

Incubation Temperature and Maternal Resource Provisioning, but Not Contaminant Exposure, Shape Hatchling Phenotypes in a Species with Temperature-Dependent Sex Determination

SAMANTHA L. BOCK^{1,2}, MATTHEW D. HALE^{1,2,3}, THOMAS R. RAINWATER^{4,5},
PHILIP M. WILKINSON⁵, AND BENJAMIN B. PARROTT^{1,2,*}

¹Eugene P. Odum School of Ecology, University of Georgia, 140 E. Green Street, Athens, Georgia 30602; ²Savannah River Ecology Laboratory, P.O. Drawer E, Aiken, South Carolina 29803; ³Department of Biology, University of Virginia, 485 McCormick Road, Charlottesville, Virginia 22904; ⁴Belle W. Baruch Institute of Coastal Ecology and Forest Science, Clemson University, Georgetown, South Carolina 29442; and ⁵Tom Yawkey Wildlife Center, Georgetown, South Carolina 29440

Abstract. The environment experienced during embryonic development is a rich source of phenotypic variation, as environmental signals have the potential to both inform adaptive plastic responses and disrupt normal developmental programs. Environment-by-embryo interactions are particularly consequential for species with temperature-dependent sex determination, a mode of sex determination common in non-avian reptiles and fish, in which thermal cues during a discrete period of development drive the formation of either an ovary or a testis. Here we examine the impact of thermal variation during incubation in combination with developmental exposure to a common endocrine-disrupting contaminant on fitness-related hatchling traits in the American alligator (*Alligator mississippiensis*), a species with temperature-dependent sex determination. Using a factorial design, we exposed field-collected eggs to five thermal profiles (three constant temperatures, two fluctuating temperatures) and two environmentally relevant doses of the pesticide metabolite dichlorodiphenyldichloroethylene; and we quantified incubation duration, sex ratios, hatchling morphometric traits, and growth (9–10 days post-hatch). Whereas di-

chlorodiphenyldichloroethylene exposure did not generally affect hatchling traits, constant and fluctuating temperatures produced diverse phenotypic effects. Thermal fluctuations led to subtle changes in incubation duration and produced shorter hatchlings with smaller heads when compared to the constant temperature control. Warmer, male-promoting incubation temperatures resulted in larger hatchlings with more residual yolk reserves when compared to cooler, female-promoting temperatures. Together, these findings advance our understanding of how complex environmental factors interact with developing organisms to generate phenotypic variation and raise questions regarding the mechanisms connecting variable thermal conditions to responses in hatchling traits and their evolutionary implications for temperature-dependent sex determination.

Introduction

The incubation period represents a critical life stage in oviparous species during which interactions between genetic factors, environmental cues, and non-genetic parental effects shape organismal phenotypes with long-term consequences for fitness. The developmental environment is a particularly potent source of phenotypic variation in reptiles (Noble *et al.*, 2018). In fact, many reptile species, including all crocodilians, many turtles, and some lizards, exhibit temperature-dependent sex determination (TSD), a system in which incubation temperature determines whether offspring become male or female (Lang and Andrews, 1994; Kohno *et al.*, 2014). Species with TSD provide intriguing systems to understand the role of environmental conditions in contributing to fitness-related phenotypes.

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* To whom correspondence should be addressed. Email: benparrott@srel.uga.edu.

Abbreviations: DDE, dichlorodiphenyldichloroethylene; EDC, endocrine-disrupting contaminant; FPT, female-promoting temperature; GLMM, generalized linear mixed model; MPT, male-promoting temperature; ODR, optimal developmental range; SVL, snout-vent length; TSD, temperature-dependent sex determination; TSP, thermosensitive period.

Temperature not only dictates sex ratios in these taxa but also influences incubation duration and a suite of phenotypes, including behavioral, morphological, and physiological traits (Noble *et al.*, 2018). Such environmental sensitivity is also associated with susceptibility to anthropogenic disturbances, such as climate change and environmental contaminants (Janzen, 1994; Guillette *et al.*, 1995; Bock *et al.*, 2020b).

The phenotypic consequences of thermal variability during incubation can be parsed into effects resulting from differences in mean incubation temperature or those resulting from differences in thermal fluctuations around similar means. Our understanding of TSD in reptiles is primarily derived from experiments using constant temperatures; and these studies demonstrate a clear influence of incubation temperature on hatchling size (*i.e.*, length and mass), with both the magnitude and the directionality of these effects being dependent on species, population, and even clutch in some instances (Allsteadt and Lang, 1995; Piña *et al.*, 2007; Noble *et al.*, 2018; Bodensteiner *et al.*, 2019). However, experiments incorporating thermal oscillations reflective of those encountered in nature have revealed clear distinctions between the effects of constant and fluctuating temperature profiles (Bowden *et al.*, 2014). For example, studies in lizards, turtles, and a crocodilian demonstrate that fluctuating incubation temperatures not only influence offspring sex ratios, when compared to constant temperature controls, but also impact other hatchling traits, such as size and performance (Les *et al.*, 2007; Warner and Shine, 2011; Simoncini *et al.*, 2019). It is clear from this work that thermal variation is a consequential component of the developmental environment, and it raises fundamental questions regarding how thermal fluctuations influence organismal responses to other environmental factors.

