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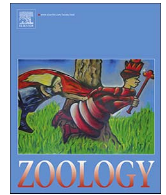

Hydric environmental effects on turtle development and sex ratio

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A B S T R A C T

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

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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 ($\approx$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

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