (Table 3, $\alpha < 0.05$); error bars represent 95% confidence intervals. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture.

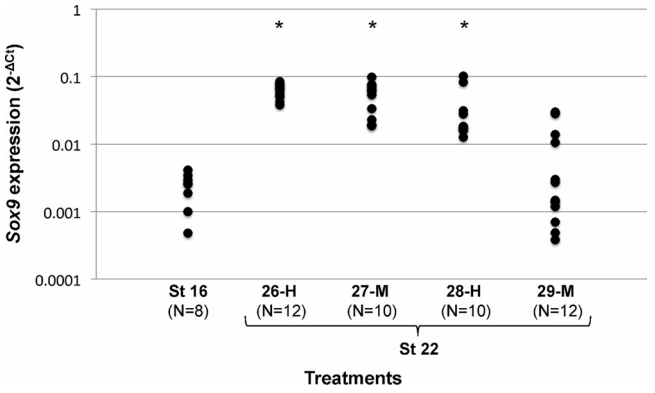

Fig. 5. *Sox9* relative expression levels of undifferentiated embryos (stage 16) and sex differentiated embryos (stage 22). Stage 16 embryos from the four treatments were considered as the control group since low *Sox9* expression was expected. At st 22, the four treatments were analyzed separately. Each point represents an individual. * Denotes statistically significant differences by ANOVA (Table 2) and post hoc Holm-Sidak test (Table 4, $\alpha < 0.05$); error bars represent 95% confidence intervals. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture.

between substrate moisture and nest temperature makes it difficult to fully separate one factor from the other. Nevertheless, our results highlight the importance of an experimental design that considers this cooling effect when studying the effects of temperature and moisture.

Table 4
Holm-Sidak analyses to identify sources of statistical difference in *Sox9* expression. Relative *Sox9* expression levels of sex determined embryos (st 22) in the four different treatments were compared against undifferentiated embryos (st 16). Stage 16 embryos from the four treatments were considered as the control group since low *Sox9* expression was expected. 26-H: ≈26 °C, high moisture; 27-M: ≈27 °C, moderate moisture; 28-H: ≈28 °C, high moisture; 29-M: ≈29 °C, moderate moisture. Significant differences are in bold.

Multiple contrast	Holm-Sidak t-stats	P-value
St 16 vs. 26-H	6.5351	< **0.001**
St 16 vs. 27-M	5.4324	< **0.001**
St 16 vs. 28-H	3.3377	< **0.001**
St 16 vs. 29-M	0.5888	0.558

By simultaneously measuring the sand temperatures and H_2O content, we were able to distinguish differences in the embryo's response to these environmental factors.

4.1. Egg mass

We found that eggs with st 16 embryos tended to gain mass regardless of sand moisture or temperature; while eggs with st 22 embryos, gained more weight at the high moisture treatments regardless of temperature (Fig. 1). Increase in egg mass is primarily due to water uptake during development by the parchment-shelled eggs (Packard et al. 1982). Our results suggest that, water uptake of the eggs did not depend on the temperature experienced inside the nest but rather depend on developmental stage and water availability. Stage 16 embryos (Fig. 6A) have distinct limb buds, head, and tail, and are characterized by a pigmented eye, a digital plate with a smooth periphery with slight indications of digital ridges and the intestinal loop is herniated (Greenbaum, 2002). They also have contractile pre-cardiac vessels, and the yolk sac is much larger in diameter than embryo length (personal observations). These characteristics suggest that catabolism is still low. At st 22 (Fig. 6B), the embryo's length is similar to yolk sac diameter (personal observations) and it has a shell with scutes, and well-developed limbs with claws (Greenbaum, 2002). Between st 16 and st 22 embryos increase yolk utilization, a process that requires greater volumes of water to meet the demands of the larger embryo (Packard and Packard, 2001). This differential requirement for moisture through development has been reported previously on other species with flexible-shelled eggs (Delmas et al., 2008) and also in species with rigid-shelled eggs (Booth, 2002). The authors reported a significant positive relationship between substrate moisture and egg water uptake especially during the last third of incubation period, which is consistent to our results.