Environmental contaminants also have bearing on developmental processes, and TSD reptiles are particularly sensitive to a class of compounds that interfere with normal hormone signaling, termed endocrine-disrupting contaminants (EDCs) (Crain and Guillette, 1998). Dichlorodiphenyltrichloroethane (DDT) is a widespread and persistent organochlorine pesticide, and its primary metabolite, dichlorodiphenyldichloroethylene (DDE), is capable of disrupting androgen signaling (Kelce *et al.*, 1995). Impaired reproductive function is associated with EDC exposure (including elevated levels of DDE) in a wild alligator population in Florida (Guillette *et al.*, 1994, 1999; Milnes and Guillette, 2008; Milnes *et al.*, 2008), and experimental work has demonstrated that these alterations to reproductive function originate during development (Moore *et al.*, 2012; Hale *et al.*, 2019; Hale and Parrott, 2020). In laboratory studies, *in ovo* exposure to DDE induces female-biased sex ratios in turtles and alligators (Willingham and Crews, 1999; Milnes *et al.*, 2005b). Interestingly, this effect emerges only at temperatures producing mixed sex ratios, pointing to the potential importance of interactions between thermal environment and EDC exposure in determining phenotypic outcomes. Given the focus on reproductive and endocrine perturbation, effects of EDCs and their

interactions with other environmental factors on hatchling size and growth are often overlooked. Yet these basic morphometric traits are likely important for hatchling survival and highly integrative with respect to the influence of climatic factors, contaminant exposure, and maternal effects.

Offspring traits depend on maternal effects and the developmental environment, as well as their complex interactive effects. Here we address the extent to which each of these factors contribute to variation in incubation length, morphology, body condition, early growth, and residual energy reserves in the American alligator (*Alligator mississippiensis*), a long-lived oviparous reptile with TSD. The temperature experienced during a discrete period of development, which commences prior to gonadal differentiation and lasts three to four weeks, determines sex in the alligator (Lang and Andrews, 1994; McCoy *et al.*, 2015). During this thermosensitive period, embryos experiencing lower (<32 °C) and higher (>34 °C) temperatures develop ovaries, and embryos incubated at intermediate temperatures develop testes (Lang and Andrews, 1994). Using a factorial design, we incubated field-collected eggs under three constant and two fluctuating temperature regimes in combination with exposure to two environmentally relevant doses of DDE, in order to address the following research questions: (1) How do thermal regime and EDC exposure influence hatchling traits other than those related to reproductive function? (2) Is there an interactive effect of DDE exposure and thermal regime on hatchling morphology? (3) What is the relative influence of different components of thermal regime (mean temperature vs. thermal variability) on hatchling traits? Findings from this study advance our understanding of how developmental environments affect offspring phenotypes, with attendant implications for conservation and basic evolutionary ecology.

Materials and Methods

Field collections

The nesting period of the American alligator (*Alligator mississippiensis* (Daudin, 1802)), during which eggs are laid and embryos develop, extends from early June to early September (Joanen and McNease, 1989). In June 2017, alligator nests were located at the Tom Yawkey Wildlife Center (Georgetown, SC) via helicopter surveys and subsequently visited on foot. We collected 10 full clutches and 11 partial clutches of eggs within 48 hours of oviposition. Eggs were then transported in their natal nest material to the Savannah River Ecology Laboratory (Aiken, SC).

Laboratory incubation experiment

Within 12 hours of arrival at the laboratory, a representative embryo from each clutch was staged according to Ferguson (1985). All eggs were candled to determine viability (Ferguson, 1985), weighed, and transferred to damp sphagnum

moss, where they were kept at a constant 31.2 °C in programmable incubator chambers (model I36NLC, Percival Scientific, Perry, IA) until stage 15, as determined according to the established relationship between temperature and developmental rate (Kohno and Guillette, 2013). At stage 15, the earliest stage at which alligator sex determination responds to temperature (McCoy *et al.*, 2015), eggs within each clutch were randomly distributed across 5 different thermal regimes: a constant female-promoting temperature (30 °C), a constant male-promoting temperature (33 °C), a constant intermediate temperature (31.2 °C), or 1 of 2 fluctuating thermal regimes, which incorporated a daily fluctuation with an amplitude of ± 0.6 °C (minor flux) or ± 2.8 °C (major flux) around a mean of 31.2 °C (Fig. 1; Lang and Andrews, 1994; Gonzalez *et al.*, 2019). The shape of the fluctuating treatments was designed based on empirically derived nest temperature profiles to mirror natural fluctuations of wild nests, as described in Bock *et al.* (2020a); and the constant intermediate temperature was based on the overall mean nest temperature at Tom Yawkey Wildlife Center in 2015. The constant female- and male-promoting temperatures were selected because each of these temperatures reliably produces unisexual or near-unisexual outcomes ($\sim 100\%$ females and $\sim 100\%$ males, respectively), with very little variation observed across studies or populations (Lang and Andrews, 1994; Gonzalez *et al.*, 2019). At stage 19, eggs were topically dosed with one of three treatments: vehicle control ($0.2 \mu\text{g g}^{-1}$ egg weight absolute ethanol), low-dose DDE ($0.1 \mu\text{g g}^{-1}$ egg weight; ~ 100 ppb; 4,4'-DDE Pestanal, Sigma Aldrich, St. Louis, MO), or high-dose DDE ($1 \mu\text{g g}^{-1}$ egg weight; ~ 1 ppm) (Kohno and Guillette, 2013). Doses were chosen based on levels measured in field-collected alligator eggs from contaminated lakes and associated with impaired reproductive function (Rauschenberger *et al.*, 2009). Eggs were then maintained at their respective thermal regimes until hatching. During the entire incubation period, Onset UTBI-001 HOBO temperature loggers (Bourne, MA), preprogrammed to

record temperature at five-minute intervals, were kept in the substrate adjacent to eggs and were checked daily to ensure that actual incubation temperatures conformed to the experimental thermal regimes. As embryos approached later stages (\sim stage 28), eggs were checked twice daily for the initiation of hatching (pipping); once hatchlings had pipped, they were removed from sphagnum and transferred to individual glass jars. Embryos that had not completed the hatching process within 48 hours of pipping were manually assisted in order to limit hatchling loss.

