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EFFECTS OF THE DEVELOPMENTAL ENVIRONMENT ON OXIDATIVE
DAMAGE AND ANTIOXIDANTS IN RED-EARED SLIDER
TURTLE (*TRACHEMYS SCRIPTA ELEGANS*)
HATCHLINGS

Lisa A. Treidel

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Developmental environments influence many individual phenotypes. However, currently we have a limited understanding of how the developmental environment influences oxidative stress resistance phenotypes. Oxidative stress is defined as a physiological state during which the production of harmful free radicals exceeds the protective capabilities of antioxidants. Variations among adults in susceptibility to oxidative stress can have important consequences for life history strategies and fitness related traits. Our work was aimed at providing insight in to the role that the developmental environment plays on oxidative stress phenotypes in the model oviparous reptile the red-eared slider turtle (*Trachemys scripta elegans*). Here, we conducted three independent experiments to determine if hatchling levels of oxidative damage and antioxidant activities vary following manipulations of the incubation thermal environment. First, to investigate if temperature fluctuations elicit oxidative stress during incubation, eggs from clutches were randomly assigned to a constant temperature (29.5°C) or daily sinusoidal 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; $28.7 \pm 3^\circ\text{C}$ sinusoidal fluctuations every 12 (Hyper), 24 (Normal), or 48 hours (Hypo). Third, we tested the influence of average incubation temperature on hatchling oxidative damage and total antioxidant capacity (TAC). To test this, eggs were incubated in daily sinusoidally fluctuating incubation temperature regime with a mean temperature of 26.5°C (Low), 27.1°C (Medium), or 27.7°C (High). We report that regardless of any thermal manipulation, no treatment effects on hatchling accumulation of oxidative damage were observed. This finding suggests that *T. scripta* hatchlings have sufficient antioxidant defenses that effectively protect individuals from temperature induced oxidative stress during incubation. However, hatchling TAC was influenced by both temperature fluctuation frequency and average incubation temperature. Following incubation with a low frequency of temperature fluctuations individuals had a reduced TAC, while incubation at a lower average temperature was associated with enhanced TAC. These results indicate that hatchling TAC is likely sensitive to the developmental thermal environment and may have important future consequences for hatchling fitness. In addition to temperature, we also saw that both oxidative damage and TAC were significantly related to clutch identity suggesting that there are either strong maternal or genetic factors influencing early life oxidative status of *T. scripta*.

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HATCHLINGS

LISA A. TREIDEL

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

2015

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HATCHLINGS

LISA A. TREIDEL

COMMITTEE MEMBERS:

Rachel M. Bowden, Chair

Steven A. Juliano

Mark F. Haussmann

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CHAPTER I

EARLY DEVELOPMENT AND OXIDATIVE STRESS

Introduction

An individual's life history strategy is delineated by trade-offs between fitness defining behaviors and physiological functions (Ricklefs & Wikelski 2002). Consequently such trade-offs represent constraints to the evolution of life history strategies (Stearns 2000). While extensive work has demonstrated the ubiquity of trade-offs among life history traits, the proximate mechanisms underlying them are poorly understood (Roff & Fairbairn 2007). One potential proximate mediator of life history strategies and trade-offs, which has recently gained considerable attention is oxidative stress. Oxidative stress occurs when the generation of free-radicals by cells exceeds the defensive capabilities of available antioxidants. Oxidative stress may be a common cost associated with the major life history traits (e.g. growth and reproduction), making it a prime candidate for mediating trade-offs (Dowling & Simmons 2009; Monaghan, Metcalfe, & Torres 2009; Constantini et al. 2010; Selman et al. 2012).

Recent work performed in wild populations provides support for a central role of oxidative stress in life-history trade-offs. For example, in a wild population of Alpine Swift (*Apus melba*) high resistance to oxidative stress co-varied with higher survival of males and higher fecundity in females (Bize et al. 2008). Similarly, a role for oxidative

stress in the trade-off between growth and maintenance was supported by work done in nestling red-winged blackbirds (*Agelaius phoeniceus*) (Hall et al. 2010). In accordance with these findings, inter-species comparative work within avian (Galván et al. 2012), mammalian (Brunet-Rossinni 2004), and reptilian (Bronikowski 2008) classes have demonstrated that increased resistance to oxidative stress is often associated with a “live slow, die old” life history strategy.

