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RESEARCH ARTICLE

Temperature experienced during incubation affects antioxidant capacity but not oxidative damage in hatchling red-eared slider turtles (*Trachemys scripta elegans*)

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ABSTRACT

Our understanding of how oxidative stress resistance phenotypes are affected by the developmental environment is limited. One component of the developmental environment, which is likely central to early life oxidative stress among ectothermic and oviparous species, is that of temperature. We investigated how incubation temperature manipulations affect oxidative damage and total antioxidant capacity (TAC) in red-eared slider turtle (*Trachemys scripta elegans*) hatchlings. First, to determine whether temperature fluctuations elicit oxidative stress, eggs from clutches were randomly assigned to either a constant (29.5°C) or daily fluctuating temperature incubation (28.7±3°C) treatment. Second, to assess the effect of temperature fluctuation frequency on oxidative stress, eggs were incubated in one of three fluctuating incubation regimes: 28.7±3°C fluctuations every 12 h (hyper), 24 h (normal) or 48 h (hypo). Third, we tested the influence of average incubation temperature by incubating eggs in a daily fluctuating incubation temperature regime with a mean temperature of 26.5°C (low), 27.1°C (medium) or 27.7°C (high). Although the accumulation of oxidative damage in hatchlings was unaffected by any thermal manipulation, TAC was affected by both temperature fluctuation frequency and average incubation temperature. Individuals incubated with a low frequency of temperature fluctuations had reduced TAC, while incubation at a lower average temperature was associated with enhanced TAC. These results indicate that although sufficient to prevent oxidative damage, TAC is influenced by developmental thermal environments, potentially because of temperature-mediated changes in metabolic rate. The observed differences in TAC may have important future consequences for hatchling fitness and overwinter survival.

KEY WORDS: Fluctuating temperature, Oxidative stress, Development, Reptile

INTRODUCTION

An individual's phenotype is determined by direct contributions of the genotype, the environment, and the interaction between the two. During early development, both abiotic (i.e. temperature, humidity, photoperiod) and biotic (i.e. parental care, hormones, egg composition) environmental components are known to have long-lasting influences on a wide variety of phenotypes (Lindström, 1999). For example, many traits such as body size, growth, locomotor performance, metabolic rate, immune response and early survival are influenced by incubation temperature in reptiles (reviewed by Deeming, 2004; Booth, 2006; DuRant et al., 2013; Bowden et al., 2014). Although the role of developmental environments in shaping many fitness-related traits has received much attention, relatively little work has been conducted to determine how they impact susceptibility to oxidative stress in either vertebrates or invertebrates, despite known consequences of oxidative stress (Metcalfe and Alonso-Alvarez, 2010; Selman et al., 2012; Marasco et al., 2013; Constantini, 2014).

Oxidative stress is defined as a shift in the redox status of a cell in favor of an oxidizing environment, which occurs when pro-oxidant generation exceeds antioxidant protective capabilities (Dröge, 2002). Among aerobic organisms, highly unstable pro-oxidants known as reactive oxygen species (ROS) are frequently produced (Finkel and Holbrook, 2000; Balaban et al., 2005). Although low levels of ROS serve as critical cellular signaling molecules, ROS also interact with and oxidize cellular macromolecules (e.g. lipids, proteins and DNA), resulting in a type of cellular damage referred to as oxidative damage (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2007). Oxidative damage accumulation threatens cellular functioning and can eventually cause cell death (Halliwell and Gutteridge, 2007). To avoid both oxidative stress and oxidative damage, organisms utilize a suite of enzymatic and dietary antioxidants that neutralize excessive ROS (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2007).

The vast majority (up to 90%) of pro-oxidants are generated during aerobic cellular respiration at the mitochondria (Balaban et al., 2005). Consequently, the onset of oxidative stress often coincides with environmental challenges associated with metabolic shifts such as exercise (Nakamoto et al., 2007), an acute stress response (Haussmann et al., 2012), and recovery from hypoxia or anoxia (Willmore and Storey, 1997). Variability in ambient temperature also commonly elicits changes in metabolic rates which, in turn, can affect ROS production rates (Constantini, 2014). Thus, temperature-induced oxidative stress appears to be widespread. Increases in tissue oxidative damage following challenges with either acute cold or acute heat has been observed in a variety of taxa (marine: Parihar et al., 1996; Heise et al., 2006; Bagnyukova et al., 2007; An et al., 2010; Ibarz et al., 2010; Castro et al., 2012; Rosa et al., 2012; birds: Mujahid and Furuse, 2009; Constantini et al., 2012; insects: Lalouette et al., 2011; reptiles: Ballen et al., 2012). Changes in enzymatic antioxidant production and activity during thermal stress also occur, but are highly variable and context dependent (Constantini, 2014). Following extended cold exposure, similar increases in some antioxidant enzymes have been observed in mammals (Spasić et al., 1993; Selman et al., 2000; Kaushik and Kaur, 2003) and zebrafish (*Danio rerio*) (Malek et al., 2004). In contrast, although antioxidant enzymes increased in

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Received 20 July 2015; Accepted 1 December 2015

Journal of Experimental Biology (2016) 219, 561-570 doi:10.1242/jeb.128843

List of abbreviations

CHE	cumene hydroperoxide equivalents
CTE	constant temperature equivalent
CUPRAC	cupric reducing antioxidant capacity
FOXO	ferrous oxidation-xylenol orange method
M-β-CD	methyl-β-cyclodextrin
ROS	reactive oxygen species
TAC	total antioxidant capacity

response to acute heat stress in tobacco plant seeds, antioxidant systems were negatively affected by long-term heat stress and high indices of oxidative stress were observed (Sgobba et al., 2015). Thus, associations between environmental temperature and oxidative stress will vary over different temporal scales.