Water exchange by eggs is affected by several physical characteristics of the substrate such as, water content, water potential, temperature, and thermal conductivity (Packard, 1991; Booth, 2002); but also by several aspects of the egg itself. Among these characteristics are the egg size (which determines the area of the eggshell making contact with the substrate) (Ackerman et al., 1985), and the eggshell structure (e.g., porosity, thickness) that modifies its water conductance (Booth, 2002; Rimkus et al., 2002). Interestingly, these characteristics change depending on how long the eggs remain in wet or dry conditions, that is, the longer they spend in a wet substrate, the bigger and thinner-shelled the eggs will be due to the water uptake (Booth, 2002); which in turn will favor the continuous exchange of water throughout incubation. Indeed, in our study, water uptake was enhanced later in development (st 22) and it was higher in wetter treatments. Moreover, the growing embryo can also modify the egg sensitivity to water uptake. Booth (2002) showed that infertile eggs did not experience any change in water uptake during the length of incubation. Normally, as the embryo grows and the yolk is consumed and converted into tissue, the osmotic concentration changes, which in turn, modifies the water

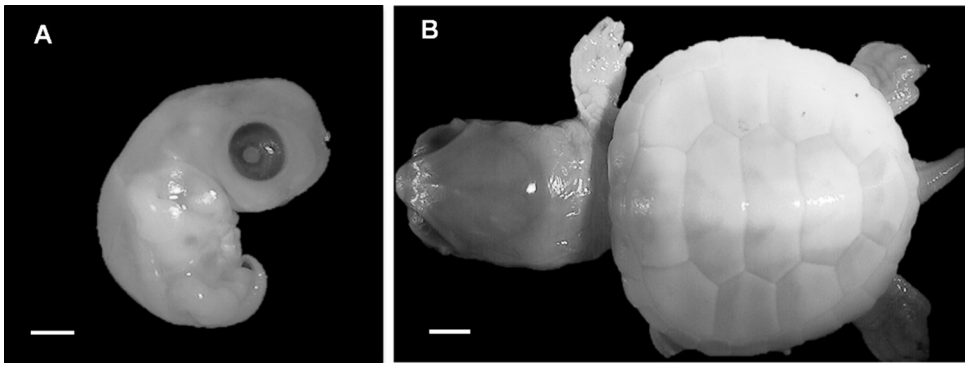

Fig. 6. *Trachemys scripta elegans* embryos at two developmental stages. (A) Lateral view of a stage 16 embryo. (B) Dorsal view of a stage 22 embryo. Scale bars = 2 mm.

potential difference across the eggshell inducing egg water uptake (Delmas et al., 2008). The increased blood circulating in the chorioallantois along the inside surface of the shell allowing gas exchange and the metabolic heat produced from embryos at later stages might also favor water exchange (Rimkus et al., 2002).

Thus, the difference in the water uptake we found at st 22 in high moisture treatments is likely due to a combination of factors. These include higher water content in the substrate, an increase in eggshell water conductance caused by shell thinning, increased shell surface area as the egg swells over incubation time, and finally, by a change in the chemical composition (less lipid content as yolk is consumed), and functioning (greater gas exchange) of the egg's internal environment as the embryo grows. While not determined in this study, the combination of these effects could result in heavier eggs in the treatments with high moisture, independent of the temperature (Fig. 1).

4.2. Developmental trajectory

Our results showed that the developmental trajectory, up to the time it takes the developing embryos to reach st 22, is affected mostly by temperature. Warmer temperatures were associated with faster development, which is consistent with previous studies (Yntema, 1978) and thermal effects were not overridden by different moisture regimes. The effect of temperature on the developmental rate of embryos has been widely studied in a variety of taxa (Gillooly and Dodson, 2000; Gillooly et al., 2002). In reptiles, the thermal environment can influence embryonic growth rate (Packard et al., 1977; Deeming and Ferguson, 1989; Georges et al., 2005; Ligon and Lovern, 2012) and therefore, incubation duration (Yntema, 1978; Lolavar and Wyneken, 2015). It is not surprising that the speed of development is positively correlated to temperature (as long as it is within the normal range of incubation temperatures) because physiological and biochemical rates are generally positively correlated with temperature (Birchard, 2004).

However, while temperature is clearly the overriding factor impacting embryonic development, moisture played an important role as well in that it modified the temperature of the nest substrate in all treatments, and thus affected the pace at which the embryos developed. Longer incubation periods are correlated with wetter substrates at constant condition (Packard et al., 1989; Mcgehee, 1990; Packard et al., 1991; Janzen et al., 1995) or under fluctuating conditions of wet and dry, where incubation period increases with longer time spent in a wet substrate (Delmas et al., 2008).

The effects of moisture and temperature, where moisture cools down the sand and thus influences the developmental rate, have also been reported in field studies. An increase in substrate moisture by rainfall events extended the incubation period of three species of fresh water turtles (*Graptemys* spp.; Bull, 1985), the painted turtle (Bodensteiner et al., 2015) and also the loggerhead sea turtle (Lolavar and Wyneken, 2015). Although we cannot reject the possibility that moisture may have other, more direct effects on developmental rate, our results suggest that this effect is at least in large part through a cooling effect, likely due to the evaporation of water from the sand at higher moisture conditions and perhaps also by intake by the eggs of water vapor (Ackerman, 1997).