Hatchling morphometrics

Upon hatching, neonates were weighed; and snout-vent length (SVL), total length, tail girth (circumference of tail at vent), and both head and snout length and width were measured. Hatchlings were individually identified with 2 unique Monel tags (National Band and Tag Co., Newport, KY) attached between the middle digits of both of the hindlimbs; and they were kept in a temperature-controlled indoor facility in custom-built fiberglass tanks, with areas for swimming and basking, for 9–10 days. Neonates were randomly assigned to tanks in order of hatching and were not fed during this period. At days 9–10 post-hatch, the morphometric measurements described above were repeated for each individual, and a blood sample was taken from the postcranial sinus. After blood sampling, hatchlings were euthanized and necropsied to collect tissues for transcriptional analysis as part of a separate study. Hatchling sex was assessed at the time of dissection by the presence or absence of the Müllerian duct. During necropsy, the residual yolk reserve was excised and weighed.

Statistical analyses

All statistical analyses were conducted in R statistical software version 3.6.1. (R Core Team, 2019). Hatchlings with missing data for any of the dependent variables of interest were excluded from all analyses, in addition to outliers clearly resulting from measurement error. Analyses of the dependent variables incubation duration, hatchling mass, total length, SVL, tail girth, and head area (head width multiplied by head length) were conducted in two ways. In the first analysis, we tested the effect of average incubation temperature only in hatchlings from the constant temperature treatments (female-promoting, male-promoting, and intermediate temperatures). In the second analysis, we tested the effect of the degree of temperature fluctuation by including only hatchlings incubated under one of the three intermediate temperature treatments (constant intermediate temperature, minor flux, or major flux). In each of the analyses above, generalized linear mixed models (GLMMs) were fit using the lme4 package, with temperature treatment, DDE treatment, the additive effect of temperature and DDE, and the interactive effect of temperature and DDE included as fixed effects and clutch included as a random effect (Bates *et al.*, 2019). Log-ratio tests were conducted to test which explanatory variables

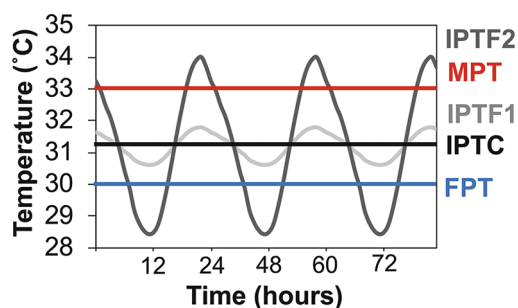


Figure 1. Experimental thermal regimes. American alligator (*Alligator mississippiensis*) embryos were incubated under one of five thermal regimes from Ferguson stage 15 to hatch. The constant incubation temperatures included a female-promoting temperature (FPT; 30 °C), an intermediate temperature (IPTC; 31.2 °C), and a male-promoting temperature (MPT; 33.5 °C). The fluctuating temperature treatments exhibited daily thermal fluctuations with an amplitude of either 0.6 °C (IPTF1; minor flux) or 2.8 °C (IPTF2; major flux) around the same mean temperature (31.2 °C) as IPTC.

improved model fit. Variables that did not significantly improve model fit were subsequently removed (according to a significance threshold of $P < 0.005$), and the P -values for variables included in the final models were derived from the lmerTest package, using Satterthwaite's degrees of freedom method (Kuznetsova *et al.*, 2017).

To assess the interactive effects of temperature and DDE exposure on hatchling growth, GLMMs were also fit for dependent variables describing the absolute change in mass and SVL between day 0 and days 9–10 post-hatch. The same independent variables were included in these models as described for the morphological traits. The mass of the residual yolk reserve at 9–10 days post-hatch, an indication of the remaining maternally derived energy reserves available following development, was also included as a dependent variable; and a GLMM was fit using the same independent variables as described above. Further, the relationship between egg mass and hatchling mass was fit using a linear regression with the lm function in R. We extracted the residuals of this relationship and treated the residuals as another dependent variable in a GLMM with the same independent variables as above.

Results

Hatching success and sex ratios

A total of 484 viable eggs were assigned to incubation temperature and DDE-exposure treatment groups. Of those eggs assigned to treatment groups, 418 individuals hatched successfully, either with or without manual assistance, within 48 hours of pipping (86.4% overall hatch rate). After filtering out all individuals with missing data and/or data outliers clearly due to measurement error, 400 hatchlings were included in further analyses (Table 1). Interestingly, all but one hatchling (336 of 337) from the constant female-promoting temperature, constant intermediate temperature, minor flux, and major flux treatment groups were female (as determined by the presence of the Müllerian duct at dissection). In contrast, all but one hatchling (62 of 63) from the constant male-promoting temperature treat-

ment group were male. The two hatchlings exhibiting the minority sex were from the female-promoting temperature-DDE high dose and male-promoting temperature-control treatment groups, respectively. Neither DDE treatment nor fluctuating thermal profile had any effect on sex ratios under the conditions tested.

Incubation duration and hatchling morphometrics

Consistent with previous findings, constant temperature significantly influenced incubation duration ($F_{2,220.25} = 745.66$, $P < 0.0001$; Fig. 2A; Table 2), with hatchlings from the warmer, male-promoting temperature hatching an average of 9.6 days earlier than hatchlings from the cooler, female-promoting temperature. In addition, the degree of thermal fluctuation also significantly influenced incubation duration ($F_{2,245.31} = 5.7207$, $P = 0.00373$; Fig. 2A; Table 2). Relative to individuals incubated at the constant intermediate temperature, individuals experiencing the minor temperature fluctuation hatched earlier (by about 0.4 days), while individuals experiencing the major temperature fluctuation hatched later (by about 0.2 days). Exposure to DDE did not significantly influence incubation duration.

