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Effects of Incubation Temperature on Sex Determination in the Endangered Magdalena River Turtle, *Podocnemis lewyana*

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ABSTRACT. – **In species with temperature-dependent sex determination, the embryo commits to its sexual fate during a time window called the thermosensitive period (TSP). Although substantial research has focused on the effect of the temperature during this canonical TSP, the effect of temperatures experienced before this time (pre-TSP) on the onset and duration of the TSP is less understood. Here, we incubated eggs of the endangered Magdalena River turtle, *Podocnemis lewyana*, under 3 relatively constant temperatures and 6 shift-twice treatments. Constant treatments included two 100% masculinizing temperatures that fostered a relatively slower and faster embryo development and one 100% feminizing temperature. The shift treatments included a masculinizing temperature and later a feminizing temperature for a period of time at different incubation stages. Sex ratios were determined by a geometric-morphometric sexing approach developed for this species and validated by gonadal histology. This technique is a nonlethal sexing alternative; thus, it contributes to the conservation of this endangered species. The sex ratios obtained under constant temperatures were more feminized than the sex ratio of another population native to a warmer climate. Shift-twice experiments revealed that pre-TSP temperatures affected the duration of the TSP and as such influenced sex determination. Specifically, at 29°C pre-TSP, the TSP lasted 20 d (28% of the incubation period), whereas at 31°C pre-TSP, the TSP was extended for 30 d (52% of the incubation period). An approximation of developmental stages based on the congeneric *Podocnemis expansa* is provided. Conservation programs should monitor temperatures throughout the entire incubation period; otherwise important effects on sex ratios and other traits may be obscured.**

KEY WORDS. – **Colombia; conservation of endangered reptilian species; embryonic developmental rates; temperature-dependent sex determination; geometric–morphometric sexing technique; sex-ratio monitoring**

RESUMEN. – **En especies con determinación sexual termo-dependiente el embrión define su sexo durante una ventana de tiempo llamada período termosensible (PTS). Muchas investigaciones se han enfocado en el efecto de la temperatura durante este PTS canónico, pero el efecto de temperaturas ocurridas en estadíos más tempranos (pre-PTS) sobre el inicio y duración del PTS ha sido poco estudiado. Nosotros incubamos huevos de la Tortuga del Río Magdalena, *Podocnemis lewyana*, bajo tres temperaturas relativamente constantes y seis tratamientos de cambio térmico doble. Los tratamientos de temperaturas constantes incluyeron dos temperaturas 100% masculinizantes que inducen un desarrollo embrionario lento y rápido respectivamente, y una temperatura 100% feminizante. Los tratamientos de doble cambio incluyeron dichas temperaturas masculinizantes (pre-PTS) y un pulso de temperatura feminizante en diferentes momentos del período de incubación. Las proporciones sexuales fueron calculadas mediante una técnica de morfometría geométrica desarrollada para esta especie y validadas usando histología gonadal. Esta técnica es una alternativa de sexaje no letal que contribuye a la conservación de esta especie amenazada. Las proporciones sexuales obtenidas a temperaturas constantes fueron más feminizadas que las reportadas en otra población que habita en un clima más cálido. En los tratamientos de doble cambio las temperaturas pre-PTS afectaron la duración del PTS, y por tanto la determinación sexual. Específicamente, a 29°C pre-PTS el PTS duró 20 días (28% del periodo de incubación), mientras que a 31°C pre-PTS, el PTS se extendió a 30 días (52% del período de incubación). Los estadíos embrionarios alcanzados durante la incubación fueron estimados usando como modelo la especie congenérica, *Podocnemis expansa*. Sugerimos que los programas de conservación monitoreen las temperaturas durante todo el período de incubación para evitar descartar importantes efectos sobre el sexo y otros fenotipos inducidos por las temperaturas tempranas.**

PALABRAS CLAVE. – Colombia; conservación de reptiles en peligro de extinción; determinación sexual; periodo termosensible; temperatura de incubación; tortuga del Río Magdalena; sexaje por morfometría geométrica

For many reptiles, sex is determined by the incubation temperature experienced during embryonic development, independent of the zygotic genotype (Bull 1980). Temperature-dependent sex determination (TSD) is present in all crocodile species studied to date, most turtles, and some lizards (Johnston et al. 1995; Valenzuela and Lance 2004; The Tree of Sex Consortium 2014). In TSD type Ia, females are produced at high temperatures and males at low temperatures, whereas the opposite occurs in TSD type Ib (Ewert and Nelson 1991). In TSD type II, females develop at low and high temperatures and males at intermediate temperatures.

In species with TSD, the time when the embryo is responsive to environmental temperatures and commits to male or female development is called the thermosensitive period (TSP; Yntema 1978, 1979; Bull 1980). Specifically, the TSP is the time window when the incubation temperature has a biasing effect on the resulting sex ratio. Several parameters of the TSP must be considered to understand sex determination by temperature in TSD taxa. The canonical (overall) TSP encompasses the middle third or half of the incubation period in some freshwater turtles and other reptiles (Wibbels et al. 1991, 1994; Young et al. 2004). However, because the incubation temperature affects the rate of embryo development (Yntema 1978, 1979; Bull and Vogt 1981), and thus the duration of the incubation period, the temperature experienced early in development (pre-TSP temperature) may not only influence sex ratios but also the onset and duration of the canonical TSP (Yntema 1978; Valenzuela 2001; Georges et al. 2005).

Several studies support the notion that the onset and duration of the TSP are temperature-sensitive and that these thermal effects may be species-specific (Table 1). Namely, although the TSP is generally considered to occur during the middle third of the incubation period in turtles with TSD (Wibbels et al. 1991, 1994; Young et al. 2004), discrepancies in the onset and duration of the TSP have been detected among taxa depending on the temperature being tested. For instance, the TSP in *Trachemys scripta* (slider turtles) starts during earlier embryonic stages at feminizing temperatures than at masculinizing temperatures (Wibbels et al. 1991). In contrast, ovarian development occurred at a later embryonic stage than testicular development in a sea turtle, *Natator depressus* (Hewavisenthi and Parmenter 2002). Differences between populations have also been reported. For instance, the TSP of *Podocnemis expansa* turtles was reported to occur during the middle of incubation in some populations (Valenzuela 2001) and during the second half in others (Bonach et al. 2011). Likewise, shift-twice experiments (feminizing–masculinizing–feminizing; 30°–26°–30°C and 20°–26°–20°C) in *Chelydra serpentina* affected the onset but not the duration of the TSP, whereas another treatment (masculinizing–feminizing–masculinizing; 26°–30°–26°C) delayed and shortened the TSP (Yntema 1979). More recently, ovaries were found to develop earlier (stage 18) than testes (stages 19–20) in *C. serpentina,* and the duration of the TSP was affected by the thermal regime (longer at male-producing temperatures; Rhen et al. 2015). In *Emys orbicularis*, ovaries developed between stages 16 and 21, whereas testes developed between stages 16 and 22 (Pieau and Dorizzi 1981). Thus, the embryonic stages at which ovaries and testes developed may differ within and among species.