4.3. Embryo mass and length

While the influence of incubation conditions on the resulting hatchling traits has been widely studied, fewer studies examine the embryo's reaction to the environmental factors inside the nest (Ligon and Lovern, 2012; Dormer et al., 2016). The mechanisms by which the environment influences the turtle embryo during different developmental stages is less understood.

As development progresses it is expected that the embryo regulatory process mature and change (Ackerman 1997). In our study, embryo length and weight were significantly affected by temperature at st 16; bigger and heavier embryos were produced in cooler temperatures (Figs. 3 and 4, respectively). But later in development (st 22), moisture tended to have a greater effect in both traits (Figs. 3 and 4).

Turtle embryos develop successfully over a narrow range of temperatures with a lower and upper lethal limit (Ackerman, 1997; Du and Ji, 2003) and they are especially sensitive to temperature changes at early stages, when crucial developmental processes including histogenesis and organogenesis take place (Yntema, 1968; Birchard, 2004). Temperature affects these aspects of morphogenesis by changing the rate of development of certain organs and traits (Dormer et al., 2016) or even by the inhibition of successful development, leading to phenotypic abnormalities (Telemeco et al., 2013). Furthermore, sex determination, part of histogenesis and organogenesis, takes place in the middle third of development and depends on temperature (Bull, 1987; Pieau et al., 1999; Merchant-Larios, 2001). A change in temperature as subtle as 0.5 °C can alter the offspring sex ratio within a *Natator depressus* clutch (Hewavisenthi and Parmenter, 2002). In our study, this embryo sensitivity to temperature at early stages was also evident. Interestingly, the differences we found at st 16 in embryo SCL and mass were influenced by temperature and not by the substrate moisture as it occurred at st 22. To our knowledge, there is no report of moisture influencing crucial developmental events like organogenesis except for sex determination (but see discussion below).

Once organogenesis is completed, the embryo is now committed to increase its body size before hatching. Most of embryonic somatic growth occurs during the final 50% of development at the end of incubation (Birchard, 2004). Embryonic growth is a metabolic process. Fuel, stored in the yolk by the female, is moved from the yolk to cells where it is transformed into energy (Ackerman, 1997). Packard and Packard (2001) proposed that embryos with access to relatively large amounts of water seemingly sustained higher rates of metabolism and growth than embryos having access to relatively small amounts of liquid. In agreement with this idea, Morris et al. (1983) and Booth (2002) found that eggs that absorbed larger amounts of water tended to have smaller residual yolk mass and bigger hatchlings, suggesting that the amount of yolk converted to tissue during embryonic development is

influenced by the amount of water absorbed by eggs during incubation. Therefore differences in metabolism and growth drive by water availability are translated into observed differences in size (wetter the substrate, larger the embryos) and are evident later in development when the embryo is increasing its body size in preparation for hatch.

An additional factor that affects embryo water availability is the osmotic potential. Salinity of the substrate during incubation has been found to have clear effects on the development of *C. expansa* (Bower et al., 2013). Since negative water potential is created by solutes in water, salty sand will cause eggs to dehydrate. Therefore the morphological effects of a saline substrate mirrored those of turtles incubated in drier media, that is, larger residual yolk sac of hatchings, smaller carapace size and smaller yolk free mass of hatchlings (Bower et al., 2013), supporting the role of moisture during embryonic growth.

However, in a study with loggerhead eggs, Mcgehee (1990) showed that there is an optimum range of substrate moisture, where moisture levels lower than 25% and higher than 50% by volume produced smaller embryos. Similar results were found for *Chelydra serpentina* (Finkler, 2006), a freshwater species that also has pliable-shelled eggs. Increasing the water content of the substrate beyond the optimal range, leads to O_2 depletion (Booth, 1998) because pore spaces contain less air. Responses to chronic hypoxia have been characterized at the molecular, morphological, and functional level in reptile embryos (Kam, 1993; Crossley and Altimiras, 2005; Eme et al., 2013) and hatchlings (Wearing et al., 2016). At the morphological level, continuous exposure to hypoxia (10% O_2) beginning early in incubation reduced embryonic mass but increased relative heart mass later in incubation in both American alligator (*Alligator mississippiensis*) and common snapping turtle embryos (Crossley and Altimiras, 2005; Eme et al., 2013, respectively). Interestingly, embryonic exposure to hypoxia appears to affect embryonic growth more than developmental events, since hypoxic embryos were smaller but hatched at the same time as normoxic embryos (Kam, 1993).

We suggest that the embryo's sensitivity to the surrounding environment changes depending on the developmental process. At earlier stages, when vital developmental processes such as organogenesis and histogenesis are occurring, embryos are more responsive to temperature because small changes in temperature can induce permanent effects in the phenotype of the hatchlings. Therefore, embryos must have a mechanism in place to respond rapidly to thermal fluctuations. Later in development, once the embryo has all major structures and organ systems, and the predominant processes are exponential growth and yolk metabolism, moisture (water availability) and O_2 play a larger role than temperature in enhancing metabolism and growth. During incubation, all physical factors are intimately related to form the nest environment; this interaction provides the suitable conditions for proper embryonic development. Our study suggests that embryos are able to respond to these factors in a stage dependent manner.