Thermal regime also significantly influenced multiple hatchling morphometric traits. Both constant temperature and the degree of thermal fluctuation significantly influenced hatchling total length (constant temperature: $F_{2,215.57} = 31.209$, $P < 0.0001$; fluctuation: $F_{2,250.67} = 32.597$, $P < 0.0001$; Fig. 2D; Table 2) and head area (constant temperature: $F_{2,214.89} = 12.093$, $P < 0.0001$; fluctuation: $F_{2,250.67} = 11.083$, $P < 0.0001$; Fig. 2F; Table 2). Constant temperature, but not the degree of thermal fluctuation, also influenced hatchling mass ($F_{2,211.45} = 13.174$, $P < 0.0001$; Fig. 2B; Table 2), tail girth ($F_{2,213.99} = 6.6855$, $P = 0.00153$; Fig. 2C; Table 2), and SVL ($F_{2,215.93} = 14.916$, $P < 0.0001$; Fig. 2E; Table 2). Notably, individuals incubated at the male-promoting temperature were larger, on average, in every morphometric trait compared to individuals incubated at the female-promoting temperature. In addition, individuals incubated under the major temperature fluctuation tended to be smaller in total length and head area

Table 1

Sample size for each temperature and dichlorodiphenyldichloroethylene (DDE)-treatment combination used in the morphometric analyses of American alligator (*Alligator mississippiensis*) hatchlings

Incubation temperature	DDE treatment		
	Control (0.2 $\mu\text{g vehicle g}^{-1}$)	Low (0.1 $\mu\text{g DDE g}^{-1}$)	High (1 $\mu\text{g DDE g}^{-1}$)
FPT (30 °C)	23 (0.92)	21 (0.88)	25 (1)
IPTC (31.2 °C)	44 (0.87)	26 (0.93)	25 (0.86)
IPTF1 (31.2 °C \pm 0.6 °C)	40 (0.79)	24 (0.86)	25 (0.93)
IPTF2 (31.2 °C \pm 2.8 °C)	41 (0.85)	20 (0.79)	23 (0.86)
MPT (33 °C)	23 (0.88)	21 (0.85)	19 (0.80)

Numbers in parentheses indicate the proportion of eggs that successfully hatched. FPT, female-promoting temperature; IPTC, constant intermediate temperature; IPTF1, intermediate temperature with minor fluctuation; IPTF2, intermediate temperature with major fluctuation; MPT, male-promoting temperature.

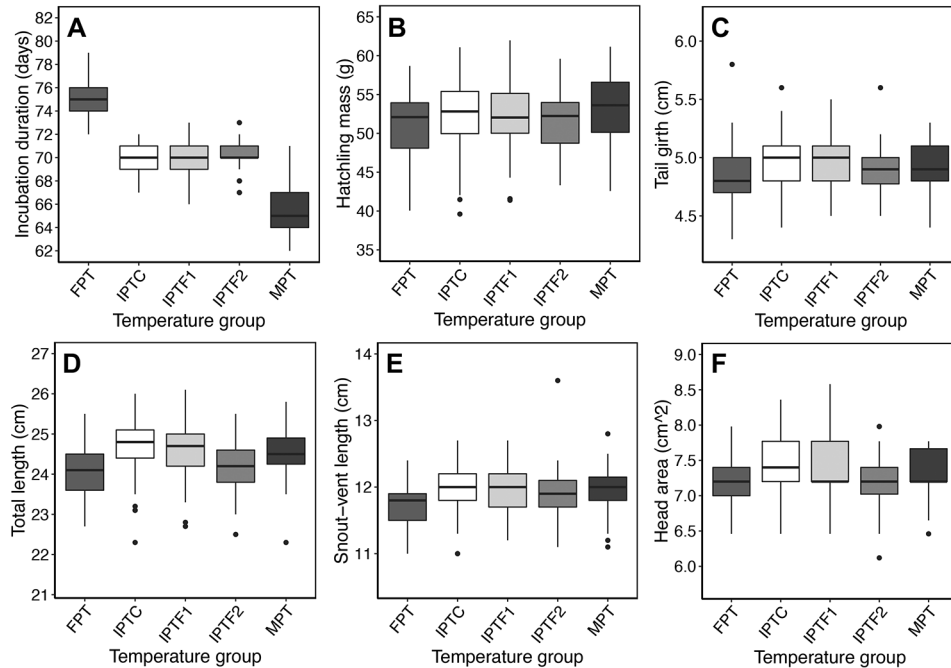


Figure 2. Thermal effects on incubation duration and hatchling traits. Effects of thermal regime on incubation duration (A) and hatchling morphometric traits: hatchling mass (B), tail girth (C), total length (D), snout-vent length (SVL; E), and head area (F) in American alligator (*Alligator mississippiensis*) hatchlings. There was a significant effect of both constant temperature and thermal fluctuations on incubation duration, total length, and head area. In addition, there were also constant temperature effects on mass, tail girth, and SVL. Central lines of boxplots depict median, boxes depict interquartile range (IQR), and vertical lines depict the maximum and minimum values. Values that deviate from the median by more than $1.5 \times \text{IQR}$ are depicted as points. FPT, female-promoting temperature; IPTC, constant intermediate temperature; IPTF1, intermediate temperature with minor fluctuation; IPTF2, intermediate temperature with major fluctuation; MPT, male-promoting temperature.

compared to the other intermediate temperature conditions. In striking contrast to thermal regime, DDE exposure did not significantly influence any of the morphometric traits examined.