Offspring of oviparous species likely encounter either acute or prolonged thermal challenges during embryonic development (DuRant et al., 2013), making temperature a candidate environmental factor for influencing oxidative stress resistance early in life. Developing embryos are generally passive inhabitants of their thermal environments, with limited ability for behavioral or physiological thermoregulation. As a result, their ability to avoid temperature-induced oxidative stress prenatally is constrained. Periods of oxidative stress may result either from nonlinear relationships between physiological traits and temperature (Ruel and Ayres, 1999) or from exposure to extreme temperatures. Additionally, ontogenic studies suggest that maturation of the antioxidant system is not completed until early in adulthood and enzymatic antioxidant activity is often lacking until the very end of embryonic development (Khan and Black, 2003; Barata et al., 2005; Fontagné et al., 2008; Rizzo et al., 2007). Thus, during early development, individuals have reduced defenses against oxidative stress and are potentially reliant on dietary antioxidants available in the yolk, including carotenoids, vitamin E and vitamin A (Thompson et al., 1999; Speake et al., 2001; Surai et al., 2001; Dierenfeld et al., 2002; Weiss et al., 2011).

Importantly, given that embryonic thermal challenges may coincide with the development of metabolic and antioxidant systems, long-term effects of oxidative stress on metabolic or antioxidant programming are also likely to occur. Consistent with this prediction, both pre- and postnatal stress experience has been related to variability in adult redox status (Constantini et al., 2012; Marasco et al., 2013; Noguera et al., 2015; Romero-Haro and Alonso-Alvarez, 2015; Zimmer and Spencer, 2015). For instance, following an intense heat stress in adulthood, zebra finches (*Taeniopygi guttata*) that had been exposed to a mild heat stress as juveniles acquired less oxidative damage than individuals with no prior exposure to heat stress (Constantini et al., 2012). Similarly, plasticity of oxidative stress resistance and antioxidant defenses has also been observed following prenatal glucocorticoid exposure (Marasco et al., 2013; Zimmer and Spencer, 2015) and limited post-hatch nutrient availability (Noguera et al., 2015; Romero-Haro and Alonso-Alvarez, 2015). The apparent priming of redox systems by early life experiences has been suggested to arise through epigenetic modifications of gene expression or post-translational alterations of enzymes (Romero-Haro and Alonso-Alvarez, 2015; Noguera et al., 2015) and may result in context-dependent fitness consequences (Marasco et al., 2013; Constantini et al., 2014; Noguera et al., 2015; Zimmer and Spencer, 2015).

Despite the potential for developmental thermal environments to affect oxidative stress, to date only a few studies have evaluated the effects of incubation temperatures on early-life accumulation of oxidative damage and antioxidant defenses (Rosa et al., 2012; Yalçin et al., 2012; Loyau et al., 2014), and these studies utilized unnatural incubation temperatures. We therefore determined whether offspring redox status is influenced by average incubation temperatures commonly encountered in the wild by an oviparous freshwater turtle, the red-eared slider [*Trachemys scripta elegans* (Wied 1839)]. Turtles offer a number of advantages for studying the effects of incubation temperature on early-life redox status. Unlike many avian species, turtles do not provide any post-laying parental care and embryos develop under ambient thermal conditions in the nest. Differences in incubation temperature resulting from nest location, depth or climate (Weisrock and Janzen, 1999; Shine and Elphick, 2001; Booth, 2006; Warner and Shine, 2008; Paitz et al., 2010a; Micheli-Campbell et al., 2012) can influence the development of numerous fitness-related traits, such as sex, locomotor performance, body size, growth and early survivorship (Deeming, 2004; Booth, 2006; DuRant et al., 2013; Bowden et al., 2014). Importantly, embryos are exposed to a broad range of daily and seasonal temperature fluctuations that represent an ecologically and physiologically relevant challenge faced during development. In comparison to artificial constant temperature incubations, the experience of incubation temperature fluctuations have notably different effects on offspring phenotypes (Schwarzkopf and Brooks, 1985; Shine and Harlow, 1996; Demuth, 2001; Webb et al., 2001; Ashmore and Janzen, 2003; Du and Ji, 2006; Les et al., 2007; Du and Feng, 2008; Lin et al., 2008; Patterson and Blouin-Demers, 2008; Du et al., 2009; Paitz et al., 2010b; Li et al., 2013; Horne et al., 2014). In terms of oxidative stress, daily exposure to higher temperatures during even small fluctuations may be sufficient to cause a shift in an embryo's redox status (Booth and Astill, 2001). Thus, development under temperature fluctuations that are large in either frequency or amplitude is potentially more oxidatively challenging compared with incubation in thermally stable conditions.

In the present study, we performed three experiments to evaluate the effects of thermal manipulations on *T. s. elegans* hatchling redox status. Using a split-clutch experimental design, we asked: (1) do *T. s. elegans* hatchlings incubated in fluctuating temperature regimes experience different levels of oxidative stress than hatchlings incubated at a constant temperature (constant versus fluctuating); (2) does changing the frequency of incubation temperature fluctuations, which thereby alters the frequency of metabolic shifts, affect levels of oxidative stress experienced by *T. s. elegans* hatchlings (fluctuation frequency); and (3) what is the effect of mean temperature on the redox status of *T. s. elegans* hatchlings (mean temperature)? We quantified oxidative damage via the measurement of lipid hydroperoxides and total antioxidant capacity (TAC) as an integrative measure of available antioxidants in hatchling liver samples (Halliwell and Gutteridge, 2007; Somogyi et al., 2007).

We hypothesized that during the development of an ectothermic turtle, exposures to high temperatures (Booth and Astill, 2001) during temperature fluctuations experienced throughout incubation would cause increases in metabolism and, consequently, oxidative stress. Based on this hypothesis, we predicted that hatchlings incubated with temperature fluctuations would experience greater amounts of oxidative stress in comparison to a constant temperature incubation regime. Additionally, we predicted that a higher frequency of temperature fluctuations or mean temperature would result in increases in oxidative stress. In all experiments, we expected that the experience of oxidative stress during embryonic development would result in either a reduction of TAC or increased levels of oxidative damage in hatchlings.