Oxidative stress acts throughout an individual’s lifetime to influence phenotype and shape life-history trajectories (Metcalf & Alonso-Alvarez 2010). However, challenges faced during embryonic development and early-life likely have long-lasting effects on one’s ability to resist oxidative stress and subsequent fitness. As a result, it’s important to gain an understanding of the roles that environmental and genetic determinants play to influence an individual’s susceptibility to oxidative stress. Studying the factors that influence the development of redox physiology may thus provide important insight into trade-offs and the evolution of life-history strategies. However, to date limited work has been focused on understanding the development of oxidative stress resistance (Metcalf & Alonso-Alvarez 2010; Constantini 2013). Accordingly, the work presented here is aimed at gaining insight into how the developmental environment influences early-life oxidative stress in a model reptilian species, the red-eared slider turtle (*Trachemys scripta*). Specifically, we sought to answer two questions:

1. How does the thermal environment of embryonic development influence hatchling oxidative damage and total antioxidant capacity (TAC) in *T. scripta*?
2. Are hatchling *T. scripta* measures of oxidative damage and TAC related to season, clutch size, or maternal age?

Reactive Oxygen Species, Oxidative Damage, Antioxidants, and Oxidative Stress

Aerobic organisms rely on the use of oxygen for the efficient production of energy in the form of ATP. Counter intuitively, while aerobic organisms rely on oxygen for survival, they frequently also encounter endogenously produced oxygen forms known as reactive oxygen species (ROS), which are toxic. Damage caused by ROS activity is referred to as oxidative damage and can occur on a daily basis to all major macromolecules. In order to deal with the constant threat of ROS and to prevent oxidative damage all aerobic organisms have developed a protective antioxidant system. Normally, activity of the antioxidant system is in balance with ROS production rates so that no damage is acquired by cells (Monaghan et al. 2009). However, during metabolic challenges such as heat or cold shock (Parihar et al. 1996; Heise et al. 2006; Mujahid & Furuse 2009; An et al. 2010; Ibarz et al. 2010; Lalouette et al. 2011; Castro et al. 2012; Ballen et al. 2012a; Rosa et al. 2012), exercise (Nakamoto et al. 2007), an acute stress response (Haussmann et al. 2012), or recovery from hypoxia or anoxia (Willmore & Storey 1997), ROS production rate is likely to exceed antioxidant activity, resulting in acquired oxidative damage. The period during which an organism experiences an imbalance between ROS and antioxidants is termed oxidative stress. Individuals typically respond to oxidative stress by up regulating their antioxidant system. Additionally, damage acquired during periods of oxidative stress can often be repaired afterwards through the use of repair mechanisms, most notably DNA Base Excision Repair (Monaghan et al. 2009).

Assessments of ROS, oxidative damage, and antioxidants have proved challenging. Part of the difficulty arises due to the fact that in actuality there are many

different types or forms of ROSs, oxidative damage, and antioxidants that could be present in different tissues at any given time. The types of ROS encountered can fall into two separate subcategories; free radicals and non-radical oxidants. Free radicals refer to compounds containing an unpaired electron in their valence shell or outer-most orbits. Free radicals are extremely biologically reactive and unstable. When produced, free radicals most often initiate cascades of damage by borrowing electrons from a presently stable molecule. Common examples of free radicals produced in organisms include superoxide (O_2^-), hydroxyl ($^{\cdot}OH$), and nitric oxide ($NO^{\cdot}$). Non-radical oxidants while more stable, cause similar oxidative damage in biological molecules to that of free radicals. Common examples of non-radical oxidants are hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and hypochlorous acid ($HClO$) (Finkel & Holbrook 2000; Monaghan et al. 2009).

Similarly to having subdivisions of ROSs there are also three general types of oxidative damage caused by ROS, each classified based on the biological macromolecule damaged; lipid peroxidation, protein damage, and DNA damage. Lipid peroxidation threatens cell membrane stability and composition (Monaghan et al. 2009). A number of markers can be detected as indicators of lipid peroxidation. Measurements of conjugated dienes can be used as an early sign of lipid peroxidation. However, more frequently measured indicators of lipid peroxidation are the longer lasting byproducts, lipid hydroperoxides and malondialdehyde (MDA) (Willmore & Storey 1997). Oxidative damage to proteins most often results in structural and functional alterations of proteins and enzymes. The most common means of assessing protein oxidative damage is the measurement of protein carbonyl formation. Carbonyls added to proteins following