Hatchling growth and metabolism

Alligator hatchlings tended to decrease in mass and increase in SVL over the first 9–10 days post-hatching (Fig. 3). Constant temperature significantly influenced the change in mass between hatching and 9–10 days post-hatching ($F_{2,218.94} = 9.32$, $P = 0.00013$; Fig. 3A; Table 2), but not the change in SVL (Fig. 3B; Table 2). On average, individuals incubated at the female-promoting temperature lost the least mass post-hatching (mean = -2.35 g), while individuals incubated at the male-promoting temperature lost the most mass post-hatching (mean = -3.14 g). There were no significant effects of thermal fluctuations or DDE exposure on either of the growth parameters assessed.

The mass of the residual yolk reserve is another critical trait that reflects the amount of maternally derived energy resources available for growth and maintenance in the early post-hatching period. Both constant temperature and the degree of thermal fluctuation influenced residual yolk mass at days 9–10 post-hatch (constant temperature: $F_{2,215.71} =$

47.395, $P < 0.0001$; fluctuation: $F_{2,249.68} = 32.451$; Fig. 4; Table 2). Among the constant temperatures, individuals incubated at the male-promoting temperature had the most residual yolk mass (mean = 2.32 g), while individuals incubated at the female-promoting temperature had the least (mean = 1.63 g). Further, among the intermediate temperatures, individuals that experienced the major thermal fluctuation had the most residual yolk mass (mean = 2.11), while individuals that experienced the minor thermal fluctuation had the least (mean = 1.64 g). Consistent with the rest of the hatchling traits, DDE exposure did not significantly influence residual yolk mass.

Interactions between thermal regime and maternal resource provisioning

Egg mass reflects maternal resource allocation on a per-offspring basis and can vary widely between clutches. We detected a strong, linear relationship between egg mass and hatchling mass ($y = 10.57 + 0.53x$; $R^2 = 0.79$, $P < 0.0001$; Fig. 5A). Interestingly, even after including clutch as a random factor, constant temperature was significantly related to the residuals of this linear relationship ($F_{2,210.05} = 18.84$, $P < 0.0001$; Fig. 5B). Individuals incubated at the male-promoting

Table 2

Summary of statistical tests assessing the effect of constant temperature and temperature fluctuations on American alligator (*Alligator mississippiensis*) hatchling phenotypes

Hatchling phenotypes	Constant temperature effects		Temperature fluctuation effects	
	Test statistic	P-value	Test statistic	P-value
Incubation duration (days)	$F_{2,220.25} = 745.66$	<0.0001	$F_{2,245.31} = 5.7207$	0.00373
Morphological traits				
Mass (g)	$F_{2,211.45} = 13.174$	<0.0001	NS	NS
Tail girth (cm)	$F_{2,213.99} = 6.6855$	0.00153	NS	NS
Total length (cm)	$F_{2,215.57} = 31.209$	<0.0001	$F_{2,250.7} = 32.597$	<0.0001
SVL (cm)	$F_{2,215.93} = 14.916$	<0.0001	NS	NS
Head area (cm ²)	$F_{2,214.89} = 12.093$	<0.0001	$F_{2,250.67} = 11.083$	<0.0001
Growth and metabolism				
Delta mass (g)	$F_{2,218.94} = 9.32$	0.00013	NS	NS
Delta SVL (cm)	NS	NS	NS	NS
Residual yolk mass (g)	$F_{2,215.71} = 47.395$	<0.0001	$F_{2,249.68} = 32.451$	<0.0001

NS, non-significant; SVL, snout-vent length.

temperature tended to be larger than expected based on their egg size, while individuals incubated at the female-promoting temperature tended to be smaller than expected based on their egg size.

Discussion

The thermal environment experienced during development, and its interaction with maternal resource provisioning, is a significant contributor to phenotypic variation observed in American alligator hatchlings. Both mean constant temperature and the degree of thermal fluctuation influenced incubation dura-

tion and a suite of morphological traits in neonatal alligators. In contrast, exposure to the pesticide metabolite DDE produced no apparent effects on the incubation period or hatchling morphology. We also did not detect any interactions between DDE exposure and thermal regime, contrary to previous studies examining reproductive endpoints. While morphological differences have been observed in alligator hatchlings from sites conferring different contaminant profiles (including EDCs such as DDE) (Milnes *et al.*, 2001, 2005a), it remains unclear whether these differences are the direct result of contaminant exposure or whether they are due to other factors. This is, in part, the result of a lack of investigations into the explicit effects of developmental