4.4. Sex ratio

Studies suggest that moisture affects sex ratios in turtles with TSD. In a series of experiments performed with the painted turtle, eggs incubated at several constant temperatures with differing moisture treatments; wetter substrates produced males, while drier substrates produced both males and females (Gutzke and Paukstis, 1983). Later studies with the same species were not able to confirm those results at either constant (Packard et al., 1989) or fluctuating incubation temperatures (Paukstis et al., 1984; Packard et al., 1991). Nevertheless, in a more recent study with a closely related species, *T. scripta*, Leblanc and Wibbels (2009) showed that eggs sprayed with H_2O during the thermosensitive period had male-biased sex ratios compare to controls. At a constant incubation temperature, a daily application of room temperature water to the eggs resulted in more males compared to the control group, not receiving the water treatment. However, in this last study, eggs were incubated on racks without any substrate in incubators of the same temperature. Such experimental conditions do not mimic a normal nest, since the physical characteristics of the substrate play an important role in the thermodynamics of the eggs and in nest heat and moisture retention.

This effect of moisture on sex ratio has also been observed in field studies. Green (*Chelonia mydas*) and leatherback (*Dermochelys coriacea*), as well as loggerhead (*C. caretta*) sea turtles nests that experienced greater rainfall had a higher production of males (Godfrey et al., 1996; Wyneken and Lolavar, 2015).

This is the first study in which *Sox9* was used as a transcriptional molecular marker to estimate sex ratio. *Sox9* is a transcription factor implicated in testis differentiation in mammals, birds and reptiles (Western et al., 1999; Moreno-Mendoza et al., 1999; Koopman, 2001; Western and Sinclair, 2001; Shoemaker et al., 2007). Thus in turtles with TSD, *Sox9* expression levels are higher in differentiated gonads incubated at MPT than in differentiated gonads at FPT and undifferentiated gonads from both MPT and FPT (Moreno-Mendoza et al., 1999; Rhen et al., 2007; Shoemaker et al., 2007). In the present study we found that all st 22 embryos from 26-H, 27-M and 28-H expressed significantly higher levels of *Sox9* compared to st 16 embryos. Actual sand temperatures in those treatments ranged from 25.84 to 27.88 °C; therefore, in those treatments where temperatures were below the PT (29 °C) and *Sox9* levels were high, we assigned the resulting embryos to be 100% males. However in 29-M, where the mean sand temperature was very close to the PT at 28.85 °C, only 5 out of 12 embryos expressed *Sox9* at high levels (5-15 fold higher than st 16 embryos). Consequently, the embryos in 29-M were assigned as 42% males

Together, these results are particularly interesting, because they show how moisture affects sex ratios by changing the temperature inside the nest. However, while moisture per se may not override the effect of temperature, the cooling effect can result in significant alterations to sex ratios vs. air temperatures measured alone. Air temperatures of 31 °C, that alone would suggest a high percentage of female embryos produced in the nest, instead resulted in 100% males at higher moisture levels and nearly 50% males at moderate moisture levels, as the moisture reduced the temperature to near pivotal for this species. In addition, our focus on one sex-specific gene, *Sox9*, showed no modification of its expression with moisture *per se*. Temperature has long been shown to be the major cue of TSD not only appearing as a phenotypic effect but also showing its influence in the molecular network responsible for sex determination. The temperature response of sex-related genes in gonads of turtles has been addressed using approaches studying individual genes (Moreno-Mendoza et al., 1999; Torres-Maldonado et al., 2002; Murdock and Wibbels, 2003; Rhen and Schroeder, 2010; Shoemaker-Daly et al., 2010; Sifuentes-Romero et al., 2010, 2013; Gómez-Picos et al., 2014) and more recently with broad, unbiased approaches using transcriptomic analysis (Czerwinski et al., 2016; Radhakrishnan et al., 2017). In these studies with constant temperatures or even with shifts within the temperature treatments, the authors have shown that temperature is able to trigger a genetic response.

This study is the first to report an effect of moisture on sex ratios in combination of empirical measures of temperature inside the nest, and demonstrate that the substrate temperature varies according to the water content. This lab study is consistent with field studies of freshwater turtles (Bull, 1985; Bodensteiner et al., 2015) and sea turtles (Godfrey et al., 1996; Lolavar and Wyneken, 2015; Wyneken and Lolavar, 2015). We suggest that the influence of moisture on sex ratios is through the temperature, which in turn will modify the molecular mechanisms responsible for sex determination.