oxidation are the byproduct directly responsible for protein function disruption following oxidative stress (Monaghan et al. 2009). Finally, oxidative damage to DNA often results in single strand breaks or the formation of 8-oxo-7,8-dihydroguanine (8-oxoGua) which, is a lesion that promotes base-pair mismatching during DNA replication. Damage to DNA cannot be measured directly, but 8-oxoGua lesions can be detected using either High-performance liquid chromatography coupled with electrochemical detection or single cell gel electrophoresis (Comet assay) (Cadet et al. 2003). Similar to both lipid peroxidation and protein damage, ROS damage to DNA, if unrepaired, can have devastating effects for cell survival via the promotion of genome instability, mutation accumulation, and apoptosis. Unfortunately, there is no single assay that can be used to quantify all three types of oxidative damage at once. As a result in assessing oxidative damage multiple methods are required to quantify levels of damage in each macromolecule separately (Monaghan et al. 2009).

Perhaps most complicated are classifications of the antioxidants that act to protect cells from oxidative damage caused by ROS. Generally speaking however, antioxidants are often classified as either being exogenously or endogenously derived. There are a large number of diverse antioxidants that vary in specificity and activity (Monaghan et al. 2009). Antioxidant activity is often affected by the characteristics of the cellular environment, most notably the partial pressure of oxygen. Additionally, when several types of antioxidant compounds are concurrently present, they act either additively or synergistically to more effectively protect cells from oxidative damage (Surai, Speake, & Sparks 2001). While it is not possible to measure an organism's total oxidative damage, we are capable of estimating their total antioxidant capacity (TAC). However, more often

than not different antioxidants are assessed individually in different tissues (Monaghan et al. 2009). By measuring a number of different antioxidants separately, it is possible to gain insight into specific strategies utilized in the face of oxidative stress challenges that may not be obvious when looking only at TAC. For example, an individual can shift its reliance on exogenous antioxidants to endogenous antioxidants or vice versa if more energetically favorable based on current prevailing environmental conditions. As a result it is important to have a basic familiarity with the key types of antioxidants and their activity.

The main classes of exogenous or dietary antioxidants are carotenoids and vitamin E. In general dietary lipophilic antioxidants act as chain-breaking antioxidants, to directly neutralize ROS (Monaghan et al. 2009). More specifically, antioxidant activity of carotenoids involves the direct incorporation of ROS into the molecule structure itself. In contrast, Vitamin E neutralizes free radicals by the direct contribution of an electron or proton (Surai, Speake, & Sparks 2001). Based on studies conducted in mammals and birds we know that organisms are capable of the selective uptake and tissue specific deposition of both vitamin E and carotenoids (Traber 2002; Kotake-Nara & Nagao 2011; Surai, Speake, & Sparks 2001). Once within cells, vitamin E and carotenoids are seen to act mainly in the lipid membrane to prevent lipid peroxidation (Monaghan et al. 2009). Of the two major classes of dietary antioxidants the most diverse group and possibly also the most important is the carotenoids. Carotenoids can be further classified based on their biochemical structure as either with or without an oxygen molecule in their hydrocarbon structure, to be a carotene or xanthophyll respectively. While representing only a small structural discrepancy among carotenoids, there are important biologically relevant

implications of this difference in that carotenes are completely internalized in the cell membrane, while xanthophylls can transverse the membrane. This positional difference in turn is directly related to slight variations in carotenoid activity with xanthophylls being more active under normal conditions compared to carotenes. Importantly, in addition to their antioxidant activities, carotenoids are also involved in the maintenance of vitamin A and vitamin E. The carotenoid β -carotene can be converted directly into retinol or vitamin A. Carotenoids also act to reduce and regenerate active vitamin E following the donation of an electron or proton to ROSs by vitamin E (Surai, Speake, & Sparks 2001).

Similar to exogenous antioxidants, there is a large variety of endogenous or enzymatic antioxidants. Generally speaking, enzymatic antioxidants can act either to directly neutralize a specific ROS or repair oxidative damage. Some of the most commonly measured specific enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX), glutathione S-transferase (GST), glutathione reductase (GR), and alkyl hydroperoxide reductase (AHR). Briefly, SOD acts along with metal binding proteins to reduce the free radical superoxide (O_2^-) to a non-reactive form of hydrogen peroxide (H_2O_2) or singlet oxygen (O_2). Both CAT and GPOX act in separate parts of the cell to neutralize high and low levels of hydrogen peroxide respectively by converting it directly to water (Willmore & Storey 1997; Monaghan et al. 2009). In the process of detoxifying hydrogen peroxide, GPOX acts by taking an oxygen molecule from hydrogen peroxide and adding it to glutathione. GST works in the same manner but simply to detoxify products of lipid peroxidation. Following the oxidation of glutathione by either GPOX or GST, GR is required to reduce and regenerate the

standard version of glutathione (Hermes-Lima & Storey 1993). Finally, AHR acts along with NADH to repair the lipid peroxidation byproduct, lipid hydroperoxides (Willmore & Storey 1997).

