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Intrinsic and extrinsic factors interact during development to influence telomere length in a long-lived reptile

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Abstract

The mechanisms connecting environmental conditions to plasticity in biological aging trajectories are fundamental to understanding individual variation in functional traits and life history. Recent findings suggest that telomere biology is especially dynamic during early life stages and has long-term consequences for subsequent reproduction and survival. However, our current understanding is mostly derived from studies investigating ecological and anthropogenic factors separately, leaving the effects of complex environmental interactions unresolved. American alligators (*Alligator mississippiensis*) are long-lived apex predators that rely on incubation temperature during a discrete period during development and endocrine cues to determine sex, making them especially vulnerable to current climatic variability and exposure to anthropogenic contaminants interfering with hormone function. Here, we combine field studies with a factorial design to understand how the developmental environment, along with intrinsic biological variation contribute to persistent telomere variation. We found that exposure to a common endocrine disrupting contaminant, DDE, affects telomere length, but that the directionality is highly dependent upon incubation temperature. Variation in hatchling growth, underlies a strong clutch effect. We also assess concentrations of a panel of glucocorticoid hormones and find that contaminant exposure elicits an increase in circulating glucocorticoids. Consistent with emerging evidence linking stress and aging trajectories, GC levels also appear to trend with shorter telomere length. Thus, we add support for a mechanistic link between contaminants and glucocorticoid signalling, which interacts with ecological aspects of the developmental environment to alter telomere dynamics.

KEYWORDS
aging, developmental environment, elomeres, life history, stress, temperature-dependent sex determination

Junsoo Bae and Emily M. Bertucci contributed equally to this study.

1 | INTRODUCTION

Interactions between organisms and their environment affect life history and aging processes with ultimate consequences for organismal fitness. Telomeres are non-coding DNA repeats located at the ends of eukaryotic chromosomes and serve critical roles in maintaining genome integrity (Blackburn, 1991). Due to incomplete DNA replication at chromosome ends, telomeric sequences become shorter with cell division in the absence of active telomere elongation, and in many organisms, telomere length (TL) decreases with age (Blackburn, 2005; Gomes et al., 2010). Recent studies suggest that extrinsic factors affect telomere dynamics (Cram et al., 2017; De Felice et al., 2012; Epel et al., 2004; Haussmann & Marchetto, 2010; Noguera & Velando, 2019; Vriens et al., 2019; Zota et al., 2015), and indicate that early life environments, including those experienced during embryonic development are especially influential (Entringer et al., 2018; Stier et al., 2020). Additionally, intrinsic factors including genetics, maternal effects, and oxidative state are also key determinants of telomere dynamics (De Meyer et al., 2007; Dugdale & Richardson, 2018; Horn et al., 2011; Njajou et al., 2007; Reichert & Stier, 2017; Tissier et al., 2014). Individual variation in TL is associated with functional traits and survival, even after correcting for chronological age (Bize et al., 2009; Dunshea et al., 2011; Eastwood et al., 2019; Monaghan, 2014; Wilbourn et al., 2018) suggesting that the effects of environmental factors on TL may have consequences on fitness. Together, these findings raise intriguing questions regarding the relative impacts of intrinsic and extrinsic factors on TL, especially with respect to the proximal mechanisms responsible for integrating developmental environments into organismal function and life history variation.

Environmental conditions appear to interact with intrinsic factors like genetics and parental age to affect telomere dynamics, making estimates of heritability for TL highly variable (Bauch et al., 2019; Dugdale & Richardson, 2018; Horn et al., 2011; Njajou et al., 2007; Unryn et al., 2005). The environmental factors reported to negatively influence TL range from exposure to predator cues to exposure to contaminants (Blévin et al., 2016; Guzzardi et al., 2016; Martens et al., 2017; Matzenbacher et al., 2019; McLennan et al., 2016; Noguera & Velando, 2019; Zota et al., 2015). In general, harsh environmental conditions occurring early in life are associated with shorter TL, and these observations have led to the "foetal programming of telomere biology hypothesis" in which complex organism-by-environment interactions during development influence TL and attendant aging trajectories (Entringer et al., 2012, 2018; Haussmann et al., 2012; Stier et al., 2020). However, we lack a broad understanding of how anthropogenic contaminants such as endocrine disrupting chemicals (EDCs) effect TL (Michels et al., 2020). Exposure to specific EDCs in utero are associated with both shorter and longer TL in offspring, and these effects are generally sex specific (Michels et al., 2020). As oestrogen stimulates telomerase expression (Kyo et al., 1999), EDCs with oestrogenic properties may negatively affect telomeres due to increased levels of reactive oxygen species (Xu et al., 2013) and promote their elongation via expression of telomerase.

The effects that environmental conditions have on TL appear to be in part mediated by glucocorticoid (GC) signalling (Angelier et al., 2018; Monaghan, 2014). GC hormones are involved in the physiological and organismal response to environmental stress and in most studies, increased levels of GCs are associated with reduced TL (Angelier et al., 2018); however, positive effects have also been observed (Noguera et al., 2020). A causal role for GC signalling in regulating telomere shortening is further supported by experimental studies in which treatment with corticosterone results in advanced telomere attrition (Angelier et al., 2018). Most recently, evidence suggests that GC hormones mediate telomere attrition after exposure to environmental contaminants (Powolny et al., 2020). However, our understanding of the interplay between telomeres and GC signalling is mostly derived from ecological studies in avian systems and biomedical studies focused on human disease. Further, the effects of secondary GCs (i.e., corticosterone in humans) and precursors in GC biosynthesis are often not analysed, leaving a somewhat narrow understanding of how endocrine pathways influence telomere biology.

In this study, we examined the influence of the developmental environment on GC levels and TL in hatchling alligators. Alligators display age-dependent telomere attrition (Scott et al., 2006) and embryos are exquisitely sensitive to environmental cues. For example, similar to many nonmammalian vertebrates, the thermal environment experienced during incubation irreversibly determines sex in alligators (Ferguson & Joanen, 1982; McCoy et al., 2015). Temperature also affects growth rate and body size in ectotherms which may result in both direct effects of temperature on TL via increases in oxidative stress and indirect effects due to the effect of growth rate and body size (Miller et al., 2014; Olsson et al., 2018; Vedder et al., 2018). The stability of temperatures during incubation have also been shown to negatively affect TL in birds (Stier et al., 2020), highlighting the important role of the thermal environment in the determination of TL which has the potential to be especially prominent in ectotherms. Additionally, alligators are long-lived apex predators and maternal deposition of common environmental contaminants into yolk, such as 1,1′-(2,2-dichloroethene-1,1-dyl)bis(4-chlorobenzene) (*p*,*p*′-DDE), referred to from here forward as DDE, alters reproductive development and subsequent function in offspring (Crain et al., 1997; Guillette, 1994; Hale et al., 2019; Moore et al., 2010). While disrupted oestrogen signalling appears to underlie many of the observed reproductive abnormalities (Hale et al., 2019; Hale & Parrott, 2020), DDE has the potential to affect additional endocrine pathways as well as other cellular processes.