A better understanding of sex determination and the factors that affect the activation and length of the TSP is crucial for the conservation of endangered species with TSD, such as the Magdalena River turtle, *Podocnemis lewyana*. Species with TSD are particularly vulnerable to climate change because higher temperatures could bias natural sex ratios (Janzen 1994; Neuwald and Valenzuela 2011). The TSP has not been studied in *P. lewyana*; yet, artificial incubation of eggs is a current conservation practice to avoid egg mortality in some populations (Romero 2011; Ceballos et al. 2014b), and the effect that this may have on sex determination is unknown. Furthermore, the TSP is ideally evaluated based on embryonic stages (Yntema 1978, 1979; Bull and Vogt 1981; Pieau and Dorizzi 1981); however, such destructive sampling is not always possible in endangered species (Le Blanc et al. 2012). Herein, we used incubation time as a proxy combined with an approximation of developmental embryonic stages based on statistical models of a congeneric species (Valenzuela 2001).

Studying sex determination in species with TSD requires information about sex ratios resulting from various incubation conditions. In species that lack a strong enough sexual dimorphism that can be observed by the naked eye at early life stages, sex information must be obtained destructively (e.g., by gonadal histology) or via indirect methods (e.g., morphometric or molecular approaches; reviewed in Literman et al. 2014). Thus, conservation efforts for endangered species benefit from indirect methods that are nonintrusive and inexpensive. Among the latter, geometric–morphometric approaches provide an excellent alternative that is powerful enough to detect very subtle sexual dimorphisms in the shape of hatchlings at an early age. Geometric–morphometrics has been applied successfully to estimate the sex of hatchlings produced under constant and fluctuating temperature from artificial incubation and from natural nests in *P. expansa* and other turtles, as well as snakes (Valenzuela et al. 2004; Ceballos and Valenzuela 2011; Henao and Ceballos 2013; Ceballos et al. 2014a).

Table 1. Empirical studies with different effects of incubation temperatures on the onset and duration of the thermosensitive period (TSP) in several turtle species. * = authors used 2 feminizing incubation temperatures; ** = authors used 2 masculinizing incubation temperatures.

	Embryonic stage thermosensitive (effect on the onset and/or duration of the TSP)				
	Shift once:		Shift twice:		
Species	Masculinizing to feminizing	Feminizing to masculinizing	Masc – Femin – Masc	Femin – Masc – Femin	Reference
Chelydra serpentina	14–19 (TSP onset not affected)	14–16 or 19 (TSP onset not affected, but duration was either shorter or not affected)*	14–19 (TSP onset not affected)		Yntema (1979)
Chrysemys picta	Laying to stage 16 (TSP duration shorter)	Laying to stage 22 (TSP duration extended)			Bull and Vogt (1981)
Emys orbicularis	< 16–21 (TSP onset not affected)	< 16–19 (TSP onset not affected, but duration was shorter)	16 (TSP onset not affected, but duration was shorter)	16 (TSP onset not affected but duration was longer)	Pieau and Dorizzi (1981)
Graptemys ouachitensis	Laying to stage 16 (TSP ends earlier)	Laying to stage 22 (TSP ends later)			Bull and Vogt (1981)
Malaclemys terrapin			16 (TSP onset hastened)	23 (TSP onset delayed)	Burke and Calichio (2014)
Natator depressus	15–17 or 18 (TSP onset did not change but was shorter or did not change)**	15–18 (TSP onset did not change)			Hewavisenthi and Parmenter (2002)
Trachemys scripta	< 17 (TSP onset delayed)	< 16 (TSP onset hastened)	TSP lasted 1.5 embryonic stages (TSP shorter)	TSP lasted 3 embryonic stages (TSP longer)	Wibbels et al. (1991)

We studied the effect of incubation temperature on the initiation and duration of the TSP in *P. lewyana*. This species exhibits TSD type Ia: 100% males are produced below 33°C, 100% females above 34.7°C, and 1:1 sex ratios at a pivotal temperature of 33.4°C (Páez et al. 2009). *Podocnemis lewyana* is endemic to Colombia and is listed as Endangered (Castaño-Mora 2002; International Union for Conservation of Nature [IUCN] 2015), although the continuing population reduction attributable to human predation and habitat degradation may require relisting it as Critically Endangered (Vargas-Ramírez et al. 2007; Restrepo et al. 2008; Páez et al. 2012).

We incubated eggs under temperatures that were 100% feminizing and 100% masculinizing in a different *P. lewyana* population (Páez et al. 2009) to test whether sex ratios differed in our population (Ceballos et al. 2014b) and to obtain a baseline for comparison with our shift experiments. We estimated the onset and duration of the TSP using 6 shift-twice treatments (masculinizing–feminizing–masculinizing temperatures) as described below. Treatments were designed to induce either a relatively slower or faster developmental rate early in incubation by using a lower or higher masculinizing temperature, followed by a feminizing temperature pulse starting at 1 of 3 different times during incubation (Table 1). Destructive sampling for the direct identification of embryonic stages was precluded in this study because *P. lewyana* is a highly endangered species. Instead, we used time as a proxy of embryonic development and calculated approximate developmental stages based on information from a congeneric species, *P. expansa*, using the cumulative temperature units that *P. lewyana* experienced in this study as a predictor (Valenzuela 2001). Additionally, we developed a nonintrusive geometric–morphometric sexing method for this endangered species and validated its effectiveness to estimate the sex of hatchlings.

METHODS

Study Site. — This study was conducted in the Claro Cocorná Sur River, bordering the town Estación Cocorná in the Municipality of Puerto Triunfo, Department of Antioquia, Colombia. The site is located at an altitude of 150 m above sea level, with average temperatures ranging between 26° and 28°C and annual mean rainfall surpassing 2,000 mm (Caballero-Acosta et al. 2001).

Egg Collection. — During the December 2012 nesting season, we monitored 14 river beaches (Ceballos et al. 2014b) and collected 227 freshly laid eggs from 10 nests. Daily boat trips were made every morning from approximately 0500 to 0800 hrs to collect eggs laid the night before. Eggs were taken to our field station located by the river in the town of Estación Cocorná. Once in the lab, eggs were individually marked, measured (length and width) with a caliper to the nearest 0.1 mm, and weighed with an electronic balance with 0.1 g precision.

Table 2. Experimental design indicating actual incubation temperatures (ºC), incubation period, hatching success, and sex ratio (% females and 9% sexing error). The 2 lighter shadings indicate the 2 masculinizing temperatures while the darkest shading indicates the feminizing temperature. Numbers in bold indicate the time of the TSP (when the confidence interval of % female hatchlings obtained does not encompass zero). * = sex ratio values not statistically different from zero given the error rate of the sexing method.