However, to completely reject or accept an influence of moisture on the molecular mechanism of sex determination and sex ratios; it is crucial to separate the effect of temperature and moisture, and take the next steps to include FPTs. Such an approach will allow us to investigate the molecular mechanisms involved in sex determination such as gene regulation and epigenetics.

5. Conclusions

Results of experimental and field studies indicate that temperature and moisture affect hatchling phenotype through their combined effect on embryonic development and water relationships with the egg. We observed stage-related differences in the response of the embryos to moisture and temperature. At earlier stages of embryogenesis, the effects of temperature are more evident, reflected by developmental trajectory and sex determination. However, in later stages, when the embryo is larger and more complex and growth is the dominant process, moisture may have a greater effect by sustaining higher rates of metabolism that support yolk utilization.

The results may provide valuable insight into development in TSD species but also herald caution when attempting to predict sex ratios based on incubation temperature in natural nests that may be subjected to locally variable and periodic rain events. Our results are relevant when considering nesting phenology in the wild because conditions such as temperature and rainfall often vary depending across the nesting season.

We strongly suggest the incorporation of other environmental factors such as moisture, in to existing models that attempt to estimate either embryo growth or sex ratios to increase the accuracy of model predictions. Improving accuracy is particularly important when trying to assess the impact of climate change in species with TSD and other forms of environmental sex determination.

Competing interests

The authors declare no competing or financial interests.

Acknowledgements

We thank N. Tempel, C. Jasper, and K. Detwiler for logistical assistance. A. Lolavar, V. Erb and J. Lasala provided constructive discussion that improved this study. M. Girondot and his laboratory's work with embryo growth models inspired this study. The research was supported financially by the National Save the Sea Turtle Foundation to B.T. and J.W., the McGinty Scholar Fund to S.L.M., and Fulbright García-Robles and CONACyT (No 263646) fellowships granted to I.S.R., as well as personal funds.

References

Ackerman, R.A., Seagrave, R.C., Dmi'el, R., Ar, A., 1985. Water and heat exchange between parchment-shelled reptile eggs and their surroundings. Copeia 1985, 703–711.

Ackerman, R.A., 1997. The nest environment and the embryonic development of sea turtles. In: Lutz, P.L., Musick, J.A. (Eds.), The Biology of Sea Turtles. CRC Press, Florida, pp. 83–106.

Birchard, G.F., 2004. Effects of incubation temperature. In: Deeming, D.C. (Ed.), Reptilian Incubation: Environment, Evolution and Behavior. University Press, Nottingham, pp. 103–123.

Bodensteiner, B.L., Mitchell, T.S., Strickland, J.T., Janzen, F.J., 2015. Hydric conditions during incubation influence phenotypes of neonatal reptiles in the field. Funct. Ecol. 29, 710–717.

Booth, D.T., 1998. Nest temperature and respiratory gases during natural incubation in the broad-shelled river turtle, *Chelodina expansa* (Testudinata: Chelidae). Aust. J. Zool. 46, 183–191.

Booth, D.T., 2002. Incubation of rigid-shelled turtle eggs: do hydric conditions matter? J. Comp. Physiol. B 172, 627–633.

Booth, D.T., Burgess, E., McCosker, J., Lanyon, J.M., 2004. The influence of incubation temperature on post-hatching fitness characteristics of turtles. Int. Congr. Ser. 1275, 226–233.

Bower, D.S., Hodges, K.M., Georges, A., 2013. Salinity of incubation media influences embryonic development of a freshwater turtle. J. Comp. Physiol. B 183, 235–241.

Bull, J.J., 1985. Sex ratio and nest temperature in turtles: comparing field and laboratory data. Ecology 66, 1115–1122.

Bull, J.J., 1987. Temperature-dependent sex determination in reptiles: validity of sex diagnosis in hatchling lizards. Can. J. Zool. 65, 1421–1424.

Crossley, D.A., Altimiras, J., 2005. Cardiovascular development in embryos of the American alligator *Alligator mississippiensis*: effects of chronic and acute hypoxia. J. Exp. Biol. 208, 31–39.

Czerwinski, M., Natarajan, A., Barske, L., Looger, L., Capel, B., 2016. A time course analysis of systemic and gonadal effects of temperature on sexual development of the red-eared slider turtle *Trachemys scripta elegans*. Dev. Biol. 420, 166–177.

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.

Delmas, V., Bonnet, X., Girondot, M., Prévot-Julliard, A.C., 2008. Varying hydric conditions during incubation influence egg water exchange and hatchling phenotype in the red-eared slider turtle. Physiol. Biochem. Zool. 81, 345–355.

Dormer, J., Old, J.M., Van Dyke, J.U., Spencer, R.J., 2016. Incubation temperature affects development order of morphological features and staging criteria in turtle embryos. J. Zool. 299, 284–294.