Using a factorial design, we exposed nine clutches of alligator eggs to four variable, ecologically informed thermal regimes producing both males and females and an environmentally relevant dose of DDE, a primary metabolite of the pesticide 1-chloro-4-[2,2,2-trichlorio-1-(4-chlorophenyl)ethyl]benzene (DDT) (Figure 1). Circulating levels of eight steroid stress hormones were analysed in hatchlings using a recently developed mass spectrometric approach (Koal et al., 2012) and the relationship of clutch, body condition, hormone levels,

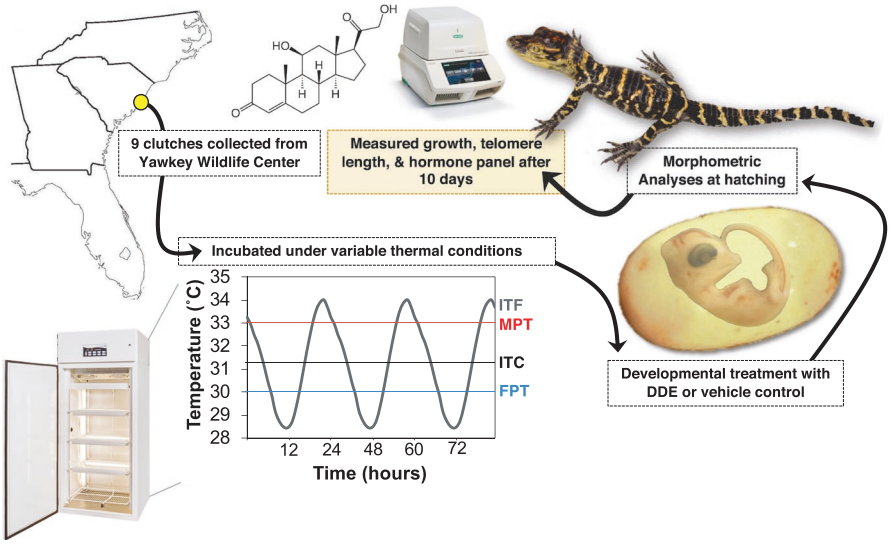

FIGURE 1 Schematic of the experimental design. Alligator eggs were collected from Yawkey Wildlife Center in coastal South Carolina and then distributed across incubation temperature regimens and exposure treatment groups. Upon hatching, neonatal alligators were measured and allowed to grow for 9–10 days at which point alligators were remeasured and telomere and hormone levels were assessed

thermal regime, and DDE exposure to TL was modelled. Given the sensitivity of alligators to developmental temperatures and the positive relationship between temperature and growth rate, we predicted that both warmer incubation temperatures and thermal variability would result in faster growth and shorter TL. Based on previous studies demonstrating negative relationships between TL and contaminant exposure, we also predicted that exposure to DDE would result in shorter TL and that this effect may be in part mediated by GC signalling. Further, we predicted that contaminant exposure and incubation temperature were likely to interact, with warmer temperatures exacerbating DDE-mediated telomere shortening, resulting in a male-biased decrease in TL. Collectively, the findings presented here advance our understanding of how complex developmental environments interact with intrinsic organismal processes to affect telomere dynamics in early life.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Nine clutches of alligator eggs (n = 36–45 per clutch) were collected from the Tom Yawkey Wildlife Center Heritage Preserve between June 12–30, 2017 within 48 h of oviposition (McCoy et al., 2015) and transported to the Savannah River Ecology Laboratory (Aiken County). All collections were made under permits from the South Carolina Department of Natural Resources, and all work involving animals was performed using protocols approved by the University of Georgia's Institutional Animal Care and Use Committee (protocol #A2017 05-005-Y3-A1). A representative embryo from each clutch was staged following collection in order to confirm oviposition date and stage according to Ferguson (1985). All eggs were maintained in damp sphagnum moss at 31.2°C (intermediate temperature-constant; ITC) until reaching stage 15, as determined according to Kohno and Guillette (2013). At stage 15, the beginning of the thermosensitive period, eggs from each clutch were equally distributed across four different incubation groups: a constant male-promoting temperature (MPT; 33°C; n = 30) predicted to produce all males, a constant female promoting temperature (FPT; 30°C; n = 28) predicted to produce all females, an environmentally informed intermediate temperature (31.2˚C, ITC; n = 30) predicted to produce female-biased sex ratios, or a fluctuating thermal regime (ITF; n = 32), which incorporated daily fluctuations (±2.8°C) around ITC (Lang & Andrews, 1994). The ITC and ITF incubation regimes were derived from a previous study measuring eight natural nest temperature profiles (Bock, Hale, et al., 2020), of which the average temperature was 31.2°C. The ITF was determined using the average deviation from the mean nest temperature for every 5 min of the day during the thermosensitive period (Ferguson stage 15–24), and then increasing that deviation by a factor of 7.7 (Bock, Hale, et al., 2020). This thermal fluctuation exhibited the same periodicity of a wild nest and a daily amplitude within the scope of that observed in nature (Bock, Lowers, et al., 2020). Within a daily cycle, the ITF treatment experienced temperatures known to promote both male and female fates. As sex ratio predictions are based on reaction norms constructed from constant incubation temperatures and experiments incorporating fluctuating thermal profiles have not been performed in alligators, we were uncertain as to what the effect on sex ratios would be in the ITF treatment.

TABLE 1 Sample sizes for statistical analyses

Incubation condition	Control	DDE
FPT	13 (6)	15 (4)
ITC	13 (6)	17 (9)
ITF	15	17
MPT	17 (8)	13 (6)

Note: Sample sizes for telomere analyses are shown, sample sizes for hormone analyses are shown in parentheses. Sample sizes for telomere analyses do not include outliers (n = 5). Hormone sample size is the total number of individuals with hormone data available. Prior to analysing extrinsic factors affecting hormone levels, outliers were removed on a per hormone basis (n = 9 total, n = 0–3 per treatment group).

Eggs were maintained at these temperatures until reaching stage 19, just prior to the onset of gonadal differentiation, at which point they were distributed by clutch and temperature treatment into groups which received a single topical application to the eggshell of either a vehicle control (0.2 µl/g egg weight absolute ethanol) or DDE (1 µg/g egg weight; 4,4′-DDE Pestanal, Sigma Aldrich) (Kohno & Guillette, 2013). This resulted in eight treatment groups, and the sample sizes of each are reported in Table 1. The dose of DDE is based on levels previously measured in field collected eggs from contaminated lakes (Rauschenberger et al., 2009). Following dosing, eggs were returned to their temperature treatments and incubated until hatching.