Treatments (eggs, clutches)	Incubation period (d)							Incubation period	% Hatching success (*n*)	% Females (confidence interval)
	0–10	11–20	21–30	31–40	41–50	51–60	61+			
Control 29°C (31, 4)	29.2 ± 0.8							81.4 ± 0.85	84 (26)	8 (0–17)*
Control 31°C (30, 4)	31.2 ± 0.4							64.3 ± 0.77	57 (17)	18 (9–27)
Control 34.7°C (30, 4)	34.6 ± 1.2							51.1 ± 0.56	73 (22)	86 (77–95)
Slow 20–30 d (20, 2)	29.3 ± 1.5		**35.5 ± 1.2**		29.3 ± 1.5			70.4 ± 0.5	95 (19)	37 (28–46)
Slow 30–40 d (25, 2)	29.4 ± 1.7			**35.1 ± 1.4**	29.4 ± 1.7			70.8 ± 0.4	100 (25)	20 (11–29)
Slow 40–50 d (24, 2)	29.5 ± 1.7				35.3 ± 1.8	29.5 ± 1.7		71.8 ± 0.48	100 (24)	4 (0–13)*
Fast 20–30 d (19, 2)	31.3 ± 1.3		**35.5 ± 1.2**	31.3 ± 1.3				57.1 ± 0.33	100 (19)	58 (49–67)
Fast 30–40 d (24, 2)	31.4 ± 1.3			**35.1 ± 1.4**	31.4 ± 1.3			56.5 ± 0.77	79 (19)	32 (23–41)
Fast 40–50 d (24, 2)	31.3 ± 1.3				**35.3 ± 1.8**	31.3 ± 1.3		59.5 ± 2.22	100 (24)	67 (58–76)

Experimental Design. — Eggs were selected randomly from the clutches as they were being collected in the field and allocated systematically to the incubation treatments until all eggs from the same clutch were used. We first filled the 3 constant-temperature treatments (following Páez et al. 2009) and then the 6 shift-twice treatments. Temperature in the constant treatments varied slightly such that the temperatures that eggs experienced were 29.2°C ± 0.8°C (Control 29°C), 31.2°C ± 0.4°C (Control 31°C), and 34.6°C ± 1.2°C (Control 34.7°C).

To estimate the onset and duration of the TSP, we used a shift-twice design: eggs were exposed to a masculinizing temperature at the beginning of incubation and groups of eggs were later switched to a feminizing temperature starting at different times during the incubation period, then returned to the original masculinizing temperature until hatching (Table 2). Two alternatives were tested by using 2 masculinizing temperatures: 29°C (which promoted a slower embryonic development) and 31°C (which promoted a relatively faster embryonic development). For both masculinizing temperatures, the feminizing temperature pulse was applied during 1 of 3 10-d periods: days 21–30, 31–40, or 41–50 of incubation, each of which falls within the middle third of the incubation period (Bull 1980; Wibbels et al. 1994; Valenzuela 2001).

We incubated eggs in plastic containers using the original sand where eggs were laid in the nesting beach as incubation substrate (Ceballos et al. 2014b). Although the moisture content of the sand was not quantified, the initial moisture level was maintained by weekly replenishing lost water as determined by the weight of the container. To account for any potential temperature clines within the incubators, we rotated the containers weekly, from front to back and in clockwise fashion. We monitored the temperature within the incubators hourly throughout the incubation period using 3–4 HOBO JR5 Temp dataloggers (Onset Computer Corporation; accuracy 0.1°C). Hatchlings were removed from the incubators once their remnant yolk was internalized (between 5 and 7 d after pipping), weighed and measured (straight-line length and width of the carapace and plastron), and photographed individually. Hatchlings were tagged by attaching 3 colored beads to the 10th marginal scute of the carapace (Galbraith and Brooks 1984), raised in captivity for 3–4 mo, and released back in their natal river.

Development of a Geometric–Morphometric Sexing Method. — The sex of 20 individuals incubated at constant temperatures was assessed by gonadal histology 3.5 mo after hatching, and results matched the expectations (Sánchez-Ospina et al. 2014): all 10 individuals from the feminizing temperature (34.7°C) were females, and all 10 from the 2 masculinizing temperatures (5 from 29°C and 5 from 31°C) were males. These individuals were then used to estimate the sex of the remaining hatchlings whose sex was unknown. For this, we quantified the shape of the carapace, plastron, and anal notch of the 20 individuals of known sex (prior to dissection) using geometric morphometrics and calculated discriminant functions based on their shape to distinguish males from females (Valenzuela et al. 2004; Ceballos et al. 2014a).

To quantify the shape of the carapace, plastron, and anal notch, all turtles were photographed at 7 d (hereafter referred to as hatchlings) and 3.5 mo of age (hereafter referred to as juveniles) with an Olympus SP-500 UZ digital camera. The number of hatchlings (n = 164) differed from the number of juveniles (n = 147) because some individuals escaped from the enclosure or were depredated by birds. We used these photographs to digitize 29 fixed landmarks at the intersections of the scutes of the carapace, 21 fixed landmarks in the plastron, and 7 fixed and 12 sliding landmarks (Bookstein 1996) in the contour of the anal notch (Fig. 1). Five individuals with deformities or supernumerary scutes were excluded from analysis. We then performed a Generalized Procrustes

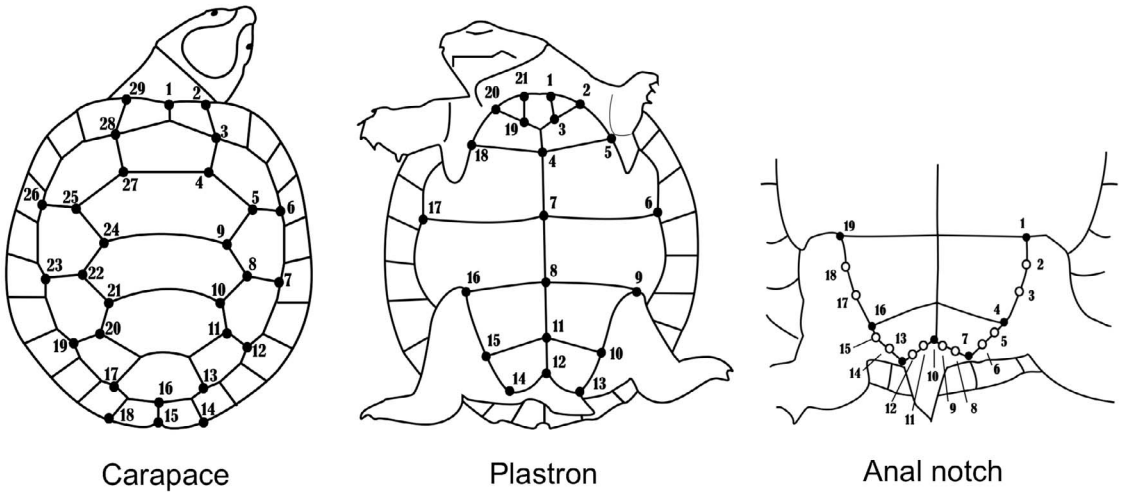

Figure 1. Landmarks digitalized in photographs of the carapace, plastron, and anal notch of *Podocnemis lewyana* hatchlings and juveniles used to quantify shapes with geometric morphometrics. Filled symbols indicate fixed landmarks and open symbols sliding landmarks (modified from Ceballos et al. 2014a).