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MATERIALS AND METHODS

Egg collection and incubation

Eggs were collected from red-eared slider turtles during the 2013 and 2014 nesting seasons at Banner Marsh State Fish and Wildlife Area (Canton, IL, USA). All clutches were obtained either via excavation of nests laid within 6 h prior to collection or from gravid females caught in baited hoop traps. Trap-caught females were brought back to the laboratory, where an oxytocin injection was used to induce oviposition (Ernst and Lovich, 2009). Over the 2 yr, a total of 45 clutches ranging in size from seven to 22 eggs were collected to conduct three independent experiments. Prior to incubation treatment assignment, all eggs were weighed to the nearest 0.01 g and marked to indicate clutch and individual. Eggs were collected under Illinois Department of Natural Resources permits NH13.2084 and NH14.2084.

Our first experiment was conducted to determine whether temperature fluctuations have different consequences for the redox status of red-eared slider hatchlings than constant temperature incubation (constant versus fluctuating). This experiment included six early season (4–9 June 2013) and five late season (16–19 June 2013) clutches of *T. s. elegans* eggs (n=132). Collecting across the nesting season ensured that our projects captured the full range of potential variation in egg composition (Harms et al., 2005) present in our population. Eggs from each clutch were randomly assigned to a constant temperature incubation (n=69; constant) or fluctuating temperature incubation group (n=63; fluctuating) in a split-clutch design. Constant temperature incubation occurred at 29.5°C, while fluctuating eggs were incubated at temperatures sinusoidally fluctuating ±3°C around a mean of 28.7°C (Fig. 1A). This fluctuating regime has a constant temperature equivalent (CTE) of 29.4°C, closely approximating the constant temperature treatment (Georges et al., 1994). The CTE model is commonly employed to estimate the sex ratios of species with temperature-dependent sex determination during fluctuating temperatures and accounts for the fact that embryonic developmental rate is positively related to temperature (Georges et al., 2005). As such, the constant temperature incubation regime is equivalent to the temperature in the fluctuating regime above and below which approximately half of development occurs (Georges et al., 2005). Further, these treatments expose eggs to temperatures well within the range experienced in natural nests of this species (A.W.C., unpublished data) and are suitable for development (Les et al., 2009). For example, for *T. s. elegans* nests at our field site in 2014, the average range of daily temperatures experienced throughout the incubation period was 5.6°C and the minimum and maximum nest temperatures recorded were 17.5 and 33.0°C, respectively (A.W.C., unpublished data).

In our second experiment, we varied the frequency of temperature fluctuations to assess the effect on hatchling redox status (fluctuation frequency). *Trachemys scripta elegans* eggs from 13 clutches (n=108) collected early in the 2013 nesting season were randomly assigned to one of three fluctuating incubation regimes: (1) 28.7±3°C sinusoidal fluctuations every 12 h (hyper, n=36), (2) 28.7±3°C sinusoidal fluctuations every 24 h (normal, n=36) or (3) 28.7±3°C sinusoidal fluctuations every 48 h (hypo, n=36) in a split-clutch design (Fig. 1B) (Bowden et al., 2014). All three of these fluctuating treatments have a CTE of 29.4°C as the total amount of time spent at any given temperature over the entire course of incubation is consistent (Georges et al., 1994). By manipulating fluctuation frequency in this manner, we were able to determine how individuals respond to deviations in environmental thermal stability and systematically control the number of metabolic changes experienced throughout incubation (Bowden et al., 2014). Furthermore, these treatments exposed individuals to warmer or cooler temperatures for different continuous durations, without affecting the overall mean and range of temperatures experienced (Fig. 1B) (Bowden et al., 2014). Thus, from this experiment we were able to establish whether the number of metabolic changes or continuous time spent at a given temperature is more important for hatchling oxidative stress phenotypes.

In the third experiment, we determined the importance of mean temperature during a fluctuating incubation regime on *T. s. elegans* redox status (mean temperature). We collected 10 clutches early in the 2014 nesting season (29 May–6 June 2014) and 11 clutches