Upon hatching, neonates were weighed and snout-vent length (SVL), total length, cloacal tail girth, head length and snout length and width were measured. Hatchlings were then given two unique numbered Monel tags in the webbing between the middle digits of both hindlimbs and transferred to custom-built fibreglass tanks housed in a climate-controlled greenhouse that permit both swimming and basking, where they were maintained at 26.7°C for 9–10 days. Neonates were randomly assigned to tanks in order of hatching and were not fed during this period. At the conclusion of this grow-out phase, neonates were then remeasured to assess changes in morphological endpoints described above, and a blood sample was collected from each animal via the postcranial sinus. Prior to blood draw, all animals were held at a constant temperature of 31.2°C for between 12–18 h. Blood draws took place between 9:00 AM and 5:00 PM. Blood samples were transferred to lithium heparin BD vacutainers and centrifuged at 1000 rcf for 10 min. Resulting plasma was collected and transferred to cryovials, whereupon it was immediately stored at −80°C. Residual pelleted blood cells were resuspended in RNAlater and also stored at −80°C. Hatchling sex was determined using gonadal morphology and the presence or absence of the Müllerian ducts at time of dissection.

2.2 | DNA extraction

DNA was extracted from nucleated erythrocytes using a modified column approach. Briefly, blood cell samples in RNAlater were thawed on ice. To remove RNAlater from the preserved cells, 500 µl of phosphate buffer saline (PBS) was added to 100 µl aliquots of each thawed sample. The samples were then centrifuged at 5000 rcf for 15 min at 4°C. Cells formed a pellet on the bottom of the tube (if a pellet did not form, the samples were centrifuged for an additional 5 min). Pelleted cells were washed again with 500 µl of PBS after supernatant removal. Samples were then homogenized in 350 µl of lysis buffer (4 M guanidinium thiocyanate, 0.01 M Tris-HCl, pH 7.5, and 2% beta-mercaptoethanol) using a sterile steel bead. The samples were homogenized using the Minibeadbeater (BioSpec) at 30 Hz for 2 min (2.0 × 1000 oc/min). Once homogenized, samples were centrifuged for 3 min at 14,000 rcf, and 350 µl of supernatant was transferred to spin columns with fibreglass filters (Epoch Life Science, Inc). The spin columns were centrifuged for 30 s at 14,000 rcf to bind DNA to the filter and washed twice with 700 µl of wash buffer (potassium acetate (162.8 mM), and tris-hydrochloric acid (27.1 mM, pH 7.5), diluted with 60% (v/v) of ethanol). DNA was eluted in 90 µl TE (pH 8.0) and both concentration and purity of DNA was assessed via Nanodrop One (Thermo-Scientific).

2.3 | Hormone measurements

Steroid hormones were measured using the AbsolteIDQ Stero17 Kit from Biocrates (Innsbruck), applied on an ultrahigh pressure liquid chromatography tandem mass spectrometry system (UHPLC-MS/MS, Waters Acquity I-Class/Xevo TQ-S micro system [Milford]). The Stero17 kit covers a panel of 17 steroid hormones including: aldosterone, androstenedione, androsterone, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol. DHEA, DHEAS, dihydrotestosterone, E2, E1, ethiocholanolone, 17-hydroxyprogesterone (17-OHP), progesterone and testosterone. The kit contains a seven-level calibrator set, a three-level quality control sample set, an isotope labelled internal standard mixture (ISTD), a calibration matrix, a steroid specific 96-well solid phase extraction (SPE) plate for the sample preparation and three liquid additive ampules for the LC-MS mobile phase. A C18-based steroid specific UHPLC column (Biocrates) is also part of the kit and up to 80 samples can be measured with one kit. The entire workflow, from sample registration through worklist generation to data processing and concentration calculation, was controlled by the MetIDQ software, version Carbon (Biocrates).

Sample preparation can be briefly described as follows: 500 µl of blank, calibrators, QC and samples were placed into individual wells of a 2-ml 96-deep well plate. Then, 10 µl reconstituted ISTD and 400 µl purified water were added to each well. This mixture was then transferred to the preconditioned SPE plate (1 ml methanol followed by 1 ml purified water) and allowed to pass through. After washing with 500 µl purified water, the SPE plate was dried under a nitrogen stream. The steroid hormones, except for DHEAS, were first eluted using dichloromethane (2 × 500 µl). DHEAS was subsequently eluted with 600 µl acetonitrile. The dichloromethane extracts were dried under a nitrogen stream, followed by reconstitution with 50 µl of 40/60 (v/v) methanol/purified water. The acetonitrile extracts were diluted with 400 µl of purified water. Both individual extract plates were then placed into the autosampler for UHPLC–MS/MS analysis.

Sixteen steroid hormones, except DHEAS, in the first SPE extracts, were chromatographically separated on the Biocrates UHPLC steroid column with a gradient starting from 30% B (at 0.0–0.5 min) to 55% B (at 3 min) to 100% B (at 4.0–4.3 min) then down to 30% B (at 4.35 min) for column re-equilibration. The total runtime was 5 min. The flow rate was 0.5 ml/min. The mobile phase was made by mixing the content of three provided additive ampoules (10 ml each) in 470 ml of purified water. Mobile phase B was a mixture of acetonitrile/methanol/purified water with a volumetric ratio of

85/10/5. The column oven was maintained at 55°C. The injection volume was 20 µl. For the analysis of DHEAS, in the second SPE extracts the same analytical column, mobile phases, flow rate and column oven temperature setting were employed. The analytical runtime was shortened to 2 min using an extremely fast gradient, starting from 30% B (at 0.0 min) up to 50% B (at 1 min) then to 100% B (at 1.1–1.3 min), then down to 30% B (at 1.4 min) for column re-equilibration. The injections of SPE extracts were carried out in alternating fashion resulting in a total analysis time of about 8 min for each sample, including the injection times.

The analytes were detected in the Xevo TQ-S micro using the highly sensitive and selective multiple reaction monitoring (MRM) technique in positive electrospray ionization mode. Settings for individual MRM transitions, such as cone voltage and collision energy, respectively, for the analytes and their corresponding isotopically-labelled internal standards, are described in detail in the Stero17 kit user manual. The following ion source settings were used: source temperature 150°C, desolvation temperature 650°C, cone gas flow 100 (L/h), and desolvation gas flow 900 (L/h). MassLynx software version 4.1 (Waters Corporation) was used for the instrument control and data acquisition.

The acquired MS/MS data were imported to the MetIDQ software. The concentrations of individual steroid hormones were calculated based on the signal ratios of the analytes and their corresponding internal standards in samples against the seven-point calibration curve of external standards. The detection limits of each hormone are shown in Table S1. The accuracy of the hormone measurement was determined using accuracy of the calibrators. Measurements deviated ≤20% from targets for all hormones which is in the normal range for this method.