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 effects of incubation temperatures on the morphology and locomotor performance of hatchling lizards (*Bassiana duperreyi*, Scincidae). Biol. J. Linn. Soc. 63, 429.

Eme, J., Rhen, T., Tate, K.B., Gruchalla, K., Kohl, Z.F., Slay, C.E., Crossley, D.A., 2013. Plasticity of cardiovascular function in snapping turtle embryos (*Chelydra serpentina*): chronic hypoxia alters autonomic regulation and gene expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 304, R966–R979.

Finkler, M.S., 2006. Does variation in soil water content induce variation in the size of hatchling snapping turtles (*Chelydra serpentina*)? Copeia 2006, 769–777.

Foley, A.M., Peck, S.A., Harman, G.R., Richardson, L.W., 2000. Loggerhead turtle (*Caretta caretta*) nesting habitat on low-relief mangrove islands in southwest Florida and consequences to hatchling sex ratios. Herpetologica 433–445.

Georges, A., Beggs, K., Young, J.E., Doody, J.S., 2005. Modelling development of reptile embryos under fluctuating temperature regimes. Physiol. Biochem. Zool. 78, 18–30.

Gilbert, S.F., Epel, D., 2015. Ecological Developmental Biology: The Environmental Regulation of Development. Health and Evolution, 2nd ed. Sinauer Associates, Sunderland.

Gillooly, J.F., Dodson, S.I., 2000. The relationship of neonate mass and incubation temperature to embryonic development time in a range of animal taxa. J. Zool. (Lond.) 251, 369.

Gillooly, J.F., Charnov, E.L., West, G.B., Savage, V.M., Brown, J.H., 2002. Effects of size and temperature on developmental time. Nature 417, 70.

Godfrey, M.H., Barreto, R., Mrosovsky, N., 1996. Estimating past and present sex ratios of sea turtles in Suriname. Can. J. Zool. 74, 267–277.

Gómez-Picos, P., Sifuentes-Romero, I., Merchant-Larios, I., Hernández-Cornejo, R., Díaz-Hernández, V., García-Gasca, A., 2014. Expression of aromatase in the embryonic brain of the olive ridley sea turtle (*Lepidochelys olivacea*), and the effect of bisphenol-A in sexually differentiated embryos. Int. J. Dev. Biol. 58, 733–741.

Greenbaum, E., 2002. A standardized series of embryonic stages for the emydid turtle *Trachemys scripta*. Can. J. Zool. 80, 1350–1370.

Gutzke, W.H.N., Paukstis, G.L., 1983. Influence of the hydric environment on sexual differentiation of turtles. J. Exp. Zool. 226, 467–469.

Hewavisenthi, S., Parmenter, C.J., 2002. Incubation environment and nest success of the flatback turtle (*Natator depressus*) from a natural nesting beach. Copeia 2002, 302–312.

Hill, J.E., Paladino, F.V., Spotila, J.R., Tomillo, P.S., 2015. Shading and watering as a tool to mitigate the impacts of climate change in sea turtle nests. PLoS ONE 10, e0129528.

Janzen, F.J., Krenz, J.G., 2004. Phylogenetics: which was first, TSD or GSD? In: Valenzuela, N., Lance, V.A. (Eds.), Temperature Dependent Sex Determination in Vertebrates. Smithsonian Institution, Washington pp. 121–130.

Janzen, F.J., Ast, T.J.C., Paukstis, G.L., 1995. Influence of the hydric environment and clutch on eggs and embryos of two sympatric map turtles. Funct. Ecol. 9, 913–922.

Kam, Y.C., 1993. Physiological effects of hypoxia on metabolism and growth of turtle embryos. Respir. Physiol. 92, 127–138.

Koopman, P., 2001. The genetics and biology of vertebrate sex determination. Cell 105, 843–847.

Leblanc, A.M., Wibbels, T., 2009. Effect of daily water treatment on hatchling sex ratios in a turtle with temperature-dependent sex determination. J. Exp. Zool. 311A, 68–72.

Ligon, D.B., Lovern, M.B., 2012. Interspecific variation in temperature effects on embryonic metabolism and development in turtles. ISRN Zool. 2012, 846136.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-2\Delta\Delta CT}$ method. Methods 25, 402–408.

Lolavar, A., Wyneken, J., 2015. Effect of rainfall on loggerhead turtle nest temperatures, sand temperatures and hatchling sex. Endang. Species Res. 28, 235–247.

Lolavar, A., Wyneken, J.W., 2017. Experimental assessment of the effects of moisture on loggerhead sea turtle hatchling sex ratios. Zoology 123, 64–70. http://dx.doi.org/10.1016/j.zool.2017.06.007.