Overall, the interactions between ROS production, oxidative damage, and antioxidants are multifaceted and extremely complex. As a consequence of the multitude of components that contribute to the antioxidant system there are many avenues individuals can manipulate in an attempt to avoid oxidative damage and stress. The system is quite plastic and gene expression modifications can often result in tissue specific modifications of individual antioxidant enzymes in response to, or in anticipation of, oxidative damage (Storey 2006). In order to more fully assess the oxidative status of an individual it is crucial to take multiple measures of both oxidative damage and antioxidants (Selman et al. 2012). Using temporal comparisons of oxidative damage and antioxidant levels we are able to detect shifts within an individual and estimate periods of time during which they are experiencing oxidative stress. Unfortunately, in natural settings, periods of oxidative stress are often difficult to predict. Additionally, oxidative stress is a transient state and the presence of oxidative damage tends to be short lived due to efficient cell repair. Thus, oxidative stress is rather difficult to detect outside of the laboratory.

It has been proposed that telomere length shortening rates can be used as a long-term indicator of oxidative stress. Telomeres are the non-coding protective ends of chromosomes that are highly susceptible to oxidative damage. However, unlike in other regions of nuclear DNA, single-strand breaks occurring in telomeres cannot be repaired and damage is eliminated through shortening instead (von Zglinicki 2002; Houben et al.

2008). Importantly, accelerated telomere shortening due to oxidative damage accumulation has been directly linked to accelerated cell senescence and cellular aging (Monaghan & Haussmann 2006).

Oxidative Stress in Development

During development, individuals frequently encounter oxidative stress challenges, as oxidative-stress appears to be an inevitable consequence of rapid growth (Monaghan et al. 2009; Metcalfe & Alonso-Alvarez 2010). For example, elevated levels of oxidative damage markers were detected in young adult Paraguayan caiman crocodiles (*Caiman yacre*) that had just completed the transition from hatchling to juvenile, but not following the transition from juveniles to adulthood. The authors attributed this result to being representative of the large oxidative stress load associated with a rapid period of growth in the hatchling to juvenile period (Furtado-Filho et al. 2007). This interpretation is also in agreement with the aforementioned noted trade-off between growth and self-maintenance (Hall et al. 2004; Foote et al. 2011; Caprioli et al. 2013). Additionally, the acceleration of growth rates due to compensatory growth is associated with an increase in oxidative damage accumulation (Alonso-Alvarez et al. 2007; Tarry-Adkins et al. 2008; Tarry-Adkins et al. 2009; Geiger et al. 2012). Finally across species, levels of yolk antioxidants are positively associated with embryonic growth rates (Deeming & Pike 2013), further supporting the idea that growth represents a significant and ubiquitous oxidative stress challenge during development at least in vertebrates.

In addition to oxidative stress associated with rapid growth, several environmentally imposed oxidative stress eliciting challenges may arise during development. For one, changes in temperature have been found to result in oxidative

stress in a number of ectothermic and endothermic species (Parihar et al. 1996; Heise et al. 2006; Mujahid & Furuse 2009; Lalouette et al. 2011; Castro et al. 2012; Ballen et al. 2012a; Vinagre et al. 2014a; Vinagre et al. 2014b). In Senegalese sole (*Solea senegalensis*) juveniles, a six degree temperature change from 12-18°C was sufficient to result in an increase in oxidative damage markers (Castro et al. 2012), suggesting that temperature may be a critical threat to ectothermic species whose metabolic rate is especially sensitive to these changes. A second potential source of early-life oxidative stress is anoxia experienced during both prolonged diving and overwintering. Overwintering of reptilian hatchlings is characterized by remaining in their natal nest after hatching until the following spring (Ernst & Lovich 2009). During the winter hatchlings experience a suppressed metabolic state and are commonly threatened by low oxygen levels (Costanzo, Lee, & Ultsch 2008). In the absence of oxygen, during anoxic or ischemic conditions there is little threat of oxidative damage. However during the recovery from anoxia and upon the return to normoxic conditions there is an associated excessive generation of ROS and oxidative stress (Storey 1996). Third, in addition to anoxia, juveniles that overwinter also face the threat of freeze thaw cycles which have been observed to result in oxidative stress (Baker, Costanzo, & Lee 2007). Fourth, oxidative stress is often associated with parasite and immune infections (Sorci & Faivre 2009). A fifth and final pervasive source of oxidative stress that can affect individuals during early development is environmental stressors such as the threat of predators or unpredictable food availability. If these stressors trigger a stress response, glucocorticoid hormones will be released and individuals will shift into a state of oxidative stress (Haussmann et al. 2012).