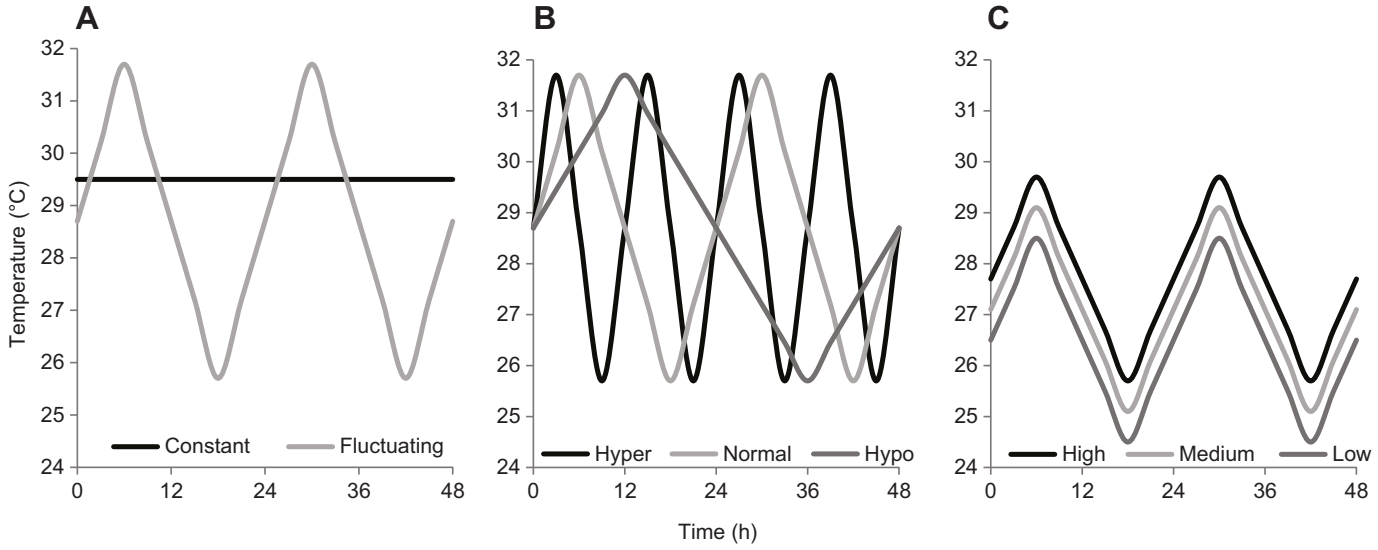

Fig. 1. Experimental design and thermal profiles of the incubation environment experienced by *Trachemys scripta elegans* embryos in each of our three split-clutch design experiments. (A) Constant versus fluctuating: eggs (n=130) from 11 clutches were incubated under either a constant (29.5°C) or fluctuating (28.7±3°C) temperature regime. (B) Fluctuation frequency: eggs (n=108) from 13 clutches experienced a 28.7±3°C sinusoidal fluctuation every 12 (hyper), 24 (normal) or 48 h (hypo). (C) Mean temperature: eggs (n=214) from 21 clutches experienced one of three sinusoidal fluctuating incubation temperature regimes: 27.7±2°C (high), 27.1±2°C (medium) or 26.5±2°C (low).

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later in the season (17–21 June 2014). Eggs within each clutch were then randomly assigned to one of three fluctuating temperature incubation regimes, all of which experienced a 'normal' 24 h fluctuation cycle: (1) 26.5±2°C (low, n=76 eggs), (2) 27.1±2°C (medium, n=78 eggs) or (3) 27.7±2°C (high, n=76 eggs), in a split-clutch design (Fig. 1C). The CTE of these treatments were 26.9, 27.5 and 28.0°C, respectively (Georges et al., 1994).

For all experiments, eggs were incubated while partially buried in moist vermiculite (−150 kPa) in plastic incubation boxes. Prior to the initiation of each experiment, a traceable thermometer was utilized to ensure the incubator temperatures were fluctuating as desired. Additionally, during incubation, i-Buttons (Maxim Integrated, San Jose, CA, USA) were placed in each incubator and recorded the temperature every hour. Boxes were weighed periodically and water loss was replaced to maintain hydric conditions near 100% humidity throughout incubation, and egg boxes were rotated within the incubators every 5 days. Day of pip was determined as the date on which the hatchling first breached the egg shell. Incubation period was defined as the number of days from the start of incubation until day of pip. Following hatching, all individuals were kept at room temperature (approximately 24–26°C).

On post-pip days 8–10, we recorded hatchling mass to the nearest 0.01 g and plastron and carapace lengths to the nearest 0.01 mm for all individuals. Hatchlings were euthanized by an intrapleuroperitoneal injection of pentobarbital sodium solution between 8 and 16 (constant versus fluctuating), 73 and 99 (fluctuation frequency), or 37 and 44 days post-pip (mean temperature). Hatchlings from the fluctuation frequency and mean temperature experiments were involved in other projects (Bowden et al., 2014; Carter et al., 2016; A.W.C., unpublished data) and could not be euthanized until these later dates, but given that hatchlings from our population overwinter in their natal nest (Ernst and Lovich et al., 2009), growth and development during this period is minimal and we would expect our hatchlings to all be at a comparable developmental state. Liver tissue samples were harvested within 15 min after injection, flash frozen in liquid nitrogen, and stored at −80°C until TAC and oxidative damage analyses were performed. All work was approved by the Illinois State University Institutional Animal Care and Use Committee (protocols 09-2013, 16-2013, 17-2013 and 08-2014).

Antioxidant and oxidative damage analyses

Antioxidant analysis

Measurement of TAC of all hatchling liver tissue samples was used to determine the combined activity of all available non-enzymatic antioxidants. Liver TAC was determined spectrophotometrically using a modified cupric reducing antioxidant capacity (CUPRAC) method. The CUPRAC method estimates TAC based on the ability of tissue samples to convert copper (II)-neocuproine to copper (I)-neocuproine (Özyürek et al., 2008). We chose to use the CUPRAC assay for liver TAC measurements for several reasons. First, unlike many other TAC assays, the CUPRAC assay is performed at the physiological relevant pH of 7.0. Second, using the CUPRAC assay the activity of both hydrophilic and hydrophobic antioxidants can be measured simultaneously. Third, the assay is readily adaptable and has been used to analyze a wide variety of biological samples including plasma and animal tissues (Özyürek et al., 2011).

Here we performed the CUPRAC assay as described by Özyürek et al. (2008) with the described modifications. Briefly, liver samples were homogenized at a 1:40 (w:v) dilution of 2% methyl-β-cyclodextrin (M-β-CD) dissolved in an 9:1 (v:v) acetone–water mixture to simultaneously extract and dissolve hydrophobic and hydrophilic antioxidants. Homogenates were centrifuged at room temperature for 10 min at 10,000 ***g*** and the supernatant was removed for use in the assay. Reactions were set up in a 96-well plate format and consisted of 75 µl of 10 mmol l^{-1} copper (II) chloride solution prepared in water, 75 µl of 7.5 mmol l^{-1} neocuproine solution prepared in 96% ethanol, 75 µl 8 mol l^{-1} urea buffer (pH 7.0) prepared in a standard Tris buffer (86 mmol l^{-1} tris, 90 mmol l^{-1} glycine and 4 mmol l^{-1} citrate, pH 8.0), and 55 µl of 2% M-β-CD prepared in an 9:1 (v:v) acetone–water mixture. All reagents were added in the order listed prior to the addition of tissue samples. Finally, 20 µl of tissue homogenate was added, bringing the total volume of each reaction to 300 µl. Reactions were incubated for 30 min at room temperature, after which absorbance was measured at 405 nm (BioTek ELx 800, Winooski, VT, USA). Liver TAC was calculated in micromolar Trolox equivalents per milligram of wet tissue weight (µmol l^{-1} Trolox equiv. mg^{-1} ww) via absorbance comparison to a standard curve that ranged from 100 to 12.5 µmol l^{-1} Trolox. The 1 mmol l^{-1} Trolox standard, which is a synthetic version of the potent antioxidant vitamin E, was prepared just prior to use in the 2% M-β-CD dissolved in 9:1 (v:v) acetone–water solution. All samples were run in duplicate. A single assay was performed for each of the three experiments and had intra-assay CVs of 6, 8 and 9%, respectively. Across all three TAC assays, the inter-assay CV was 2%.