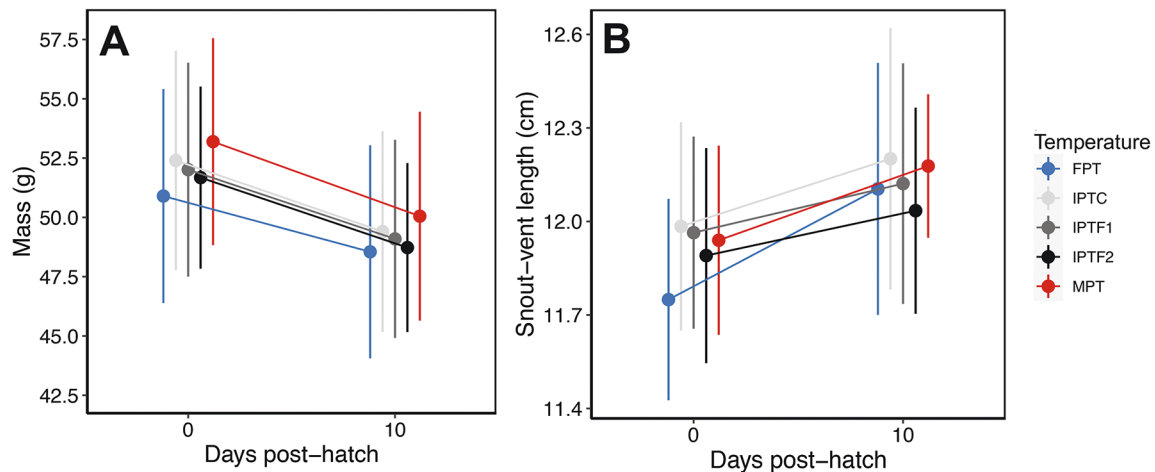


Figure 3. Effects of thermal regime on American alligator (*Alligator mississippiensis*) hatchling growth in the early post-hatching period. Hatchlings lost mass (A) and increased in length (in terms of snout-vent length [SVL]; B) over the first 9–10 days post-hatching. There was a significant effect of constant temperature on the change in mass. There were no significant effects of treatment on the change in SVL. Points depict the treatment mean, and vertical lines depict ± 1 SD. FPT, female-promoting temperature; IPTC, constant intermediate temperature; IPTF1, intermediate temperature with minor fluctuation; IPTF2, intermediate temperature with major fluctuation; MPT, male-promoting temperature.

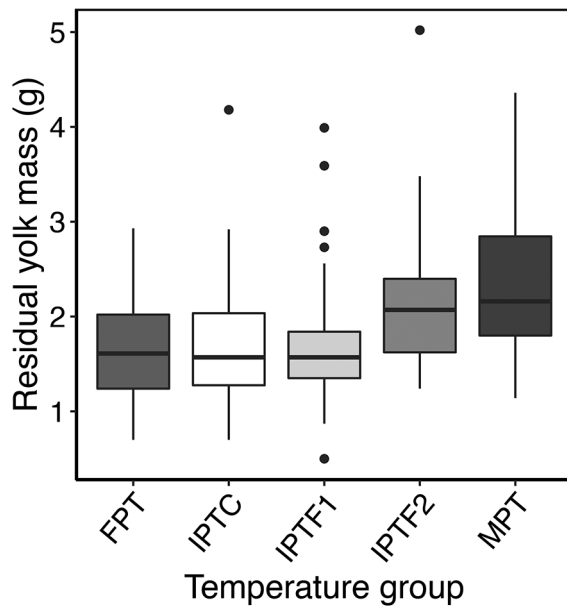


Figure 4. Effects of thermal regime on residual yolk mass at 9–10 days post-hatching in American alligator (*Alligator mississippiensis*) hatchlings. There was a significant effect of both constant temperature and the degree of temperature fluctuation on the mass of the hatchlings' residual yolk reserve. Central lines of boxplots depict median, boxes depict interquartile range (IQR), and vertical lines depict the maximum and minimum values. Values that deviate from the median by more than 1.5 IQR are depicted as points. FPT, female-promoting temperature; IPTC, constant intermediate temperature; IPTF1, intermediate temperature with minor fluctuation; IPTF2, intermediate temperature with major fluctuation; MPT, male-promoting temperature.

exposure to EDCs in experimental settings on hatchling morphology and growth. Findings presented here suggest that morphological traits are comparatively less sensitive to DDE exposure than other traits (*i.e.*, hormone signaling, reproductive function) and underscore the importance of more complex physiological and/or ecological mechanisms beyond morphological differences in contributing to reduced recruitment of hatchling alligators in historically contaminated sites (Woodward *et al.*, 1993; Guillette *et al.*, 1994).

Nest thermal environments in nature are often more dynamic than those implemented in artificial incubation experiments (Paiz *et al.*, 2010; Bowden *et al.*, 2014; Bock *et al.*, 2020a). In this study, we found that fluctuating thermal regimes yielded different outcomes from the constant temperature control for a subset of hatchling traits. Hatchling length, head area, and residual yolk mass were all significantly influenced by the degree of thermal variability that alligator embryos experienced during incubation. The duration of incubation was also influenced by the degree of thermal variability, though the effect size was small relative to the effect of differences in mean constant temperature. In some cases (*e.g.*, incubation duration, residual yolk reserves), the direction of the effect of thermal fluctuation varied depending on the amplitude of the fluctuation. These findings fit into a growing body of knowledge clarifying how ther-

mal complexity is integrated into developmental processes in reptiles (Les *et al.*, 2007, 2009; Warner and Shine, 2011; Bowden *et al.*, 2014; Simoncini *et al.*, 2019; Massey and Hutchings, 2021). Existing models suggest that the relationship between temperature and developmental rate plays a key role in explaining the effects of thermal fluctuations on hatchling phenotypes (Georges *et al.*, 1994, 2005; Bowden *et al.*, 2014). The optimal developmental range (ODR) is defined as the range of temperatures within which the relationship between temperature and developmental rate is linear, and when thermal profiles fluctuate within the ODR, a greater proportion of development occurs at temperatures above the mean than below (Georges *et al.*, 1994, 2005). In contrast, when thermal profiles span temperatures outside the ODR, development temporarily slows or pauses; and resulting phenotypes vary according to the timing of these perturbations (Georges *et al.*, 2005). This likely explains the variable phenotypic outcomes we observed in response to the minor and major fluctuation in the present experiment. The major fluctuation featured a minimum temperature of 28.4 °C, which, when employed continuously, can result in increased embryonic mortality in *Alligator mississippiensis* (Lang and Andrews, 1994) and likely resulted in transient periods of impeded development or developmental arrest, leading to the observed increase in incubation duration and decrease in somatic size (total length, head area). However, the very concept of the ODR is defined using constant incubation temperatures, and how developmental rates respond to intermittent deviations from the ODR is a lingering question.