Mcgehee, M.A., 1990. Effects of moisture on eggs and hatchlings of loggerhead sea turtles (*Caretta caretta*). Herpetologica 46, 251–258.

Merchant-Larios, H., 2001. Temperature sex determination in reptiles: the third strategy. J. Rep. Dev. 47, 245–252.

Moreno-Mendoza, N., Harley, V.R., Merchant-Larios, H., 1999. Differential expression of *SOX9* in gonads of the sea turtle *Lepidochelys olivacea* at male- or female-promoting temperatures. J. Exp. Zool. 284, 705–710.

Morgan, T.J., Mackay, T.F.C., 2006. Quantitative trait loci for thermotolerance phenotypes in *Drosophila melanogaster*. Heredity 96, 232–242.

Morris, K.A., Packard, G.C., Boardman, T.J., Paukstis, G.L., Packard, M.J., 1983. Effect of the hydric environment on growth of embryonic snapping turtles (*Chelydra serpentina*). Herpetologica 39, 272–285.

Murdock, C., Wibbels, T., 2003. Expression of *Dmrt1* in a turtle with temperature-dependent sex determination. Cytogenet. Genome Res. 101, 302–308.

Packard, G.C., 1991. Physiological and ecological importance of water to embryos of

oviparous reptiles. In: Deeming, D.C., Ferguson, M.J.W. (Eds.), Egg Incubation: Its Effect on Embryonic Development in Birds and Reptiles. Cambridge University Press, New York pp. 213-228.

Packard, G.C., Packard, M.J., 2001. Environmentally induced variation in size, energy reserves and hydration of hatchling painted turtles, *Chrysemys picta*. Funct. Ecol. 15, 481–489.

Packard, G.C., Tracy, C.R., Roth, J.J., 1977. The physiological ecology of reptilian eggs and embryos and the evolution of viviparity within the class Reptilia. Biol. Rev. 52, 71–105.

Packard, M.J., Packard, G.C., Boardman, T.J., 1982. Structure of eggshells and water relations of reptilian eggs. Herpetologica 38, 136–155.

Packard, G.C., Packard, M.J., Birchard, G.F., 1989. Sexual differentiation and hatching success by painted turtles incubating in different thermal and hydric environments. Herpetologica 45, 385–392.

Packard, G.C., Packard, M.J., Benigan, L., 1991. Sexual differentiation, growth, and hatching success by embryonic painted turtles incubated in wet and dry environments at fluctuating temperatures. Herpetologica 47, 125–132.

Paukstis, G.L., Gutzke, W.H., Packard, G.C., 1984. Effects of substrate water potential and fluctuating temperatures on sex ratios of hatchling painted turtles (*Chrysemys picta*). Can. J. Zool. 62, 1491–1494.

Pieau, C., Dorizzi, M., 2004. Oestrogens and temperature-dependent sex determination in reptiles: all is in the gonads. J. Endocrinol. 181, 367–377.

Pieau, C., Dorizzi, M., Richard-Mercier, M., 1999. Temperature-dependent sex determination and gonadal differentiation in reptiles. Cell. Mol. Life Sci. 55, 887–900.

Radhakrishnan, S., Literman, R., Neuwald, J., Severin, A., Valenzuela, N., 2017. Transcriptomic responses to environmental temperature by turtles with temperature-dependent and genotypic sex determination assessed by RNAseq inform the genetic architecture of embryonic gonadal development. PLoS ONE 12, e0172044. http://dx.doi.org/10.1371/journal.pone.0172044.

Rasband, W.S., ImageJ, U. S. National Institutes of Health; Bethesda, Maryland, USA, 1997–2016, https://imagej.nih.gov/ij/.

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., 1999. Temperature during embryonic and juvenile development influences growth in hatchling snapping turtles, *Chelydra serpentina*. J. Therm. Biol. 24, 33–41.

Rhen, T., Schroeder, A., 2010. Molecular mechanisms of sex determination in reptiles. Sex. Dev. 4, 16–28.

Rhen, T., Metzger, K., Schroeder, A., Woodward, R., 2007. Expression of putative sex-determining genes during the thermosensitive period of gonad development in the snapping turtle, *Chelydra serpentina*. Sex. Dev. 1, 255–270.

Rimkus, T.A., Hruska, N., Ackerman, R., 2002. Separating the effects of vapor pressure and heat exchange on water exchange by snapping turtle (*Chelydra serpentina*) eggs. Copeia 2002, 706–715.

Rowe, J.W., Clark, D.L., Mortensen, R.A., Commissaris, C.V., Wittle, L.W., Tucker, J.K., 2016. Thermal and substrate color-induced melanization in laboratory reared red-eared sliders (*Trachemys scripta elegans*). J. Therm. Biol. 61, 125–132.