Analysis (Rohlf and Slice 1990) to obtain centroid size (a surrogate for carapace and plastron sizes; Bookstein 1996) and calculated shape variables for each of the 3 anatomical body parts: 54 variables from the carapace, 38 from the plastron, and 34 from the anal notch (Fig. 2). Morphometric analyses were performed using TpsDig, TpsRelw, TpsUtil, and TpsSplin software (Rohlf 2001, 2003, 2004).

To estimate the sex of turtles of unknown sex, we calculated discriminant functions using the shape of the carapace, plastron, and anal notch at both ages for the 20 individuals sexed by histology and estimated the percentage of correct classification (Valenzuela et al. 2004). Cross-validation of these functions was performed by using 75% of these individuals of known sex to calculate a discriminant function that was then used to predict the sex of the remaining 25%, which was then compared with their true sex. Cross-validation analyses were done with the function "Predict" from R package MASS (Venables and Ripley 2002).

We used the morphological trait whose function had the highest cross-validation rate to estimate the sex of the remaining individuals. To assess the significance of the classification, we used a Procrustes ANOVA, which is a linear model that uses the linear distances among individuals instead of using the covariance matrix and is identical to running a permutational MANOVA (Adams and Otárola-Castillo 2013). Procrustes ANOVA is advised when sample size is low (20 individuals whose true sex is known) relative to the number of shape variables. These linear models were built using sex as main factor; carapace, plastron, or anal notch shape as response variables; and clutch of origin as covariate to account for maternal effects (Páez et al. 2009). Analyses were conducted using the function procD.lm in the statistical package Geomorph (Adams and Otárola-Castillo 2013) in software R version 3.0.1 (R Development Core Team 2013).

Ontogeny of Sexual Dimorphism. — We characterized sexual size and shape dimorphism in hatchlings (n = 164) and juveniles (n = 147) as reported for its congener *P. expansa* (Ceballos et al. 2014a) to identify subtle differences that could be used as diagnostics for the nonlethal sexing of hatchlings.

Embryonic Development. — As a proxy for the embryonic stage that *P. lewyana* may have reached in each of the 10-d periods of the experimental treatments in this study, we used a statistical model developed for the congener *P. expansa* (Valenzuela 2001) that accounts for the cumulative effect of temperature on development. Daily rates at which cumulative temperature units (CTUs) accrue is a strong predictor of the days it takes for the embryos to reach particular developmental stages in *P. expansa*, and the thermosensitive period in this species occurs approximately between developmental stages 17 and 24 (Valenzuela 2001). Thus, assuming that the developmental biology of *P. lewyana* resembles that of *P. expansa*, we calculated the daily CTUs accumulated by *P. lewyana* in each of the 10-d periods of the experimental treatments in this study and predicted whether the embryos had reached or surpassed stages 17 and 24 of development using the equations for *P. expansa* (Valenzuela 2001, fig. 8). Mean daily CTUs were calculated by subtracting 28°C (the developmental zero temperature for *P. expansa*) from each hourly temperature recorded, summing the daily CTUs, and dividing by the number of days of the period (20, 30, 40, and 50 d).

RESULTS

Nonintrusive Geometric–Morphometric Sexing and Sexual Dimorphism. — Results from our sexing procedure

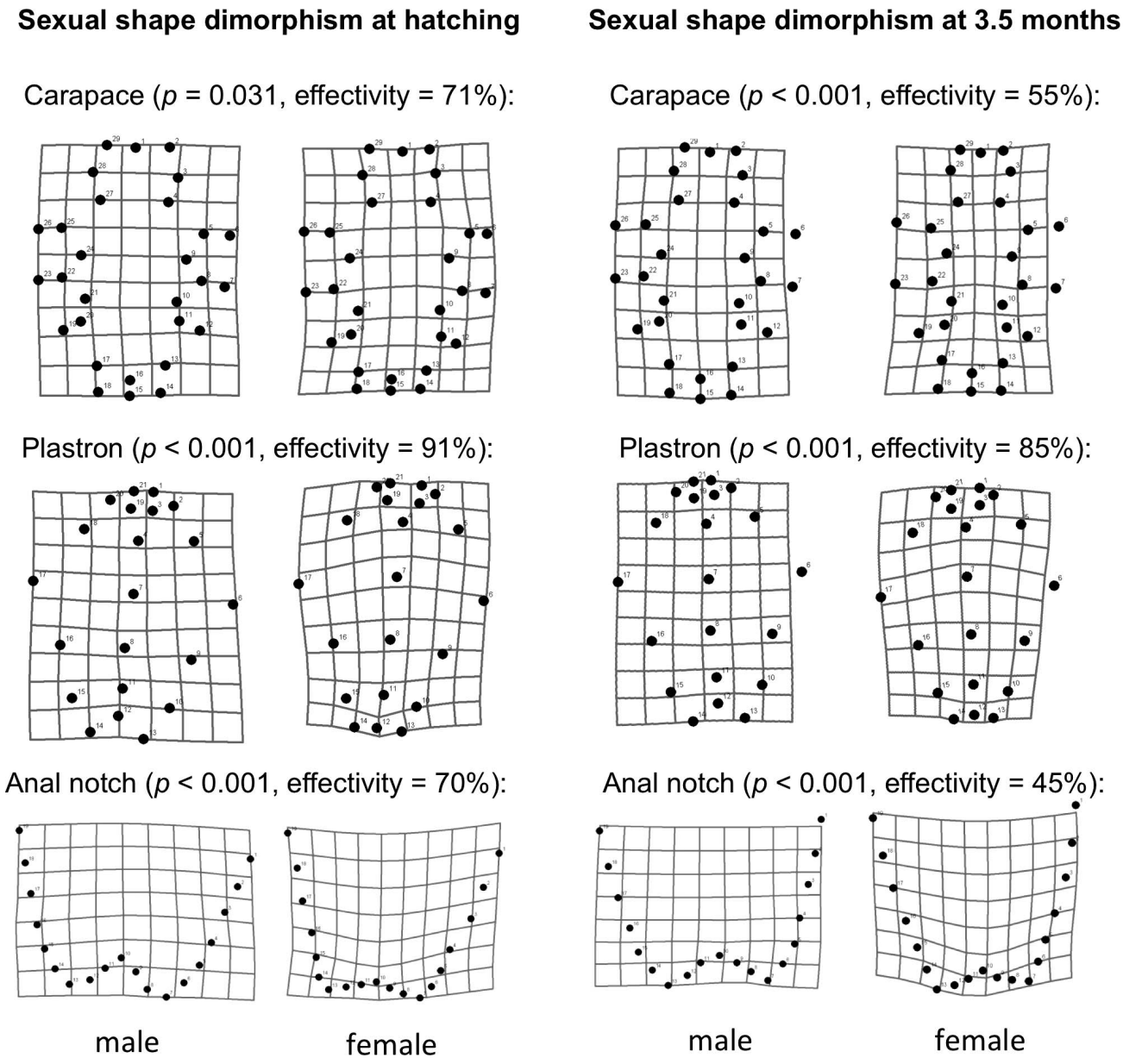

Figure 2. Sexual shape dimorphism of carapace, plastron, and anal notch at 2 ages of *Podocnemis lewyana*. These are the average shapes (×3 magnification) of individuals for which sex was histologically confirmed.