2.4 | Telomere length assay

TL for each sample was assessed using quantitative polymerase chain reaction (qPCR) according to previously described methods (O'Callaghan et al., 2008; O'Callaghan & Fenech, 2011). To normalize for genome copy number, a single copy gene (Sox9; NCBI gene ID: 102573123) was used as a reference (Western et al., 1999). A standard curve for Sox9 (10^7–10^2 copies/µl) was developed by transformation and cloning of amplicons using the PCR4 TOPO plasmid vector kit (Invitrogen 45-0030). A standard curve for the telomere sequence was developed using serial dilutions of a telomere standard oligo (5′-(TTAGG)x14-′3) according to O'Callaghan and Fenech (2011). The telomere standard oligo was ordered as an Ultramer DNA oligo from Integrated DNA Technologies (Coralville) to ensure high quality. PCR primers used for amplification were Tel1b (5′-CGG TTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTT ACCCTTACCCTTACCCTTACCCT-3′) for amplification of telomeres (Callicott & Womack, 2006) and forward sox9_199F (5′–GACAAGTTCCCCGTTTGCATC–3′) and reverse sox9_309R (5′–CGGCTTGTTCTTACTGGATCCA–3′) for amplification of sox9.

Briefly, each DNA sample was diluted to 20 ng/µl in TE (pH 8.0) and stored at −20°C in 2 µl aliquots for telomere and single copy gene reactions. For each sample, 48 µl of qPCR master mix (22 µl of filtered autoclaved water, 25 µl 2× SYBR green, 1 µl of 10 µM forward/reverse primer mix and 0.04 units/µl of AmpliTaq Gold DNA Polymerase [Thermo-Scientific]) were added to the 2 µl aliquot of DNA. Three reactions of 15 µl (containing 12 ng of DNA each) from each 50 µl aliquot were carefully pipetted onto a qPCR plate in triplicate. qPCR conditions for Sox9 were 10 min at 95°C, followed by 35 cycles of 95°C for 15 s, 64°C for 30 s, and 72°C for 30 s. qPCR conditions for the telomere primers were 10 min at 95°C, followed by 30 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s. Each run was followed by a melting curve to assure there was a single product. A standard curve, nontemplate control, and pooled DNA sample (to assess interplate variance) were run on each plate.

The data from the qPCR was processed through Bio-Rad CFX Manager (Version 3.1) to retrieve the starting quantity (SQ) values based on known quantities in the standard curve. The SQ were averaged across triplicates. The standard deviation between the triplicates were calculated in Microsoft Excel (Microsoft Corporation). Triplicates exhibiting coefficient of variation (CV) values above 15% were evaluated as potential outliers. If removal of the potential outlier brought the CV below 15%, the aberrant well was removed from the analysis (n = 16). Average CV of starting quantities per triplicate reaction was 3.4% for Sox9 and 5.3% for Tel1b/Tel2b. TL was calculated by taking the average of the triplicate SQ value of telomere (Kb of telomere sequence) and dividing it by the average of the triplicate SQ value of Sox9 (divided by two to derive number of genome copies) for each sample. Interplate CV was calculated using the TL of a control sample run on each qPCR plate. Interplate CV was 9.1% after removal of a single aberrant plate. The R^2 value for each standard curve was >0.99 across all plates. Amplification efficiencies ranged from 88.95%–98.88% (mean = 95.48%) for sox9 and 86.96%–95.03% (mean = 89.24%) for telomere plates.

2.5 | Statistical analysis

TL measurements passed tests for normality (D'Agostino's K-squared test; skewness = 0.30) and equal variance (Bartlett's test; K^2 = 8.04, df = 7, p = .33). Outliers of TL were identified using 1.5× interquartile range per treatment group and were removed from the analysis (n = 5). Statistical analysis was done by using R (Version 3.6.1) (R Core Team, 2021) utilizing the lme4 package (Bates et al., 2007; Bates et al., 2015) for liner mixed effects models (LMM). For the effects of extrinsic factors on TL and mass at hatch, we compared the effects of constant temperatures (FPT, ITC, MPT) separately from the effect of fluctuation (ITC, ITF). We compared models fit using maximum likelihood estimation (REML = FALSE) using second-order Akaike information criterion (AICc). The top model was determined by the lowest AICc value (with ΔAICc >2).

We then refit the top model using restricted maximum likelihood (REML = TRUE) for parameter estimation. *p*-values were extracted using the R package lmerTest using Satterthwaite's degrees of freedom (Kuznetsova et al., 2017). If an interaction was detected as a significant main effect, post-hoc tests were done for within group comparisons in R package emmeans using Kenward-Roger's degrees of freedom (Russel, 2021). All tests on TL used qPCR plate as a random effect.

For hormone analyses, hormone levels were tested for normality and equal variance as described above. All hormones were log base 10 transformed to bring them closer to a normal distribution. To do this a small nominal value (1.0) was added to all hormones which contained samples with zero values (17-OHP, cortisone, and 11-deoxycortisol). After log transformation, all hormones with the exception of cortisone, 17-OHP, and 11-deoxycortisol had normal distributions and all with exception of 17-OHP and cortisone had equal variances across treatment groups. Log transformed data was used for all subsequent analyses. Prior to analysing hormone levels across extrinsic treatment groups, we removed outliers which fell outside the 1.5× interquartile range (n = 9 total, n = 0–3 per hormone). Model selection for the extrinsic effects on hormone levels was performed as described above for TL with temperature used as a continuous variable. *p*-values were corrected for multiple comparisons using the false discovery rate (FDR) by implementing the "p.adjust" function in R (Benjamini & Hochberg, 1995). Whereas clutch was initially included as a random effect in all models, in three cases (17-OHP, progesterone, and 11-deoxycorticosterone) it resulted in a singular fit of the null model due to the random effect of clutch having zero variance. In these instances, the random effect of clutch was removed and linear models were used for model selection.

Individual LMMs for relationships between levels of each hormone with TL. *p*-values generated from these models were corrected for multiple comparisons using the FDR as described above.

3 | RESULTS

3.1 | Sex ratios of hatchlings

With the exception of three individuals of unknown sex, all hatchlings included in this study which were incubated at FPT, ITC, and ITF were female, while all but one hatchling produced at MPT were male. Individuals with uncertain sex assignment were removed from further analysis (n = 3).

3.2 | Clutch and telomere length

Clutch was a significant predictor of TL (p = 5.1e−06; Figure 2) while correcting for the random effect of qPCR plate and differences arising from "treatment group" which incorporated incubation temperature, temperature fluctuation, and DDE treatment. For this reason, clutch was included as a random effect in all models.