Oxidative damage analysis

As our primary measure of oxidative damage, lipid hydroperoxides were spectrophotometrically quantified using the ferrous oxidation-xylenol orange (FOXO) method (Hermes-Lima et al., 1995). Lipid hydroperoxides are long-term by-products of oxidative damage to lipids that threaten both cell membrane stability and composition (Halliwell and Gutteridge, 2007). Given that liver is the primary storage site of lipids, lipid hydroperoxides are an appropriate measure of oxidative damage. We chose to use the FOXO assay because it has been previously applied for lipid hydroperoxide measurement in a variety of adult red-eared slider turtle tissues (Hermes-Lima et al., 1995).

The FOXO assay was performed as described by Hermes-Lima et al. (1995). Lipids were extracted from tissue via homogenization in ice-cold methanol at a 1:40 (w:v) ratio. Samples were centrifuged at room temperature for 10 min at 10,000 ***g*** and the supernatant was saved. Assay reactions composed of 250 µl of 1 mmol l^{-1} $FeSO_4$, 50 µl of 500 mmol l^{-1} H_2SO_4, 100 µl of 1 mmol l^{-1} xylenol orange, 580 µl of H_2O and 20 µl of tissue extract, were set up in 1.5 ml Eppendorf tubes. All solutions were prepared just prior to use and added in the order listed. Reagent blanks were prepared by replacing the 20 µl aliquot of tissue extract with water. Following addition of tissue extract, all reactions were vortexed and allowed to incubate at room temperature for 5 h. Absorbance at 580 nm was subsequently measured for all samples and blanks. Next, 5 µl of 1 mmol l^{-1} cumene hydroperoxide was added to every reaction and 580 nm absorbance was re-measured following a 60 min incubation at room temperature. Lipid hydroperoxides are expressed in nanomolar cumene hydroperoxide equivalents (CHE) per milligram of wet tissue weight (nmol CHE mg^{-1} ww) and were calculated using the following equation: lipid hydroperoxides=$(A_{sample}-A_{blank})/(A_{sample+CHE}-A_{blank})\times5\times(1000/V)\times41$, where V is the volume of extract (Hermes-Lima et al., 1995). All samples were run in duplicate and intra-assay CVs of all replicates were below 10%. Inter-assay CVs for the constant versus fluctuating, fluctuation frequency, and mean temperature experiments were 7, 6 and 5%, respectively.

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Statistical analysis

All statistical analyses were performed using SAS 9.3 statistical software. For all analyses, treatment and season were included as fixed effects and clutch was included as a random effect. Interactions including clutch were also considered random effects. Season was included in our analyses because of potential differences in the egg composition of clutches laid early or late in the nesting season (Harms et al., 2005). Clutch was nested within season because these two variables were dependent on one another. If necessary, data were transformed prior to analysis to meet assumptions of normality and homogeneity of variances. When included as a covariate, age at euthanization was not statistically associated with hatchling liver oxidative damage or TAC (all $P>0.05$), except with liver oxidative damage in hatchlings from the constant versus fluctuating experiment ($F_{1,107}=4.18$, $P=0.043$). Although statistically significant, the estimated linear relationship between age and liver oxidative damage was close to zero ($\beta=-0.068$ nmol CHE mg^{-1} ww day^{-1}). Given this, and the fact that hatchling liver oxidative damage and liver TAC did not co-vary with age in any of the other experiments, we concluded that the one significant effect is unlikely to be biologically relevant. As such, age at euthanization was excluded from all statistical analyses.

Separate analyses for each of our experiments were performed to determine the effects of incubation conditions on measures of oxidative damage and TAC in hatchling livers. To analyze results from the first (constant versus fluctuating) and third experiments (mean temperature), we performed a mixed-effect MANOVA (PROC GLM). For the MANOVAs, we used Pillai's trace to derive our F-statistics and the standardized canonical coefficients were used to interpret relative contributions of our dependent variables to significant effects (Scheiner, 2001). All follow-up analyses of significant fixed effects were conducted using pairwise comparisons with a Tukey correction and an experimentwise $\alpha=0.05$. All significant random effects were estimated by determining the variance components via PROC MIXED.

For the analysis of the fluctuation frequency experiment, measurements of TAC could not be transformed to meet the assumptions of the MANOVA. Thus, we analyzed effects on liver oxidative damage and TAC separately. Specifically, clutch and treatment effects on oxidative damage were determined using a two-way mixed-effect ANOVA (PROC GLM). To analyze the effect of temperature fluctuation frequency on TAC, we first applied an aligned-rank transformation using ARTool (v. 1.5.1; Wobbrock et al., 2011). The aligned ranked transformation procedure can be applied for the non-parametric analysis of multi-factorial designs (Higgins and Tashtoush, 1990, 1994; Wobbrock et al., 2011). During the alignment process, contributions of all other effects are removed prior to ranking (Higgins and Tashtoush, 1990, 1994). Thus, separate ranks and ANOVAs were conducted to test for clutch, treatment, and clutch by treatment interaction effects on hatchling TAC.

Finally, as a follow-up analysis to the mean temperature experiment, we were interested in determining whether there was a significant linear relationship between hatchling liver TAC and mean incubation temperature. To do this we conducted a mixed-effect ANCOVA (PROC GLM). In the ANCOVA model, average incubation temperature was included as a covariate, TAC as the response variable, season as a fixed effect, and clutch nested within season as a random effect.