Interestingly, we did not observe any sex ratio variation resulting from the fluctuating temperature treatments. Mounting evidence suggests that thermal fluctuations in wild nests play key roles in shaping offspring sex ratios in other TSD species, such as *Trachemys scripta* (Carter *et al.*, 2018); yet we currently lack an empirically validated model to predict crocodilian sex ratios under fluctuating thermal regimes. This is in part because few experiments have implemented incubation treatments that incorporate thermal variability in these taxa (Simoncini *et al.*, 2019; Bock *et al.*, 2020a) and because the molecular mechanisms underlying TSD remain elusive, despite recent advances in this area (Czerwinski *et al.*, 2016; Yatsu *et al.*, 2016; Deveson *et al.*, 2017; Ge *et al.*, 2018). Predicting the impacts of thermal fluctuations on crocodilian sex ratios is further complicated by their unique temperature-by-sex reaction norm, in which females are produced at extreme temperatures and males at intermediate temperatures (F-M-F pattern) (Lang and Andrews, 1994). Based on this pattern, we may hypothesize that in order for male development to proceed in crocodilians, an unknown physiological threshold must be met based on the “dosage” of male-promoting temperature cues experienced during the thermosensitive period. Our findings suggest that neither of the fluctuating thermal regimes implemented in this experiment conferred exposure to sufficient male-promoting signals to meet this threshold. Further experiments implementing fluctuating thermal regimes

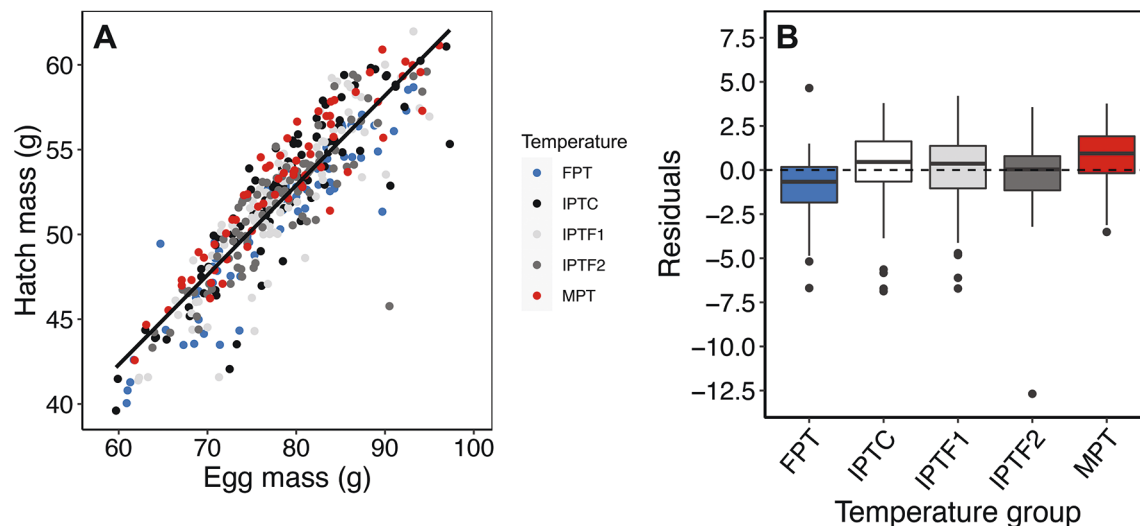


Figure 5. Interactive effects of thermal cues and maternal resource provisioning on American alligator (*Alligator mississippiensis*) hatchling phenotype. There is a strong, linear relationship between hatchling mass and maternal resource provisioning as indicated by egg mass (A). The residuals of the relationship between egg mass and hatchling mass are significantly related to constant incubation temperature, but not the degree of temperature fluctuations (B). Central lines of boxplots depict median, boxes depict interquartile range (IQR), and vertical lines depict the maximum and minimum values. Values that deviate from the median by more than 1.5 IQR are depicted as points. FPT, female-promoting temperature; IPTC, constant intermediate temperature; IPTF1, intermediate temperature with minor fluctuation; IPTF2, intermediate temperature with major fluctuation; MPT, male-promoting temperature.

with a wider range of median temperatures are needed to resolve the threshold for male development and to develop a model for predicting crocodilian sex ratios in the context of thermal variability.

The most striking phenotypic variation observed in this study resulted from the interaction of constant temperature and maternal resource provisioning. In particular, hatchlings incubated under the male-promoting temperature were larger in terms of multiple morphometric traits and hatched with more residual yolk reserve than hatchlings from any of the other thermal regimes. This contrasts with previous studies that have demonstrated trade-offs between offspring size and the quantity of residual yolk available at hatch (Allsteadt and Lang, 1995; Pezaro *et al.*, 2013; Murphy *et al.*, 2020). For example, when yolk mass was experimentally reduced early in development in *Amphibolurus muricatus*, resulting hatchlings were smaller but had comparable quantities of residual yolk at hatch, suggesting a prioritization of post-hatching energy reserves over hatchling size (Murphy *et al.*, 2020). The findings we present here suggest that male hatchlings are not larger merely because they have transformed more of their maternally provisioned resources into somatic growth, but rather that yolk utilization is more efficient at male-promoting temperatures. If this is indeed the case, this would imply that incubation temperature influences the nature of the trade-off between hatchling size and residual yolk reserve and, in turn, could have important consequences for hatchling fitness.

Recently, researchers developed a simple model to describe the temperature dependence of developmental energet-

ics in ectotherms that may provide insight into this phenomenon (Marshall *et al.*, 2020; Pettersen *et al.*, 2020). Under this model, the energetic cost of development is represented as the product of metabolic rate and incubation duration. Based on the temperature dependence of each of these traits, this model yields a single temperature at which developmental costs are minimized, which closely corresponds to natural incubation temperatures across ectothermic species from diverse phyla (Marshall *et al.*, 2020). If male-promoting temperatures more closely approximate those temperatures that minimize developmental costs in *A. mississippiensis*, this could explain why male hatchlings are larger while also possessing more residual energy reserves at hatch. However, an explicit test of this hypothesis would require data on embryonic metabolic rate at temperatures spanning the temperature-by-sex reaction norm in this species—a key piece of information that is currently lacking from the literature (Whitehead and Seymour, 1990; Crossley *et al.*, 2017).