Importantly, stemming from development-related deficits in antioxidant defense systems, individuals may be at a greater risk for oxidative stress during early-life in comparison to other life-history stages. Work focused on elucidating ontogenetic changes in the oxidative status of individuals suggests that juvenile endogenous antioxidant systems may be under-developed. Population wide analyses relating age and total antioxidant capacity have found that oxidative stress resistance follows a general parabolic pattern; the youngest and oldest individuals have the lowest total antioxidant capacities while the highest defenses are exhibited in early adulthood (Alonso-Alvarez et al. 2006; Isaksson et al. 2011). Similarly, Hermes-Lima et al. (2012) found that the hatchling to juvenile transition of Paraguayan caiman crocodiles (*Caiman yacre*) was also associated with an increase in antioxidant enzyme presence. However, once reaching elevated levels in young adulthood, antioxidants are consistent into adulthood (Hermes-Lima et al. 2012). This suggests that in some species there might be a critical period of time during development in which antioxidant defense systems develop. Finally, there are noted enzyme specific changes in endogenous antioxidant activity in fish (Fontagné et al. 2008), frog (Rizzo et al. 2007), crustacean (Barata et al. 2005), and mammalian species (Khan & Black 2003) during embryonic and juvenile development. While exact changes appear to be varied and species specific, together these studies indicate that developmental changes in antioxidant systems and consequent susceptibility to oxidative stress are widespread. Overall, juveniles appear to have less developed antioxidant systems potentially placing them at a greater risk for oxidative stress. To date the potential consequences of these ontogenetic changes in terms of oxidative damage

accumulation are unknown (Metcalf & Alonso-Alvarez 2010) and there is the possibility that exogenous dietary antioxidants may be able to compensate for this disadvantage.

Critically, the oxidative stress experienced during development can have long-term consequences for offspring fitness and life-history traits. Work done in a number of avian species has demonstrated that both nestling oxidative damage levels and telomere lengths are predictive of growth rates (Hall et al. 2004; Foote et al. 2011; Caprioli et al. 2013), survival probability (Losdat et al. 2012), and future reproductive success (Noguera, Kim, & Velando 2012). Furthermore, by utilizing a brood size manipulation in a captive zebra-finch (*Taenipygia guttata*) population, Alonso-Alvarez et al. (2006) found that development in larger broods was associated with delayed reproduction and thus a reduced lifetime reproductive success. In addition to reduced fecundity, individuals raised in crowded early-life environments also had a higher resistance to oxidative stress and a longer life-span in comparison to individuals raised in uncrowded conditions. Together these findings suggest that development conditions influence the trade-off between maintenance and reproduction (Alonso-Alvarez et al. 2006). Similarly, enhancement of the developmental environment of red-eye tree frogs (*Agalychnis callidryas*) through carotenoid supplementation was associated with an increase in growth rate and fecundity during adulthood (Ogilvy, Preziosi, & Fidgett 2012). These two experimental manipulations of developmental conditions clearly provide additional support for the importance of early-life experiences in shaping future offspring oxidative stress and life-history trait phenotypes.

In summary, individuals are both likely to encounter high amounts and be susceptible to oxidative stress during development. Substantial variability in

developmental environments may cause a large amount of individual variation in the frequency and intensity of oxidative stress challenges faced early in life. Finally, long-term consequences of excessive oxidative stress during development seems to play a key role in promoting life history trade-offs and shaping individual life-history strategies. Due to such life-long fitness consequences future work focused on gaining a better understanding of the factors influencing the development of oxidative stress resistant phenotypes in a broad range of organisms is critical and could shed light on the evolution of life history strategies.