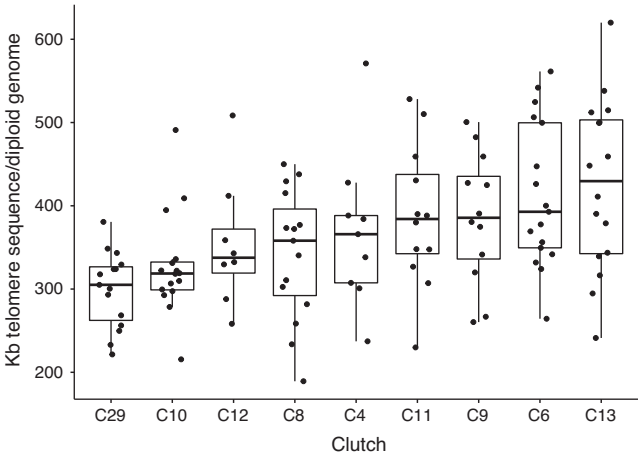

FIGURE 2 Variation in TL across clutches in hatchling alligators. Box plots show median values, upper and lower quantiles, and maximum and minimum values

3.3 | Extrinsic determinants of telomere length

To determine the extent to which extrinsic factors contributed to variation in TL, we first investigated the effects of constant incubation temperature and DDE treatment. The top-ranked model included incubation temperature, DDE, and the interactive effects of temperature and DDE (Table S2). Within this model, there was a strong interaction between DDE treatment and temperature (β = −45.32, SE = 11.40, df = 73.57, p = .0002; Figure 3a). Post-hoc tests revealed that the effect of DDE on TL is positive at FPT (β = 80.2, SE = 24.1, df = 71.8, p = .001) and at ITC (β = 57.9, SE = 23.3, df = 71.2, p = .02) while at MPT, DDE exposure has a negative effect on TL (β = −47.4, SE = 23.0, df = 70.4, p = .04; Figure 3b). Although sex and incubation temperature are confounded in this study, females at warmer temperatures (ITC) had shorter telomeres than those at cooler temperatures (FPT) (LMM; β = −36.18, SE = 17.00, df = 44.96, p = .04), suggesting that there is a direct effect of temperature in females. When temperature is treated as a continuous variable, independent LMMs in control and DDE treated animals demonstrate that constant incubation temperature has a positive effect on TL in control animals (LMM; β = 26.40, SE = 6.55, df = 31.79, p = .0003), while in DDE-treated animals TL decreases with increased incubation temperature (LMM; β = −21.35, SE = 9.86, df = 35.55, p = .04; Figure 3a).

We then separately assessed the extent to which thermal variability affects TL by comparing hatchlings incubated at ITC and ITF. The top ranked model as determined by AICc (Table S3) included effects of the thermal fluctuation and DDE. There was a positive effect of both DDE (β = 59.9, SE = 18.06, df = 51.08, p = .002) and thermal variability (β = 39.42, SE = 17.95, df = 51.05, p = .03) on TL (Figure 3b).

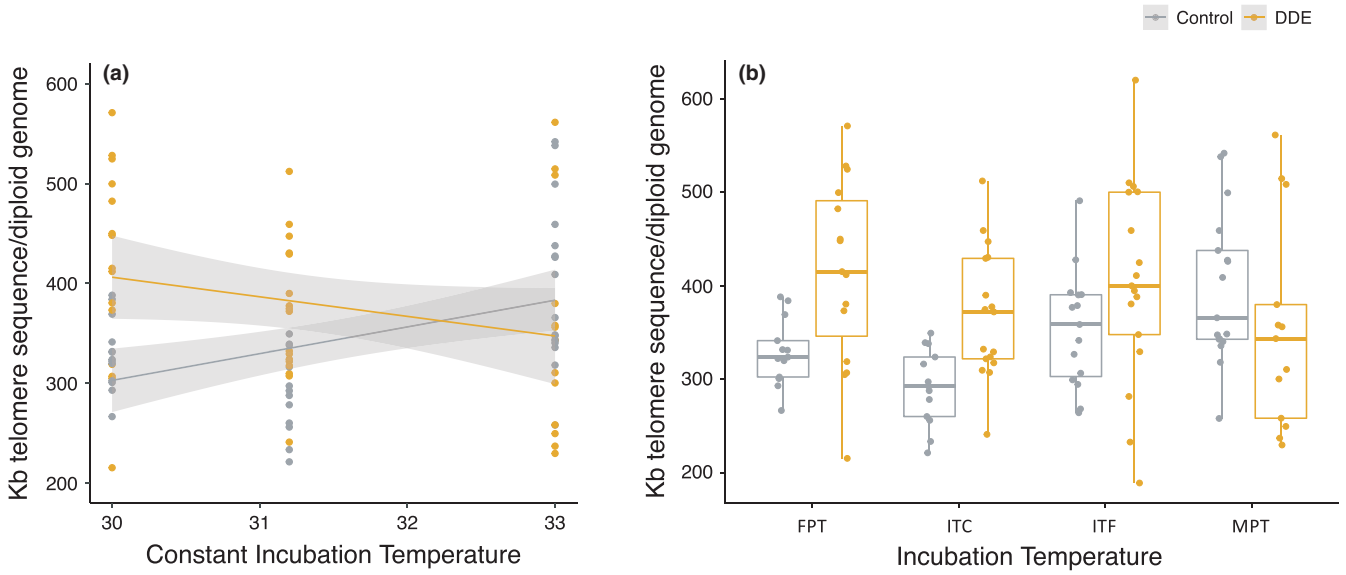

FIGURE 3 Extrinsic determinants of TL in hatchling alligators. (a) Linear regressions of TL and constant incubation temperature with DDE treatment indicated (control – grey, 1 PPM – yellow) Regression line and 95% confidence interval (shaded) are shown. (b) Box plots of TL across different incubation temperature regimes (female promoting temperature [FPT] – 30°C, intermediating temperature constant [ITC] – 31.2°C, intermediate temperature with fluctuation [ITF]– 31.2°C ± 2.8°C, and male promoting temperature [MPT] – 33°C) and DDE treatment (control – grey, 1 PPM DDE – yellow) Box plots show median values, upper and lower quantiles, and maximum and minimum values