Results from this study also suggest that egg size contributes substantially to hatchling size and post-hatching energy reserves, two traits with important consequences for offspring fitness (Janzen *et al.*, 2000; Nafus *et al.*, 2015). This raises the question: what are the proximate and ultimate drivers of variation in egg size? This topic has featured prominently in studies of reptile life-history strategies (Congdon and Gibbons, 1987; Bernardo, 1996; King, 2000; Shine, 2005; Janzen and Warner, 2009; Rollinson and Rowe, 2016; Iverson *et al.*, 2019). Both inter- and intraspecies comparisons have revealed apparent links between maternal size and reproductive traits,

including egg size (Warne and Charnov, 2008). Maternal body size scales allometrically with egg size and/or offspring size within many reptile taxa (King, 2000), including testudines (Valenzuela, 2001; Wilkinson and Gibbons, 2005; Nafus *et al.*, 2015), squamates (Warne and Charnov, 2008), and crocodilians (Larriera *et al.*, 2004), including our focal species, *A. mississippiensis* (Thorbjarnarson, 1996; Murray *et al.*, 2013). These relationships are likely underlain by a combination of factors, including, but not limited to, maternal genetics, maternal resource availability, and morphological constraints (Congdon and Gibbons, 1987; Congdon, 1989; Sinervo and Licht, 1991). Interestingly, the nature of this relationship between maternal size and reproductive traits can vary across species (Meiri *et al.*, 2015), populations (Murray *et al.*, 2013; Iverson *et al.*, 2019), and life stages (Harms *et al.*, 2005; Wilkinson and Gibbons, 2005; Bowden *et al.*, 2011). Given our results suggesting that incubation temperature can modify the relationship between egg size and hatchling size, it is interesting to consider the role of developmental plasticity in shaping the links between maternal and offspring phenotypes and, in turn, contributing to variation in maternal resource allocation strategies across environmental gradients (Sinervo, 1990; Telemeco *et al.*, 2010; Roitberg *et al.*, 2013; Du *et al.*, 2014; Lu *et al.*, 2014).

Beyond resource provisioning, other components of parental influence on hatchling phenotypes, including genetic and physiological effects, were not explicitly examined in the current study, yet they could represent important sources of fitness-related trait variation. Several studies in other TSD species, including *Chelydra serpentina* and *Chrysemys picta*, show geographic variation in the thermal sensitivity of hatchling phenotypes, including sex (Ewert *et al.*, 2005; Schroeder *et al.*, 2016) and morphological traits (Bodensteiner *et al.*, 2019). Very little is known regarding how thermal reaction norms vary across parental genotypes and populations in crocodilians. A systematic review of studies reporting temperature-by-sex reaction norms in crocodilians found evidence for latitudinal variation in the reaction norm of *A. mississippiensis* (González *et al.*, 2019). In particular, populations appear to vary in the range of male-promoting temperatures, with higher-latitude populations exhibiting a wider range of male-promoting temperatures (González *et al.*, 2019). While intriguing, such observations should be interpreted with caution because many of the studies examined were conducted prior to the discovery that the thermosensitive period of this species begins much earlier than previously thought, opening up the possibility that incubation temperatures prior to egg collection may have accounted for a proportion of the observed variation in sex ratios (McCoy *et al.*, 2015). Future investigations into the mechanisms underpinning clutch effects on thermal plasticity and those better characterizing population-level variation in thermal reaction norms are likely to reveal important insights into the adaptive potential of this group to respond to climate change.

Taken together, findings from this study raise several intriguing questions with substantial implications for our understanding of the evolutionary significance of TSD and our ability to conserve species employing this life-history strategy in the face of rapid environmental change. For one, the mechanisms by which thermal variability programs offspring phenotype independently and in concert with parental influences (including resource provisioning, physiological factors, and genetics) remain unclear. Investigations into the impacts of temperature and physiological cues, such as endocrine signals, on embryonic metabolism provide an exciting area of future inquiry, because developmental energetics might represent a general mechanism by which the environment shapes multiple hatchling traits (*e.g.*, morphological, immunological, performance). Further, few studies have quantified fitness consequences associated with phenotypic responses to incubation temperature. Many theories regarding the adaptive value of TSD rely on the presence of a sex-specific effect of incubation temperature on offspring fitness (Charnov and Bull, 1977; Valenzuela and Lance, 2004; Schwanz *et al.*, 2016). For example, in the American alligator, it is plausible that males benefit disproportionately from increased size and energy reserves resulting from intermediate incubation temperatures, because males must attain larger sizes (and older ages) in order to reach reproductive maturity compared to females (Wilkinson *et al.*, 2016; Zajdel *et al.*, 2019). As such, TSD would serve as an adaptive sex allocation strategy in this species. However, these ideas remain speculative in the absence of field data linking temperature-dependent phenotypes to components of fitness (survival and reproduction). As multiple features of the nest thermal environment (mean temperature and thermal variability) shift with climate change, information on the fitness consequences of thermal variability during incubation could also contribute valuable insight into the potential impacts of these changes on future population persistence.

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Ethical Care

All experimental procedures were approved prior to the beginning of the study by the Institutional Animal Care and Use Committee of the University of Georgia (protocol A2017 05-005-Y3-A1). Field collections were conducted under permits granted by the South Carolina Department of Natural Resources (SC-04-2017).

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