RESULTS

Experiment 1: constant versus fluctuating incubation

Incubation temperature fluctuations did not have a significant effect on measures of *T. s. elegans* hatchling oxidative status (MANOVA: $F_{2,8}=2.38$, $P=0.154$; Table 1); there were no differences in mean levels of liver lipid peroxidation or TAC of hatchlings subjected to a constant or fluctuating temperature incubation (Fig. 2A). There were also no seasonal differences in hatchling measures of liver oxidative damage or TAC, or an interaction effect between treatment and season on redox status (all $P>0.05$; Table 1). There was, however, a significant effect of clutch on liver oxidative damage and TAC (MANOVA: $F_{18,18}=9.05$, $P<0.0001$; Table 1). Based on the standardized canonical coefficients there was a stronger effect of clutch on liver oxidative damage in comparison to TAC (Table 1). Accordingly, clutch identity explained 65% of random variation in liver oxidative damage, but only 36% of the random variation in liver TAC. Within-clutch measures of liver oxidative damage and TAC were positivity related to one another such that clutches with higher levels of oxidative damage also had higher levels of TAC. Finally, the interaction between clutch and treatment was not significantly associated with either liver oxidative damage (ANOVA: $F_{9,108}=0.96$, $P=0.477$) or liver TAC (ANOVA: $F_{9,108}=1.31$, $P=0.242$), and was estimated to explain 0% and 4% of the random variation in these measures, respectively.

Experiment 2: effect of temperature fluctuation frequency

Temperature fluctuation frequency did not have a significant effect on levels of lipid hydroperoxides in hatchling livers (ANOVA: $F_{2,37.018}=0.65$, $P=0.500$; Fig. 2B). However, incubation temperature fluctuation frequency did have a significant effect on hatchling liver TAC (ANOVA: $F_{2,101}=8.30$, $P=0.0005$; Fig. 2B). Based on pairwise comparisons, median liver TAC following low-frequency (every 48 h) temperature fluctuations was significantly reduced in comparison to normal (every 24 h) and high (every 12 h) fluctuation frequencies. There were, however, no differences in the TAC of hatchlings experiencing temperature fluctuations either

Table 1. The redox status of *Trachemys scripta elegans* hatchlings does not vary between constant (29.5°C) and fluctuating (28.7±3°C) incubation temperatures

				Standardized canonical coefficients	
Source	Pillai's trace (F)	d.f.	P	Damage	TAC
Treatment	2.38	2,8	0.154	1.391	0.946
Season	0.57	2,8	0.586	0.016	0.441
Season×Treatment	1.63	2,8	0.254	0.141	–1.092
Clutch	**9.05**	**18,18**	**<0.0001**	**1.671**	**0.604**

We conducted a mixed-effect MANOVA to analyze treatment, clutch and season effects on measures of oxidative damage and total antioxidant capacity (TAC). Significant effects are in bold.

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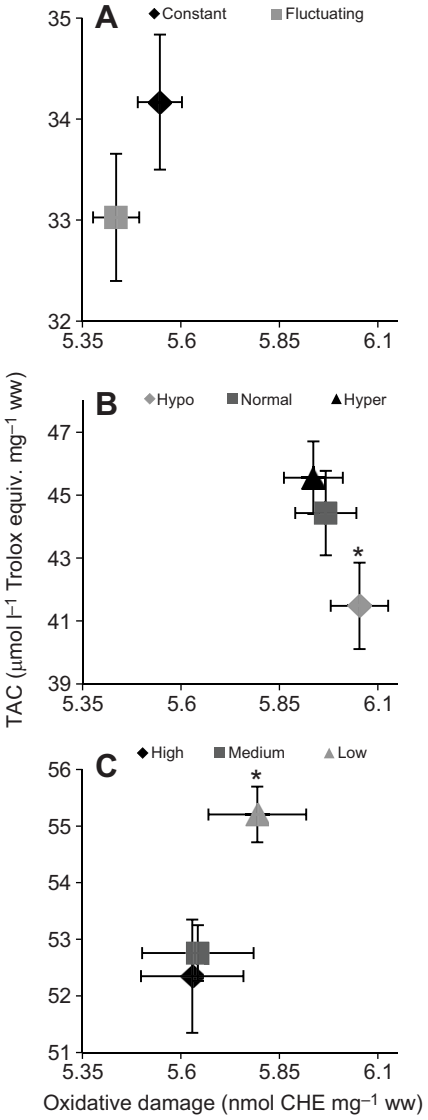

Fig. 2. Effects of incubation temperature manipulations on *Trachemys scripta elegans* hatchling oxidative damage and total antioxidant capacity (TAC). (A) Mean±s.e.m. liver lipid hydroperoxides and TAC in hatchlings from eggs incubated under either a constant (n=69) or fluctuating (n=63) incubation temperature regime. There are no significant differences (all P>0.05) in either measure between groups. (B) Median±s.e.m. liver lipid hydroperoxides and TAC in hatchlings from eggs incubated under thermal regimes that varied in fluctuation frequency (every 12, 24 or 48 h cycles; n=36 for all treatments). TAC is significantly reduced following low-frequency incubation temperature fluctuations (hypo; ANOVA: $F_{2,101}$=8.30, P=0.0005). (C) Mean±s.e.m. liver lipid hydroperoxides and TAC in hatchlings from eggs incubated under thermal regimes that varied in mean temperature [26.5°C (n=76), 27.1°C (n=78) or 27.7°C (n=76)]. TAC is significantly increased in hatchlings from eggs incubated with a low (26.5°C) average temperature (MANOVA: $F_{4,76}$=4.23, P=0.004).

every 12 or 24 h. In addition to this treatment effect, there was a significant effect of clutch on both liver lipid oxidative damage (ANOVA: $F_{12,26.1}$=14.16, P<0.0001) and TAC (ANOVA: $F_{12,91}$=11.19, P<0.0001) that explained 67% and 39% of the random variation among individuals in these measures, respectively. Finally, there was no significant interaction effect between fluctuation frequency treatment and clutch on either oxidative damage (ANOVA: $F_{24,69}$=0.89, P=0.612) or TAC (ANOVA: $F_{24,65}$=0.94, P=0.552) in hatchling livers.