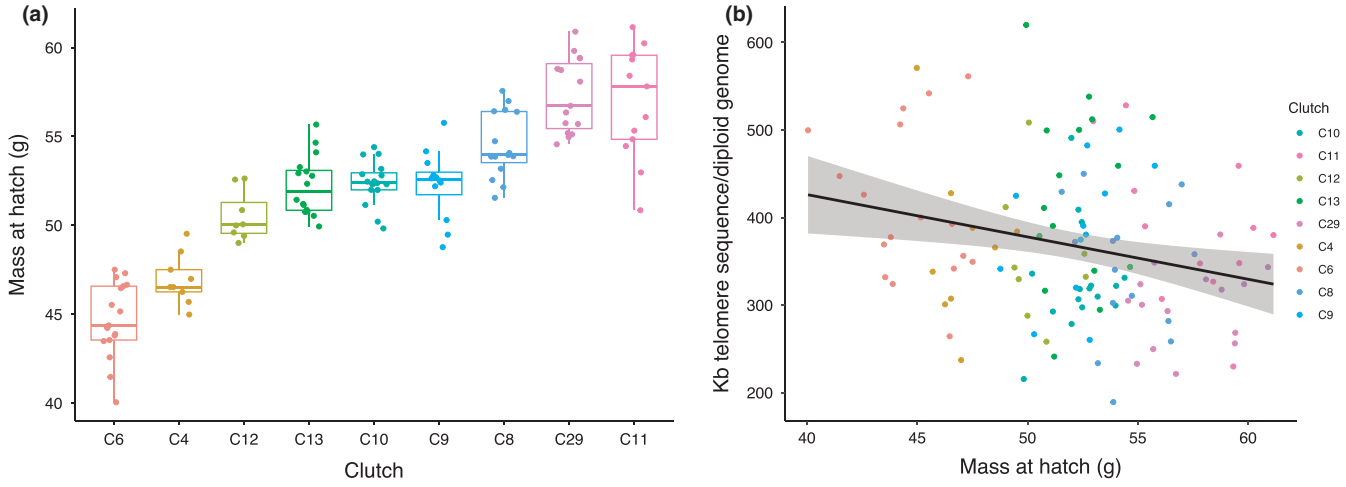

FIGURE 4 Mass at hatching underlies clutch variation in TL in hatchling alligators. (a) Box plot showing variation in mass at hatch across clutch. Box plots show median values, upper and lower quantiles, and maximum and minimum values. (b) Linear regression demonstrating the negative relationship between TL and mass at hatch. Regression line and 95% confidence interval (shaded) are shown. Individuals are coloured by clutch

3.4 | Relationship between mass at hatch and telomere length

Body size parameters, such as mass at hatching, vary significantly between clutches (LM; F = 64.03, df = 112, adjusted R^2 = 0.81, p < 2.2e−16) and might contribute to the variation in TL due to clutch (Figure 4a). To investigate this possibility, we ran LMMs testing the relationship between mass at hatch and TL. Hatchling mass was a significant predictor of TL (LMM; β = −5.06, SE = 2.33, df = 22.01, p = .04) with individuals displaying larger mass at hatching having significantly shorter telomeres (Figure 4b). Interestingly, individuals that displayed the fastest post-hatching growth rates (ΔSVL) also trended towards having the shortest TL (Figure S1).

Given the relationship between increased hatchling mass and decreased TL, we next tested the effects of incubation temperature and DDE treatment on mass at hatch. The most supported model included an interaction between incubation temperature and DDE treatment (β = 0.69, SE = 0.32, df = 76.10, p = .03), although this

model performed similarly to a model with just the effect of temperature (Table S4). In both models the increased temperature resulted in increased mass at hatch (β = 0.54, *SE* = 0.17, *df* = 78.2, *p* = .002). However, post-hoc tests revealed a more nuanced relationship whereby the effect of temperature was only significant when comparing MPT to ITC (β = 1.74, *SE* = 0.69, *df* = 74.1, *p* = .04) or FPT (β = 2.69, *SE* = 0.71, *df* = 74.1, *p* = .0009) in DDE treated animals. There was no effect of fluctuating thermal profiles (ITF) on mass at hatch when compared to ITC (Table S5).

3.5 | Effects of DDE exposure on hormone levels

In light of previous findings demonstrating relationships between contaminant exposure and glucocorticoid signalling, we hypothesized that DDE treatment might increase circulating levels of stress hormones. We tested if a panel of glucocorticoid hormones and their precursors (Figure 5a) were impacted by temperature and DDE treatments and ranked all models using AICc. DDE exposure resulted in increased levels of circulating cortisol (LMM: β = 0.20, *SE* = 0.05, *df* = 32.40, adjusted-*p* = .004; Figure 5b; Table S6). DDE exposure and an interaction between DDE and temperature also contributed to variation in levels of 17-OHP (LM: *F* = 8.10, *df* = 33, adjusted-*p* = .002; Figure 5c; Table S7), with post-hoc tests demonstrating a positive effect of DDE only at FPT (β = 0.008, *SE* = 0.002, *df* = 31, *p* = .0009) and ITC (β = 0.005, *SE* = 0.002, *df* = 31, *p* = .02). Neither DDE treatment or incubation temperature was a significant predictor for 11-deoxycortisol, progesterone, 11-deoxycorticosterone, corticosterone, or aldosterone levels (Figure 5d–h). However, in most cases DDE exposure resulted in a