indicated that the plastron shape of hatchlings was the best sex diagnostic because it had the highest cross-validation rate (91%), followed by plastron shape of juveniles (85%; Fig. 2). Thus, we used the plastron shape of hatchlings to classify individuals of unknown sex as male or female and estimated the sex ratio (% females) obtained from our 9 incubation treatments (Table 2).

Sexual size and shape dimorphism changed over time and differentially for distinct body parts. Namely, the carapace and plastron size were monomorphic at hatching ($p = 0.335$ and 0.192, respectively) but became sexually dimorphic by the third month of age ($p < 0.001$; Table 3) when females were larger than males by 4.5% for the carapace and 6.4% for the plastron. Contrastingly, the carapace, plastron, and anal notch were sexually dimorphic in shape at hatching, and differences became more pronounced by the third month of age ($p < 0.05$ and $p < 0.001$, respectively; Table 4). Specifically, males exhibited a wider carapace and plastron and a more acute angle of the anal notch than females, differences which became accentuated with age (Fig. 2).

Effect of Constant Incubation Temperatures on Sex Ratios. — The sex ratio obtained in our constant temperature treatments varied slightly from those previously reported (28°–33°C ± 0.4°C = 100% males and 34.7°C ± 0.4°C = 100% females; Páez et al. 2009). The sex ratio from our Control 29°C treatment was 7.7% ± 9% (0%–17%) females; thus, it did not differ from the expected 0% females. However, sex ratios from our Control 31°C and Control 34°C were 18% ± 9% females (9%–27%) and

Table 3. ANOVA test of the effect of sex and clutch of origin on the size of *Podocnemis lewyana* at 7 d and 3.5 mo of age.

Model	df	SS	MS	*F*	*p*	Group means (centroid size)
Carapace at 7 d						
Sex	1	6.86	6.86	0.93	0.34	Female: 96.53 Male: 96.94
Clutch	9	2122.72	235.86	32.03	< 0.001	
Residuals	168	1237.00	7.36			
Carapace at 3.5 mo						
Sex	1	1208.60	1208.58	24.99	< 0.001	Female: 128.53 Male: 122.76
Clutch	9	3986.30	442.93	91.57	< 0.001	
Residuals	151	7303.70	48.37			
Plastron at 7 d						
Sex	1	7.14	7.14	1.72	0.19	Female: 79.49 Male: 79.09
Clutch	9	1740.94	193.44	46.48	< 0.001	
Residuals	173	719.93	4.16			
Plastron at 3.5 mo						
Sex	1	1884.70	1884.69	38.46	< 0.001	Female: 109.92 Male: 102.84
Clutch	9	4829.90	536.66	10.95	< 0.001	
Residuals	156	7645.00	49.01			

86% ± 9% females (77%–95%); thus, they differed from the expected 100% males and 100% females, respectively. These results indicate that our observed temperatures (31.2°C ± 0.4°C and 34.6°C ± 1.2°C) fall within the transitional range (values producing > 0% and < 100% females) for our population (Table 2).

Effect of Shift-Twice Temperatures on Sex and the Onset and Duration of the TSP. — In general, sex ratios from the slow-development shift experiments (29°–34°–29°C) were more male-biased than those from the fast-development shift experiments (31°–34°–31°C; Table 2). The sex ratios observed in our slow-shift experiment indicate that the TSP included days 20–40 of the incubation period because some females were produced in these treatments when none were expected if the TSP had been inactive (Table 2). This TSP encompassed 28% of the incubation period (71 d) and fell roughly within the middle third. On the other hand, the sex ratios observed in the fast-shift experiment indicate that the TSP included days 20–50 of the incubation period, again because some females were produced in all 3 treatments (> 18% females produced in the Control 31°C treatment). This TSP encompassed 52% of the incubation period (58 d) and fell within the middle half.

The difference between the average baseline temperatures of the slow (29.3) and fast (31.3) treatments suggests that 2°C accelerated embryo development 13.3 d on average. Additionally, within each of these 2 treatment types, there was a trend for embryonic development to take longer with later onset of exposure to the feminizing temperature pulse (Table 2). Interestingly, the timing of exposure to the feminizing temperature had a different effect on sex ratios in the slow and fast treatments. Namely, more males were produced at delayed feminizing pulses in the 29°–34°–29°C treatments, whereas more males were produced at the intermediate pulse time in the 31°–34°–31°C treatments (Table 2).

Embryonic Development. — The approximate embryonic stages presumably attained by *P. lewyana* in this study, calculated using the model of *P. expansa*, were somewhat concordant with our sex-ratio observations obtained in the slow- and fast-shift treatments, with differences mostly about the end of the TSP (Table 5). According to our approximation: 1) *P. lewyana* embryos in the slow treatments had not reached stage 17 by day 20 and would have been between stages 17 and 24 before day 50. This result supports our finding that the TSP was active

Table 4. MANOVA tests of the effect of sex and nest of origin in the body shape of *Podocnemis lewyana* at 7 d and 3.5 mo of age.

Model	df	Pillai	$F_{num,den}$	*p*
Carapace at 7 d				
Sex	1	0.416	$1.519_{54,115}$	0.031
Clutch	9	5.719	$3.970_{486,1107}$	< 0.001
Residuals	168			
Carapace at 3.5 mo				
Sex	1	0.628	$3.069_{54,98}$	< 0.001
Clutch	9	5.872	$3.686_{486,954}$	< 0.001
Residuals	151			
Plastron at 7 d				
Sex	1	0.737	$10.044_{38,136}$	< 0.001
Clutch	9	4.843	$4.416_{342,1296}$	< 0.001
Residuals	173			
Plastron at 3.5 mo				
Sex	1	0.583	$4.388_{38,119}$	< 0.001
Clutch	9	5.111	$4.394_{342,1143}$	< 0.001
Residuals	156			
Anal notch at 7 d				
Sex	1	0.403	$2.788_{34,140}$	< 0.001
Clutch	9	3.616	$2.923_{306,1332}$	< 0.001
Residuals	173			
Anal notch at 3.5 mo				
Sex	1	0.391	$2.331_{34,123}$	< 0.001
Clutch	9	3.765	$2.771_{306,1179}$	< 0.001
Residuals	156			