Experiment 3: effect of mean incubation temperature

Interestingly, we found that hatchling oxidative status was significantly related to mean incubation temperature (MANOVA: $F_{4,76}$=4.23, P=0.004; Table 2). Based on the standardized canonical coefficients, TAC contributed more to the significant effect of temperature treatment than did oxidative damage, and the two measures were positively associated with one another (Table 2). Accordingly, follow-up analyses via multiple pair-wise comparisons revealed that there were no significant differences in average levels of liver lipid peroxidation (Fig. 2C). However, the low mean temperature treatment group was associated with a significant increase in average hatchling liver TAC in comparison to the medium and high mean temperature treatment groups (Fig. 2C). In addition to the main effect of treatment, there was a highly significant effect of clutch on the oxidative status of hatchlings (MANOVA: $F_{38,76}$=16.00, P<0.0001; Table 2), consistent with our other experiments. Both liver oxidative damage and TAC were influenced by clutch to a similar and strong degree, based on the standardized canonical coefficients (Table 2). Accordingly, clutch was able to explain 58% of random variation in levels of lipid peroxidation and 64% of random variation in TAC. Furthermore, within-clutch amounts of liver oxidative damage and TAC were directly related to one another such that hatchling livers from clutches with higher amounts of oxidative damage also tended to have a higher TAC. However, there was no significant effect of the clutch by treatment interaction on either oxidative damage (ANOVA: $F_{38,151}$=0.81, P=0.780) or TAC (ANOVA: $F_{38,151}$=0.98, P=0.516). Finally, nesting season was not significantly related to hatchling oxidative status, nor was there a significant treatment by season interaction (all P>0.05; Table 2).

Given the significant effect of incubation treatment on hatchling liver TAC, we were further interested in determining whether there was a linear relationship between mean incubation temperature and TAC. Thus, as a follow-up analysis, we conducted a mixed-effect ANCOVA with TAC as the dependent variable, season as a fixed effect, clutch nested within season as a random effect, and mean incubation temperature as a covariate. In agreement with our first MANOVA analysis, there was a significant effect of temperature (ANCOVA: $F_{1,192}$=17.26, P<0.0001) and clutch (ANCOVA: $F_{19,192}$=17.66, P<0.0001), but no effect of season on liver TAC (ANCOVA: $F_{1,19.113}$=0.49, P=0.4924). There was a significant negative linear relationship between mean incubation temperature and hatchling TAC with an estimated slope of −2.41 µmol l^{-1} Trolox equiv. mg^{-1} ww $°C^{-1}$ (t-test: P<0.0001).

DISCUSSION

In a series of three experiments we were able to gain insight into the influence of developmental thermal environments on the early life redox status of an oviparous reptile, the red-eared slider turtle. We found no effect of incubation temperature or temperature fluctuations on the accumulation of liver lipid hydroperoxides post-hatch, a finding that was consistent across all experiments. Additionally, contrary to what we expected, thermal fluctuations during incubation do not appear to influence early life oxidative stress in red-eared slider hatchlings. In our first experiment, there were no differences in hatchling levels of liver oxidative damage or TAC following incubation under a constant or fluctuating temperature regime. Thus, there is no evidence that diel temperature fluctuations, within the optimal thermal range of turtle development, elicit oxidative stress in red-eared slider hatchlings. Similarly, in our hyper treatment group, an increase in the frequency of temperature fluctuations was not associated with

Journal of Experimental Biology (2016) 219, 561-570 doi:10.1242/jeb.128843

Table 2. Average incubation temperature significantly affects the redox status of *Trachemys scripta elegans* hatchlings

Source	Pillai's trace (*F*)	d.f.	*P*	Standardized canonical coefficients	
				Damage	TAC
Treatment	**4.23**	**4,76**	**0.004**	**0.561**	**1.488**
Season	2.34	2,18	0.125	0.420	–0.265
Season×Treatment	1.54	4,76	0.198	0.901	1.287
Clutch	**16**	**38,76**	**<0.0001**	**1.021**	**1.192**

We conducted a mixed-effect MANOVA to analyze treatment, clutch and season effects on measures of oxidative damage and TAC. Significant effects are in bold.

any differences in the oxidative status of hatchlings. Although there was a significant effect of low-frequency temperature fluctuations on hatchling redox status, as discussed below, we suspect that this was not related to the experience of temperature fluctuations, but instead was a consequence of extended exposure to higher temperatures in this treatment. Overall, these experiments highlight the potential for developmental conditions to affect redox status, but also that these effects may be complex.

Under natural incubation conditions oviparous reptile embryos are normally exposed to predictable daily incubation temperature fluctuations that can vary unpredictably in magnitude (Weisrock and Janzen, 1999; Shine and Elphick, 2001; Booth, 2006; Warner and Shine, 2008; Paitz et al., 2010a; Micheli-Campbell et al., 2012). Under normal developmental conditions, transient, temperature-associated changes in enzyme production or activity may be sufficient to prevent oxidative stress and lipid hydroperoxide accumulation. Thus, *T. s. elegans* may be well adapted to cope with predictable and moderate temperature changes during embryonic development via the activation of enzymatic antioxidant and repair systems. In the present study, we were unable to measure the activity of any enzymatic antioxidants. However, previous studies in adult red-eared slider turtles report that they have exceptional antioxidant defense systems and constitutively maintain high levels of enzymatic antioxidants (Willmore and Storey, 1997, 2005). In this species, the frequent threat of oxidative challenges (e.g. prolonged submergence, super-cooling, freezing, anoxia) may have favored strong selective pressures for increased investment in cellular maintenance. Investment in the maintenance of a proficient antioxidant system to minimize oxidative damage in their cells might also underlie reptilian life history traits including an extended lifespan, reproduction throughout adulthood, and indeterminate growth (Wilbur, 1975). Our finding of low levels of oxidative damage accumulation in *T. s. elegans* hatchlings adds support to the idea that turtles consistently allocate a large proportion of resources to cellular maintenance throughout their lifetime.