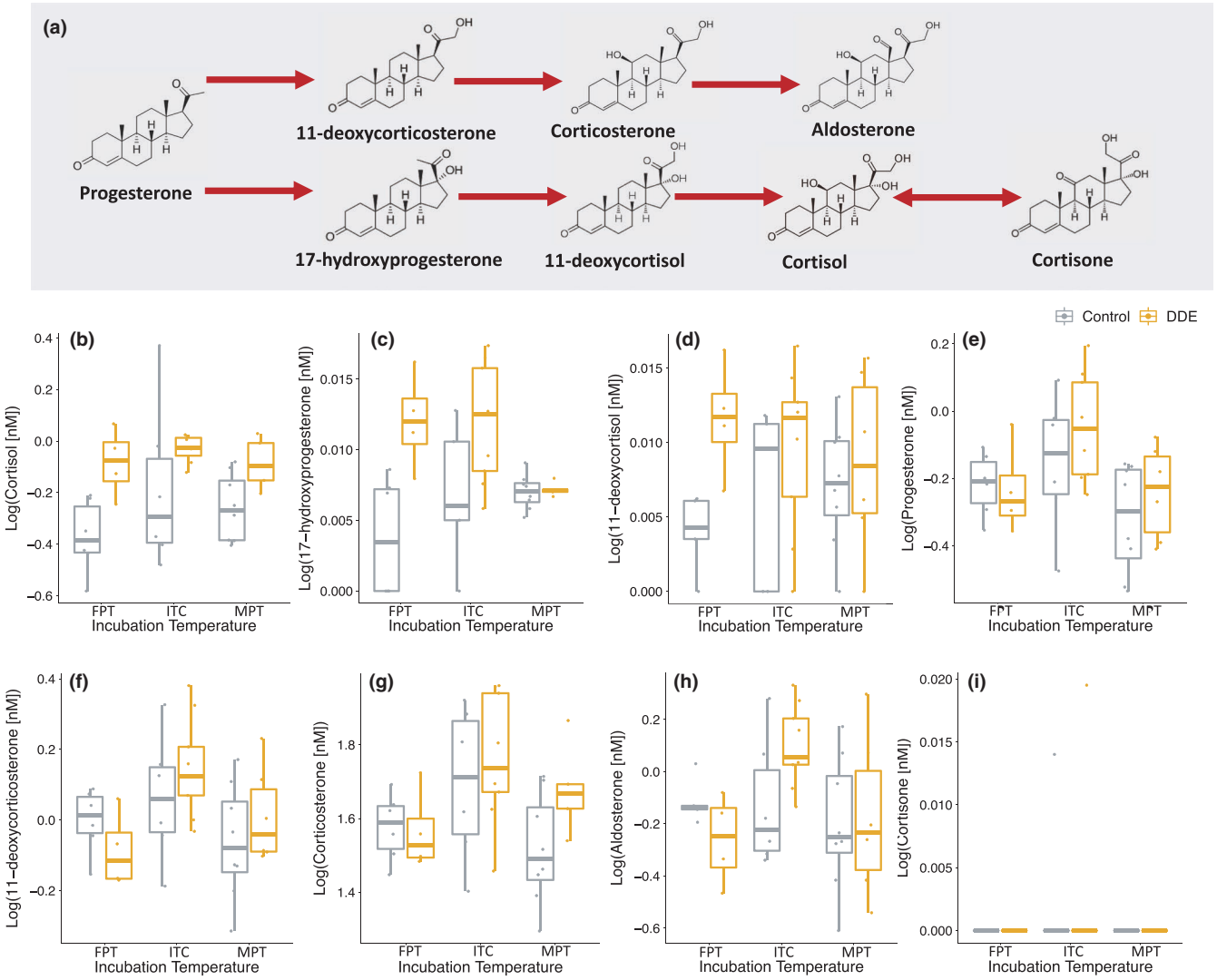

FIGURE 5 Effects of constant incubation temperatures and DDE treatment on circulating levels of glucocorticoids and their precursors in hatchling alligators. (a) Diagram of biosynthetic pathway of the measured hormones. (b–i) Box plots show hormone levels across different incubation temperature regimes (female promoting temperature [FPT] – 30°C, intermediating temperature constant [ITC] – 31.2°C, and male promoting temperature [MPT] – 33°C) and DDE treatments (control – grey, 1 PPM – yellow.) Box plots show median values, upper and lower quantiles, and maximum and minimum values

trend towards higher hormone levels. Cortisone levels were not investigated due to levels exceeding the limit of detection in only two individuals (Figure 5i).

3.6 | Relationships between hormone levels and telomere length

Given the effects of DDE exposure on cortisol levels and due to established links between glucocorticoid signalling and TL, we also investigated the relationships between circulating glucocorticoid and glucocorticoid-related hormones and TL length. We ran individual LMMs to determine if levels of the hormone panel explained variation in TL. Clutch and qPCR plate were included as random effects in all models. TL trended negatively with increased hormone levels (Figure 6a–h); however out of the eight hormones, none were significant predictors of TL.

4 | DISCUSSION

The environmental and biological factors (as well as their interactions) that affect telomere dynamics under variable ecological contexts are not resolved. However, the present study advances current understanding by combining field collections with a factorial design aimed at measuring the relative influence of intrinsic and extrinsic forces on TL in neonatal alligators. We find that both incubation temperature and embryonic exposure to a widespread endocrine disrupting contaminant affect TL. However, these effects are complex, as interactions between incubation temperature and DDE exposure appear prominently throughout our analyses. For example, embryonic exposure to DDE results in shorter telomeres in alligators incubated at the warmest temperature (MPT). At cooler temperatures (FPT, ITC, ITF), DDE exposure resulted in longer telomeres relative to vehicle exposed controls. Thus, depending on incubation temperature, the effects of DDE on TL could be considered positive or negative. Given that telomerase is known to be activated by oestrogen (Bayne et al., 2008, 2011; Kyo et al., 1999; Lin et al., 2011), it is possible that oestrogenic contaminants, such as DDE, play a similar role in promoting telomere elongation but given the dependence of sex on incubation temperature, these effects may be environment- or sex-specific in our study.

Sex and incubation temperature are inextricably linked in species with temperature dependent sex determination; thus, we were unable to directly parse the relative contributions of sex and temperature within our study design. However, an effect of incubation temperature on TL is supported by analyses comparing the females produced from the FPT and ITC incubations. In both control and DDE treated females TL trended negatively with incubation temperature, suggesting that temperature may have a direct effect on TL in females. However, the degree to which the influence of DDE exposure on TL depends on temperature or sex remains uncertain and requires additional experimentation to address.

Additionally, in contrast our prediction and to what has been demonstrated in birds (Stier et al., 2020) we report a positive effect of thermal variability on TL. This may be due to the fluctuating temperature regime (ITF) more closely replicating natural nest conditions (Bock, Hale, et al., 2020) than the constant incubation temperature (ITC) and thus being a less stressful developmental environment. However, as GCs were not measured in individuals incubated at ITF, we are unable to investigate this potential mechanism. Future studies are clearly needed to understand the long-term impacts of thermal variability on developing ectotherms.

We observed a strong effect of clutch on TL and variation in hatchling mass, both of which track negatively with TL. When taken at face value, this relationship is suggestive of a tradeoff between increased early growth occurring at the expense of longevity. There is sound support for the potential of telomere dynamics to reflect life-history tradeoffs (Young, 2018). For example, telomere attrition is enhanced by oxidative damage (Monaghan & Ozanne, 2018; Selman et al., 2012), and in the absence of telomerase, telomeric repeats are partially lost with every cell division (Blackburn, 1991; Gomes et al., 2010). Because oxidative stress and cellular proliferation are fundamental to the rapid growth occurring during development, the potential for telomere attrition is enhanced during embryonic and early life (Monaghan & Ozanne, 2018; Young, 2018). In addition to the relationship between hatchling mass and TL, an increased rate of somatic growth in neonatal alligators was also accompanied by a trend towards shorter telomeres across clutches (Figure S1). Taken together with previous studies demonstrating a relationship between TL in early life and lifespan in other archosaurs (Eastwood et al., 2019; Heidinger et al., 2012), these relationships further support the hypothesis that faster growth early in life might negatively influence TL and subsequently, longevity. Interestingly, the direct and interactive effects of incubation temperature on TL may even result in sex-specific longevity. Although little is known regarding the determinants of alligator hatchling survival, it is possible that similar to other reptiles, larger hatchlings have higher survival probabilities when compared to their smaller counterparts (Bobyn & Brooks, 1994; Civantos et al., 1999; O'Brien et al., 2005). Thus, increased hatchling size and faster growth might lead to greater survival in the short term but negatively influence traits associated with shorter telomeres later in life. These questions require additional experiments and would benefit from integrating other markers of biological aging (Parrott & Bertucci, 2019) and development (e.g., cell cycle length, oxidation state). Even so, directly testing if the decreased telomeric sequences observed in heavier and faster growing hatchlings has a bearing on individual longevity or even physiological function later in adulthood presents considerable logistical challenges in regard to the focal species here and will require long term study.