Table 5. Approximate embryonic stages attained by *Podocnemis lewyana* embryos based on models from the congeneric species *Podocnemis expansa* (Valenzuela 2001).[a]

Treatment	Period	X (daily CTUs, this study)	Y_{17} (days to reach stage 17)	Y_{24} (days to reach stage 24)	Approx. embryonic stage in *P. lewyana*	Relative TSP in *P. expansa*
Slow 20–30	0–20	17.54	31.18	66.20	< 17	Before TSP
	0–30	60.8	20.35	48.34	$\ll 24$	TSP
	0–40	53.51	21.73	50.61	$\ll 24$	TSP
	0–50	44.83	23.61	53.71	< 24	TSP
Slow 30–40	0–20	13.81	32.42	68.23	< 17	Before TSP
	0–30	13.24	32.61	68.54	~ 17	Close to TSP
	0–40	45.56	23.44	53.43	$\ll 24$	TSP
	0–50	40.94	24.53	55.23	< 24	TSP
Slow 40–50	0–20	13.81	32.42	68.23	< 17	Before TSP
	0–30	13.24	32.61	68.54	~ 17	Close to TSP
	0–40	14.25	32.27	67.98	> 17	TSP
	0–50	41.21	24.46	55.13	< 24	TSP
Fast 20–30	0–20	66.26	19.43	46.83	17	TSP
	0–30	93.28	16.39	41.82	$\ll 24$	TSP
	0–40	88.66	16.73	42.38	< 24	TSP
	0–50	83.8	17.17	43.11	$\gg 24$	After TSP
Fast 30–40	0–20	65.65	19.53	46.99	17	TSP
	0–30	64.87	19.65	47.20	$\ll 24$	TSP
	0–40	84.28	17.13	43.03	< 24	TSP
	0–50	82.01	17.36	43.41	$\gg 24$	After TSP
Fast 40–50	0–20	65.65	19.53	46.99	17	TSP
	0–30	64.87	19.65	47.20	$\ll 24$	TSP
	0–40	64.56	19.70	47.28	24	TSP
	0–50	81.46	17.41	43.51	$\gg 24$	After TSP

[a] Note: $Y_{17} = 0.0017x^2 - 0.3837x + 37.39$ and $Y_{24} = 0.0028x^2 - 0.6322x + 76.424$ (Valenzuela 2001). The TSP of *Podocnemis expansa* is active between embryonic stages 17 and 24. Interpretation: In the Slow 20–30 treatment, by day 20 embryos had experienced 17.54 CTUs daily on average, and to reach embryonic stage 17/24, they would need 31.18/66.2 d. Thus, by day 20 embryos had not even reached stage 17. Under those conditions *P. expansa* embryos would not have reached the TSP.

during days 20–40 because embryos are estimated to have reached stage 17. However our data shows that the TSP in *P. lewyana* had ended by day 40, which would be earlier than stage 24 (the end of the TSP in *P. expansa*, which would have been reached by day 50 under the rate of CTU accumulation experienced by *P. lewyana* in this treatments). 2) *Podocnemis lewyana* embryos in the fast treatments would have reached around stage 17 by day 20, were estimated to be between stages 17 and 24 until day 40, and likely surpassed stage 24 by day 50. Therefore, given the estimated developmental stages and the TSP of *P. expansa*, the TSP of *P. lewyana* should have been active between days 20 and 40. Yet our sex-ratio data show that the TSP of *P. lewyana* was active for a longer period, between days 20 and 50 (past the estimated stage 24 of *P. lewyana*). Potential explanations for these discrepancies are that developmental rates for *P. lewyana* and *P. expansa* are different, such that estimating embryonic stages in *P. lewyana* from *P. expansa* is not entirely accurate or that the TSP differs between both species.

DISCUSSION

Nonintrusive Estimation of Individual Sex by Geometric–Morphometric Techniques. — Diagnosing the sex of individuals is important to study the ecology and evolution of species, and nonintrusive methods are particularly crucial to monitor the sex ratios of endangered species. Molecular methods have been developed for some taxa (reviewed in Literman et al. 2014), including *Podocnemis* (Lance et al. 1992), but they can be cumbersome and expensive and may require highly specialized equipment. Geometric–morphometric techniques are extremely powerful to compare the shape of individuals based on photographic records alone and can distinguish very subtle differences that are undetectable to the naked eye. Here, we developed an effective geometric–morphometric sexing method for *P. lewyana* and validated its accuracy at estimating individual sex with a subset of hatchlings whose sex was identified by gonadal histology. We found that sexing via geometric–morphometric comparison of the plastron shape of hatchlings was highly accurate (91% rate of cross-validation), even more so than in previous studies in *P. expansa*, in which the correct cross-validation values ranged between 75% and 90% (Valenzuela et al. 2004; Ceballos et al. 2014a). It should be noted that a margin of error still exists (albeit small) when estimating sex ratios using this approach. Geometric–morphometric techniques have been successfully used previously to sex young hatchlings of the congener *P. expansa* obtained under 1) constant temperatures, 2) simple artificially fluctuating profiles, and 3) naturally fluctuating temperatures in the wild (Valenzuela 2001; Ceballos et al. 2014a). As such, the approach we used to sex *P. lewyana* hatchlings from our experiments should apply equally well to sexing hatchlings from natural nests.

Podocnemis lewyana *Populations Differ in Their Sex Ratio Response to Temperature*. — Despite the thermal variability experienced in the Control 29°C (28.4°–30°C) and Control 31°C (30.8°–31.6°C) treatments, these temperatures were within the range of values reported as 100% masculinizing for a northern Magdalena River population (27.6°–33.4°C in Páez et al. 2009). However, sex ratios from our study population from the Claro Cocorná Sur River, a southern tributary of the Magdalena River, were more feminized (i.e., needed less accumulated heat to produce females) than those from the north. These discrepancies are not attributable to differences in mortality rates, which were comparable between studies, and instead suggest that these populations, which are approximately 475 km of river distance apart, may differ in their thermal sensitivity.

Geographic differences in the TSD reaction norm have been reported in many turtles and may have a genetic basis (Bull et al. 1982; Ewert et al. 2005; Burke and Calichio 2014; Rhen et al. 2015). The population differences observed in the present study may reflect local adaptation, particularly considering that the northern population inhabits a region with warmer climate (mean ambient temperature 31°C, elevation 33 m a.s.l.), compared with our southern population (mean ambient temperature 28°C, elevation 150 m a.s.l.). However, previous studies have detected low genetic variability and weak population differentiation in this species, which are most likely attributable to genetic drift (Vargas-Ramírez et al. 2007, 2012), such that other causes should also be considered. For instance, differences between populations may also be attributable to variation in maternal allocation of yolk hormones, as has been reported for *P. lewyana* (Páez et al. 2015) and other turtles (Janzen et al. 1998; Bowden et al. 2000; Ramsey and Crews 2007), or even trans-generational effects of incubation temperature reported in other reptile species (i.e., inheritance of the parents' thermal sensitivity at which their sex was determined during embryonic development; Warner et al. 2013). Further research is necessary to test these hypotheses.