Segovia, Y., García, M., Gómez-Torres, M.J., Mengual, R., 2016. Ultrastructural study of retinal development in the turtle *Trachemys scripta elegans*. Zoomorphology 135, 205–216.

Shoemaker, C., Crews, D., 2009. Analyzing the coordinated gene network underlying temperature-dependent sex determination in reptiles. Semin. Cell. Dev. Biol. 20, 293–303.

Shoemaker, C., Ramsey, M., Queen, J., Crews, D., 2007. Expression of *Sox9*, *Mis*, and *Dmrt1* in the gonad of a species with temperature-dependent sex determination. Dev. Dyn. 236, 1055–1063.

Shoemaker-Daly, C., Jackson, K., Yatsu, R., Matsumoto, Y., Crews, D., 2010. Genetic network underlying temperature dependent sex determination is endogenously regulated by temperature in isolated cultured *Trachemys scripta* gonads. Dev. Dyn. 239, 1061–1075.

Sifuentes-Romero, I., Merchant-Larios, H., García-Gasca, A., 2010. *Hox* gene expression in the embryonic genital system of the sea turtle *Lepidochelys olivacea* (Eschscholtz, 1829), a species with temperature-dependent sex determination. Gene Expr. Patterns 10, 290–298.

Sifuentes-Romero, I., Merchant-Larios, H., Milton, S.L., Moreno-Mendoza, N., Díaz-Hernández, V., García-Gasca, A., 2013. RNAi-mediated gene silencing in a gonad organ culture to study sex determination mechanisms in sea turtle. Genes 4, 293–305.

Siviter, H., Deeming, D.C., Rosenberger, J., Burman, O.H.P., Moszuti, S.A., Wilkinson, A., 2017. The impact of egg incubation temperature on the personality of oviparous reptiles. Anim. Cogn. 20, 109–116.

Tang, X., Yue, F., Ma, M., Wang, N., He, J., Chen, Q., 2012. Effects of thermal and hydric conditions on egg incubation and hatchling phenotypes in two *Phrynocephalus* lizards. Asian Herpetol. Res. 3, 184–191.

Telemeco, R.S., Warner, D.A., Reida, M.K., Janzen, F.J., 2013. Extreme developmental temperatures result in morphological abnormalities in painted turtles (*Chrysemys picta*): a climate change perspective. Integr. Zool. 8, 197–208.

Torres-Maldonado, L.C., Landa-Piedra, A., Moreno-Mendoza, N., Marmolejo-Valencia, A., Meza-Martínez, A., Merchant-Larios, H., 2002. Expression profiles of *Dax1*, *Dmrt1*, and *Sox9* during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*. Gen. Comp. Endocrinol. 129, 20–26.

Treidel, L.A., Carter, A.W., Bowden, R.M., 2016. Temperature experienced during incubation affects antioxidant capacity but not oxidative damage in hatchling red-eared slider turtles (*Trachemys scripta elegans*). J. Exp. Biol. 219, 561–570.

Tucker, J.K., Filoramo, N.I., 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.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1–11.

Vervust, B., Brecko, J., Herrel, A., 2011. Temperature effects on snapping performance in the common snapper *Chelydra serpentina* (Reptilia, Testudines). J. Exp. Zool. 315, 41–47.

Wearing, O.H., Eme, J., Rhen, T., Crossley, D.A., 2016. Phenotypic plasticity in the common snapping turtle (*Chelydra serpentina*): long-term physiological effects of chronic hypoxia during embryonic development. Am. J. Physiol. Regul. Integr. Comp. Physiol. 310, R176–R184.

Western, P.S., Sinclair, A.H., 2001. Sex, genes, and heat: triggers of diversity. J. Exp. Zool. 290, 624–631.

Western, P.S., Harry, J.L., Graves, J.A.M., Sinclair, A.H., 1999. Temperature-dependent sex determination: upregulation of *SOX9* expression after commitment to male development. Dev. Dyn. 214, 171–177.

Wibbels, T., Crews, D., 1995. Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD. Gen. Comp. Endocrinol. 100, 53–60.

Wibbels, T., Bull, J.J., Crews, D., 1991. Chronology and morphology of temperature-dependent sex determination. J. Exp. Zool. 260, 371–381.

Wyneken, J., Lolavar, A., 2015. Loggerhead sea turtle environmental sex determination: implications of moisture and temperature for climate change based predictions for species survival. J. Exp. Zool. B. 324, 295–314.

Yntema, C.L., 1968. A series of stages in the embryonic development of *Chelydra serpentina*. J. Morphol. 125, 219–252.

Yntema, C.L., 1978. Incubation times for eggs of the turtle *Chelydra serpentina* (Testudines: Chelydridae) at various temperatures. Herpetologica 34, 274–277.

Zar, J.H., 1999. Biostatistical Analysis, 4th Ed. Prentice-Hall Inc., New Jersey.