In this study, we employed a mass spectrometric approach to examine the effect of temperature and DDE treatment on circulating levels of eight steroid hormones involved in GC biosynthetic and signalling pathways. Treatment with DDE both increased GC hormone levels and decreased TL. Given that previous

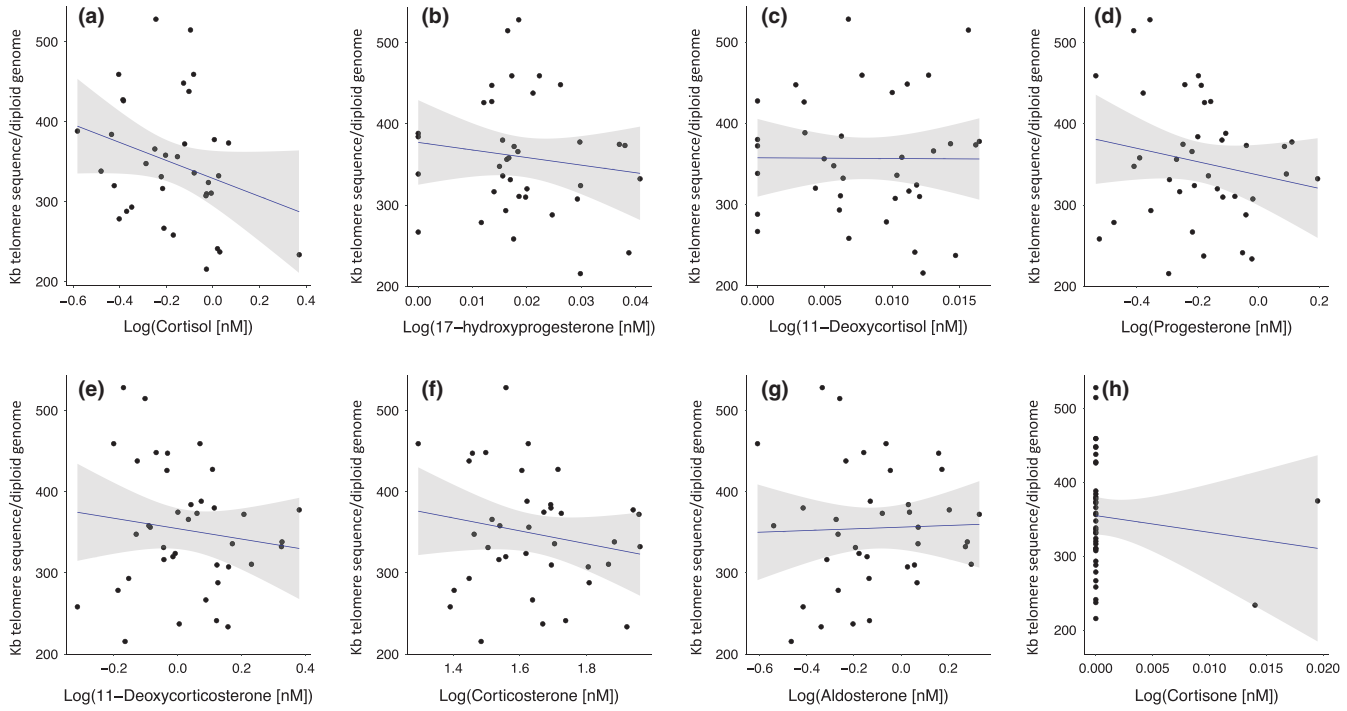

FIGURE 6 Relationship between stress hormones and TL in hatchling alligators. Linear regressions depicting relationships between each hormone and TL. Regression line and 95% confidence interval (shaded) are shown

work in birds shows similar patterns of GC signals potentially mediating contaminant-induced telomere attrition (Powolny et al., 2020), our findings partially support the hypothesis that stress hormones mediate the of the effects of anthropogenic "stressors" on TL. However, upon directly assessing the relationship between GC hormone levels and TL, we observed nonsignificant negative trends across most hormones measured. Given the generality of these trends across hormones and the limited number of individuals assayed, interpretating these results should be made with care as they do not necessarily indicate that GC signalling is uncoupled from TL in our study. Interestingly, whereas DDE treatment resulted in elevated cortisol levels, corticosterone levels were not affected. Corticosterone is considered the primary GC in reptiles; however, a study contrasting the ability of cortisol and corticosterone to activate the alligator glucocorticoid receptor in vitro demonstrated that cortisol appears to be the more potent ligand (Oka et al., 2013). Collectively, these findings suggest that cortisol might play an important role in alligator GC signalling, and that a reliance on antibody-based assays that do not distinguish between cortisol and corticosterone have the potential to skew our understanding of the relative roles of these molecules. Because almost all hormone intermediates involved in GC synthesis measured in this study appeared to trend negatively with TL, it is also possible that commonly reported negative relationships between cortisol and corticosterone levels and TL across taxa might represent a cumulative effect resulting from a number of different hormone signals. Additional studies comprehensively resolving the relationships between TL and hormones involved in GC synthesis and signalling within ecological contexts are likely to reveal novel interactions and insight into the mechanisms underlying the effects of GC signalling on telomere dynamics.

The findings reported here reveal the importance of dynamic environmental factors experienced during development on TL and provide additional support for the role of intrinsic factors, including GC hormones, in mediating telomere dynamics early in life. When contextualized within the well-documented relationships between telomere biology and longevity, reproduction, and physiological function in other vertebrates (predominantly Aves), the findings reported here suggest that organism-by-environment interactions occurring during development have the potential to influence aging trajectories with ultimate consequences for individual fitness. The current study of course is focused on one life stage and thus our ability to directly test what consequences our findings have for adult animals is limited. Yet, to our knowledge, this is one of the first studies to examine how developmental environments affect TL in a species with TSD. A relatively recently proposed theory suggests that the effects of incubation temperature on juvenile survival in combination with sex differences in age at maturity are key evolutionary drivers of TSD (Schwanz et al., 2016). Thus, the relationships between incubation temperature on TL reported here and the potential for impacts on juvenile survivorship and subsequent growth rates has the potential to inform our evolutionary understanding of TSD. Clearly additional studies connecting the developmental environment to telomere dynamics and further, linking these effects on TL to subsequent growth, survival, and reproduction are needed. We are hopeful that the current study lays the foundation for these studies as well as contributes to the growing knowledge of the early life environment as a key determinant of organismal physiology.

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AUTHOR CONTRIBUTIONS

Junsoo Bae executed telomere assays and aided in writing and editing the manuscript. Emily M. Bertucci participated in telomere assays, conducted statistical analyses, and participated in writing and editing the manuscript. Samantha L. Bock helped with DNA extractions, telomere assays, statistical analyses, and edited the manuscript. Matthew D. Hale performed animal experiments and edited the manuscript. Jameel Moore aided in DNA extractions and edited the manuscript. Phil M. Wilkinson and Thomas R. Rainwater coordinated fieldwork and edited the manuscript. Junsoo Bae, Therese Koal, and Hai PhamTuan conducted hormone assays and participated in writing and editing the manuscript. Benjamin B. Parrott oversaw all aspects of experimental design, execution, and analysis and participated in writing and editing the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been made openly available in Dryad (https://doi.org/10.5061/dryad.3bk3j9khh) at https://datadryad.org/stash/share/tbdAcGVnTrbSY76MSG7VEZuRf8yBArY9rK5V9GKqo18 (Bertucci et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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