Initial Incubation Temperatures Affect the Duration but Not the Onset of the TSP. — Duration of the TSP was influenced by the baseline temperature used in the shift experiments, such that the higher masculinizing temperature (31°C) induced a longer-lasting TSP than using 29°C as the base temperature. Similarly, in *E. orbicularis*, shifting from a lower baseline to higher temperature induced a longer TSP, and shifting from a higher baseline to a lower temperature induced a shorter TSP (Pieau and Dorizzi 1981). Furthermore, in *Malaclemys terrapin*, the TSP occurred in the middle third of incubation in eggs maintained at 25°C, but it occurred during the last third of incubation in eggs kept at 31°C (Burke and Calichio 2014).

On the other hand, the finding that the fast and slow treatments with feminizing temperature pulses at days 21–30 produced females suggests that temperatures experienced early in development affected the duration of the TSP, but we found no evidence of an effect of early temperatures on the onset of the TSP. However, it should be noted that our experimental design does not permit an absolute identification of the onset of the TSP because all of our pulse experiments produced some females. In other words, our data indicate that the TSP was active some time during days 21–30, but our data do not rule out the TSP having already been active by day 21. Thus, future research using earlier pulses and shift-one experiments will be needed to narrow down the exact onset and the end of the TSP.

Our findings have important implications for conservation because understanding sex determination is necessary to evaluate management programs of endangered reptiles with TSD to avoid or mitigate the effects of climate change. Given that the onset and duration of the TSP may vary depending on the temperatures experienced early in development as observed here, we recommend that conservation programs monitor the incubation temperature throughout the entire incubation period to better predict sex-ratio production. Our results also underscore the need for further research to understand the effect of fluctuating temperatures on sex determination in this and other endangered TSD taxa facing climate change (Neuwald and Valenzuela 2011). The geometric–morphometric approach developed here to sex *P. lewyana* should aid sex-ratio monitoring in the lab and in the wild and, thus, constitutes an important contribution to the toolkit for the conservation of this charismatic turtle.

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LITERATURE CITED

Adams, D.C. and Otárola-Castillo, E. 2013. Geomorph: an R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution 4:393–399.

Bonach, K., Malvasio, A., Matushima, E.R., and Verdade, L.M. 2011. Temperature-sex determination in *Podocnemis expansa* (Testudines, Podocnemididae). Iheringia Serie Zoologia 101: 151–155.

Bookstein, F.L. 1996. Landmark methods for forms without landmarks: localizing group differences in outline shape. In: Kavanaugh, M.E (Ed.). Proceedings of the IEEX Xplore Workshop on Mathematical Methods in Biomedical Image Analysis. San Francisco: IEEE Computer Society Press, 339 pp.

BOWDEN, R.M., EWERT, M.A., AND NELSON, C.E. 2000. Environmental sex determination in a reptile varies seasonally and with yolk hormones. Proceedings of the Royal Society of London Series B 267:1745–1749.

BULL, J.J. 1980. Sex determination in reptiles. Quarterly Review of Biology 55:3–22.

BULL, J.J. AND VOGT, R.C. 1981. Temperature-sensitive periods of sex determination in emydid turtles. Journal of Experimental Zoology 218:435–440.

BULL, J.J., VOGT, R.C., AND MCCOY, C.J. 1982. Sex determining temperatures in turtles: a geographic comparison. Evolution 36:326–332.

BURKE, R.L. AND CALICHIO, A.M. 2014. Temperature-dependent sex determination in the diamond-backed terrapin (*Malaclemys terrapin*). Journal of Herpetology 48:466–470.

CABALLERO-ACOSTA, H., DURANGO-LÓPEZ, C., AND GIRALDO-CASTRO, C.A. 2001. Los humedales del Magdalena Medio antioqueño desde la perspectiva física y sociocultural. Gestión y Ambiente 4:67–79.

CASTAÑO-MORA, O.V. 2002. Libro Rojo de Reptiles de Colombia. Bogotá: Instituto de Ciencias Naturales, Universidad Nacional, Ministerio del Medio Ambiente, Conservación Internacional, 160 pp.

CEBALLOS, C.P., HERNÁNDEZ, O.E., AND VALENZUELA, N. 2014a. Divergent sex-specific plasticity in long-lived vertebrates with contrasting sexual dimorphism. Evolutionary Biology 41:81–98.

CEBALLOS, C.P., ROMERO, I., GÓMEZ-SALDARRIAGA, C., AND MIRANDA-RÍOS, K. 2014b. Reproduction and conservation of the Magdalena River turtle (*Podocnemis lewyana*) in the Claro Cocorná Sur River, Colombia. Acta Biológica Colombiana 19: 427–435.

CEBALLOS, C.P. AND VALENZUELA, N. 2011. The role of sex-specific plasticity in shaping sexual dimorphism in a long-lived vertebrate, the snapping turtle *Chelydra serpentina*. Evolutionary Biology 38:163–181.

EWERT, M.A., LANG, J.W., AND NELSON, C.E. 2005. Geographic variation in the pattern of temperature-dependent sex determination in the American snapping turtle (*Chelydra serpentina*). Journal of Zoology 265:81–95.

EWERT, M.A. AND NELSON, C.E. 1991. Sex determination in turtles: diverse patterns and some possible adaptative values. Copeia 1991:50–69.

GALBRAITH, D.A. AND BROOKS, R.J. 1984. A tagging method for use in hatchling turtles. Herpetological Review 15:73–75.

GEORGES, A., BEGGS, K., YOUNG, J.E., AND DOODY, J.S. 2005. Modelling development of reptile embryos under fluctuating temperature regimes. Physiological and Biochemical Zoology 78:18–30.

HENAO, A.M. AND CEBALLOS, C.P. 2013. Sexual size and shape dimorphism in head characteristics in terciopelo snakes, *Bothrops asper*. Revista Colombiana de Ciencias Pecuarias 26:201–210.

HEWAVISENTHI, S. AND PARMENTER, C.J. 2002. Thermosensitive period for sexual differentiation of the gonads of the flatback turtle (*Natator depressus* Garman). Australian Journal of Zoology 50:521–527.

INTERNATIONAL UNION FOR CONSERVATION OF NATURE (IUCN). 2015. Red List of Threatened Species. Magdalena River turtle (*Podocnemis lewyana*). http://www.iucnredlist.org/details/17823/10 (5 April 2016).

JANZEN, F.J. 1994. Climate change and temperature-dependent sex determination in reptiles. Proceedings of the National Academy of Sciences 91:7487–7490.