Alternatively, because all average incubation temperatures were ecologically relevant, and temperature fluctuations were predictable, it is also possible that our thermal manipulations did not elicit oxidative stress during development. Yet, treatment effects on hatchling liver non-enzymatic antioxidants were observed in two of our experiments. Thus, although temperature fluctuations per se do not appear to pose much of an oxidative threat to reptilian embryos, the duration of continuous exposure and the absolute magnitude of temperatures experienced are likely the most critical aspects of the thermal environment influencing TAC during development in *T. s. elegans*. For example, low-frequency temperature fluctuations were associated with decreases in TAC of hatchlings. By manipulating the frequency of temperature fluctuations we were able to hold the range and average temperatures experienced by hatchlings during development constant. However, the duration over which individuals were exposed to either low or high temperatures was altered. Hatchlings experiencing low-frequency fluctuations were subjected to temperatures above and below the mean in continuous bouts that were at least two times longer than hatchlings in the normal and high fluctuation frequency groups (see thermal traces in Fig. 1B). We suspect that the extended exposure to higher temperatures was also associated with longer periods of elevated metabolic rates (Gatten, 1974; Du and Shine, 2015; Sun et al., 2015). Recently, Sun et al. (2015) reported that increasing incubation temperatures are associated with significant increases in the rate of aerobic respiration and the activity of cytochrome oxidase (involved in the electron transport chain) in reptile embryos. We propose that during periodic exposure to high temperatures during diel temperature fluctuations, alterations in mitochondrial activity can directly influence basal ROS production rates and result in the subsequent depletion of dietary antioxidants for protection. Consistent with this interpretation, higher average incubation temperatures were associated with lower TAC in hatchlings. Importantly, in our data there is no evidence of a relationship between TAC and incubation period (ANCOVA: $F_{1,327}$=0.10, P=0.750). Thus, it is likely that the observed effects of temperature on TAC were direct and not related to developmental rate.

In addition to low metabolic rates, two other non-mutually exclusive proximate mechanisms may contribute to increases in TAC following incubation at lower temperatures in the thermal neutral range. First, in turtles, incubation at lower constant temperatures is associated with a greater incorporation of yolk nutrients into hatchling tissues (Gutzke et al., 1987; Booth et al., 2004). Accordingly, the observed elevations in TAC following low-temperature incubation may be reflective of a greater deposition of unused maternally derived yolk antioxidants into hatchling liver tissue. Second, the expression of uncoupling proteins during cold exposure might also occur (Loyau et al., 2014). Uncoupling proteins act in the inner mitochondrial membrane to promote thermogenesis or heat production instead of ATP production. Consequently, increases in uncoupling protein expression have been linked to a reduction in ROS generation (Finkel and Holbrook, 2000).

Despite no significant treatment effects of low-frequency temperature fluctuations and low temperature on oxidative damage, there were significant consequences of these treatments for hatchling levels of TAC. The effects on TAC alone have the potential for long-term consequences for hatchling life history and fitness. In our population of red-eared sliders, following hatching in late summer–early autumn, hatchlings remain in their natal nest and do not emerge until the following spring (Ernst and Lovich, 2009). During this period of overwintering, hatchlings only have the resources left in their residual yolk, making metabolic depression critical for survival. However, during the winter months, several metabolic- and oxidative-stress-promoting challenges such as

recovery from freezing and anoxic environments may occur (Storey, 1996; Baker et al., 2007; Costanzo et al., 2008). Individuals with lower TAC due to the thermal incubation environment may be at a disadvantage and unable to handle such large and frequent oxidative stress challenges. If this is the case, then lower TAC may result in an increase in oxidative damage accumulation during overwintering and potentially a lower overwinter survival probability. Furthermore, if observed incubation-temperature-associated differences in TAC persists into adulthood it may play a role in mediating future life history trade-offs and survival (Alonso-Alvarez et al., 2006; Bize et al., 2008; Kim et al., 2010). Future longitudinal studies focused on determining whether there are any long-term consequences of differences in hatchling TAC are necessary to test these predictions.

Similar to many other turtle hatchling phenotypes (e.g. size, mass, locomotor performance), we found that redox status was strongly and consistently related to clutch identity in all of the experiments. These clutch effects suggest that despite exposure to identical incubation environments, some clutches may be predisposed to be more susceptible to early life oxidative stress than others. Such robust clutch effects additionally suggest that there are potentially strong genetic or maternal effects influencing oxidative stress phenotypes in *T. s. elegans*.

Our work is the first to measure levels of oxidative damage and TAC in red-eared slider turtle hatchings. We demonstrate that during incubation temperature fluctuations within natural temperature ranges, hatchlings are well protected from oxidative stress and do not accumulate excessive liver lipid peroxidation. This effect is potentially reflective of a life-long combined adaptive advantage of high antioxidant activities and metabolic plasticity. Despite this, *T. s. elegans* post-hatch liver TAC does appear to be influenced by the duration of continuous exposure and the absolute magnitude of incubation temperature. Although no short-term consequences of variability in TAC are evident in hatchlings, if persistent, these differences could have long-term effects on red-eared slider life history strategies and fitness.

Acknowledgements

We thank Justin Dillard, Piers Hunter, Sarah Marrochello, Ryan Paitz and Laura Zimmerman for all of their help in the field. In addition, we acknowledge Dr Mark Haussmann for insightful discussion on the interpretation of these results and Dr Steve Juliano for both discussion and advice on the statistical analyses of the datasets. Finally, we thank two anonymous reviewers for their comments and suggestions, which have greatly improved this article.

Competing interests

The authors declare no competing or financial interests.

Author contributions

L.A.T., A.W.C. and R.M.B. designed the experiments and performed the field work and sample collections; L.A.T. performed the laboratory work and statistical analysis; L.A.T., A.W.C. and R.M.B. all provided ideas and contributed to the writing of the manuscript.

Funding

Funding for this work was provided by a Phi Sigma Weigel Grant, a Phi Sigma Mockford-Thompson Fellowship and a Sigma Xi Grant in Aid of Research to L.A.T.; an NSF-GRFP and a Phi Sigma Weigel Grant to A.W.C.; and a College of Arts & Sciences University Research Grant and NSF-IOS 0952840 to R.M.B.

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