JANZEN, F.J., WILSON, M.E., TUCKER, J.K., AND FORD, S.P. 1998. Endogenous yolk steroid hormones in turtles with different sex-determining mechanisms. General Comparative Endocrinology 111:306–317.

JOHNSTON, C.M., BARNETT, M., AND SHARPE, P.T. 1995. The molecular biology of temperature-dependent sex determination. Philosophical Transactions of the Royal Society of London Series B—Biological Sciences 350:297–303.

LANCE, V.A., VALENZUELA, N., AND VON HILDEBRAND, P. 1992. A hormonal method to determine sex of hatchling giant river turtles, *Podocnemis expansa*: application to endangered species. Journal of Experimental Zoology 270:16A.

LE BLANC, A.M., WIBBELS, T., SHAVER, D., AND WALKER, J.S. 2012. Temperature-dependent sex determination in the Kemp's ridley sea turtle: effects of incubation temperature on sex ratios. Endangered Species Research 19:123–128.

LITERMAN, R., BADENHORST, D., AND VALENZUELA, N. 2014. qPCR-based molecular sexing by copy number variation in rRNA genes and its utility for sex identification in soft-shell turtles. Methods in Ecology and Evolution 5:872–880.

NEUWALD, J.L. AND VALENZUELA, N. 2011. The lesser known challenge of climate change: thermal variance and sex-reversal in vertebrates with temperature-dependent sex determination. PLoS One 6:e18117.

PÁEZ, V.P., CORREA, J.C., CANO, A.M., AND 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 2009:698–704.

PÁEZ, V.P., ECHEVERRI, L., BOCK, B.C., BOWDEN, R.M., AND 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.

PÁEZ, V.P., RESTREPO, A., VARGAS-RAMÍREZ, M., BOCK, B.C., AND GALLEGO-GARCÍA, N. 2012. *Podocnemis lewyana*. In: Páez, V.P., Morales-Betancourt, M.A., Lasso, C.A., Castaño-Mora, O.V., and Bock, B.C. (Eds.). V. Biología y Conservación de las Tortugas Continentales de Colombia. Bogotá: Instituto de Investigación de Recursos Biológicos Alexander von Humboldt (IAvH), pp. 375–381.

PIEAU, C. AND DORIZZI, M. 1981. Determination of temperature sensitive stages for sexual differentiation of the gonads in embryos of the turtle, *Emys orbicularis*. Journal of Morphology 170:373–382.

RAMSEY, M. AND CREWS, D. 2007. Steroid signaling system responds differently to temperature and hormone manipulation in the red-eared slider turtle (*Trachemys scripta elegans*), a reptile with temperature-dependent sex determination. Sexual Development 1:181–196.

R DEVELOPMENT CORE TEAM. 2013. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. http://www.R-project.org (1 July 2013).

RESTREPO, A., PÁEZ, V.P., LÓPEZ, C., AND BOCK, B.C. 2008. Distribution and status of *Podocnemis lewyana* in the Magdalena River drainage of Colombia. Chelonian Conservation and Biology 7:45–51.

RHEN, T., FAGERLIE, R., SCHROEDER, A., CROSSLEY, D.A., II, AND LANG, J.W. 2015. Molecular and morphological differentiation of testes and ovaries in relation to the thermosensitive period of gonad development in the snapping turtle, *Chelydra serpentina*. Differentiation 89:31–41.

ROHLF, F.J. 2001. TpsDig, version 1.31. http://life.bio.sunysb.edu/morph/.

ROHLF, F.J. 2003. TpsRelw, version 1.29. http://life.bio.sunysb.edu/morph/.

ROHLF, F.J. 2004. TpsSplin, version 1.20. http://life.bio.sunysb.edu/morph/.

ROHLF, F.J. AND SLICE, D. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. Systematic Zoology 39:40–59.

ROMERO, I. 2011. Proyecto de Conservación de la tortuga de río *Podocnemis lewyana* en la Cuenca baja del río Claro Cocorná Sur. Estación Cocorná, Colombia: CORANTIOQUIA, CORNARE, Asociación Ambientalista Futuro Verde AFUVER, 40 pp.

SÁNCHEZ-OSPINA, A., RODRÍGUEZ, B., AND CEBALLOS, C.P. 2014. Histological description of the reproductive system of male and female hatchlings of the Magdalena River turtle (*Podocnemis lewyana*). Acta Biológica Colombiana 19:427–435.

THE TREE OF SEX CONSORTIUM. 2014. Tree of Sex: a database of sexual systems. Scientific Data 1: 140015. doi:10.1038/sdata.2014.15.

VALENZUELA, N. 2001. Constant, shift, and natural temperature effects on sex determination in *Podocnemis expansa*. Ecology 82:3010–3024.

VALENZUELA, N., ADAMS, D.C., BOWDEN, R.M., AND GAUGER, A.C. 2004. Geometric morphometric sex estimation for hatchling turtles: a powerful alternative for detecting subtle sexual shape dimorphism. Copeia 2004:735–742.

VALENZUELA, N. AND LANCE, V.A. 2004. Temperature Dependent Sex Determination in Vertebrates. Washington, DC: Smithsonian Books, 200 pp.

VARGAS-RAMÍREZ, M., CHIARI, Y., CASTAÑO-MORA, O.V., AND MENKEN, S.B.J. 2007. Low genetic variability in the endangered Colombian endemic freshwater turtle *Podocnemis lewyana* (Testudines, Podocnemididae). Contributions to Zoology 76:1–7.

VARGAS-RAMÍREZ, M., STUCKAS, H., CASTAÑO-MORA, O.V., AND FRITZ, U. 2012. Extremely low genetic diversity and weak population differentiation in the endangered Colombian river turtle *Podocnemis lewyana* (Testudines: Podocnemididae). Conservation Genetics 13:65–77.

VENABLES, W.N. AND RIPLEY, B.D. 2002. Modern Applied Statistics with S. New York: Springer, 495 pp.

WARNER, D.A., ULLER, T., AND SHINE, R. 2013. Transgenerational sex determination: the embryonic environment experienced by a male affects offspring sex ratio. Scientific Reports 3:2709.

WIBBELS, T., BULL, J.J., AND CREWS, D. 1991. Chronology and morphology of temperature-dependent sex determination. Journal of Experimental Zoology 260:371–381.

WIBBELS, T., BULL, J.J., AND CREWS, D. 1994. Temperature-dependent sex determination—a mechanistic approach. Journal of Experimental Zoology 270:71–78.

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

YNTEMA, C.L. 1979. Temperature levels and periods of sex determination during incubation of eggs of *Chelydra serpentina*. Journal of Morphology 159:17–28.

YOUNG, J.E., GEORGES, A., DOODY, J.S., WEST, P.B., AND ALDERMAN, R.L. 2004. Pivotal range and thermosensitive period of the pig-nosed turtle, *Carettochelys insculpta* (Testudines: Carettochelydidae), from northern Australia. Canadian Journal of Zoology 82:1251–1257.

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