Environmental Factors Influencing the Development of Oxidative Stress Resistance

As a multifaceted and complex trait there are numerous factors that determine oxidative stress phenotypes. In general however, an adult's susceptibility to oxidative stress and antioxidant defense system appears to be shaped by a combination of genetic and environmental influences acting during early development (Constantini & Dell'Omo 2006; Kim et al. 2010; Asghar et al. 2014; Losdat et al. 2014). To date a majority of work performed to explore the role of early development on oxidative stress has focused primarily on environmental factors. The two largest contributing broad environmental influences on oxidative stress resistance appear to be maternal effects (i.e. yolk composition and post-natal rearing environments) (Surai, Speake, & Sparks 2001), and the frequency and intensity of oxidative stress challenges experienced early in life (Constantini 2013). More recently, a handful of quantitative genetic studies have suggested that there are also genetic contributions to oxidative stress phenotypes (Nordfjäll et al. 2005; Njajou et al. 2007; Olsson et al. 2008; Kim et al. 2009; Olsson et al. 2011; Ballen et al. 2012b). However, this work is limited in its scope and the exact

importance of genetic links to early life oxidative stress susceptibility in all species remains uncertain and is debated (Losdat et al. 2014). Thus, the rest of this discussion will focus only on how environmental factors act to influence the development of oxidative stress resistance phenotypes.

Of the above mentioned environmental determinants of oxidative stress, most work has been aimed at understanding the impact of maternal effects. During development mothers have the ability to manipulate both the pre- and post-natal environment in which her offspring develops. These environmental manipulations can result in changes to offspring phenotype and are known as maternal effects (Mousseau & Fox 1998). One broad and influential class of pre-natal maternal effects among oviparous species is maternal contributions to egg composition. Differential deposition of nutrients, hormones, and antioxidants into eggs can all have profound, either costly or beneficial, effects on offspring traits and fitness (Groothuis et al. 2005; Gil 2008).

In terms of the development of oxidative stress phenotypes, likely the most influential maternal effect acting is the deposition of maternally provided yolk antioxidants. A wide variety of oviparous species are observed to differentially deposit a number of lipid soluble dietary antioxidants including carotenoids (Lutein, α -carotene, β -carotene, lycopene, β -cryptoxanthin, zeaxanthin), Vitamin E (α -tocopherol, β -tocopherol, γ -tocopherol), and Vitamin A into their eggs (Surai, Noble, & Speake 1996; Surai, Sparks, & Noble 1999; Speake et al. 1999; Thompson et al. 1999; Speake, Surai, & Gore 2001; Surai et al. 2001; Royle, Surai, & Hartley 2001; Barton et al. 2002; Hrak , Surai, & Mller 2002; Dierenfeld et al. 2002; Hargitai et al. 2006; Weiss et al. 2011). Avian embryos can successfully uptake yolk antioxidants and differentially deposit them into

tissues over the course of development (Gaal et al. 1995; Surai, Noble, & Speake 1996; Surai & Speake 1998; Surai, Sparks & Noble 1999; Karadas et al. 2005). Patterns of embryonic uptake and utilization of antioxidants in non-avian species has not been studied to date.

When looking both across and within species one finds an incredible amount of variation in the absolute concentrations of deposited yolk antioxidants. Much of this variation seems to be driven by the availability of dietary antioxidants to the mother during egg production (Surai, Speake, & Sparks 2001). In support of this idea numerous studies have demonstrated that maternal dietary supplementation with antioxidants during egg production results in elevated yolk antioxidant concentrations (Surai & Speake 1998; Blount et al. 2002a; Blount et al. 2002b; Royle, Surai, & Hartley 2003; Surai et al. 2003; Biard, Surai, & Møller 2005; McGraw, Adkins-Regan, & Parker 2005; Tsai et al. 2008; Müller et al. 2012). Similarly, one study performed in the great tit (*Parus major*), found a positive correlation between maternal levels of plasma carotenoids and yolk carotenoid levels (Isaksson, Johansson, & Andersson 2008). Furthermore, suggesting that dietary antioxidants represent a limited resource to females is the observation that yolk antioxidant concentrations either decrease with laying date (Royle, Surai, & Hartley 2001; Blount et al. 2002a; Cassey et al. 2005; Rubolini et al. 2006; Williamson, Surai, & Graves 2006; Safran et al. 2008; García-Tarrasón et al. 2014) or clutch size (Weiss et al. 2011). Additionally, intra-species comparisons reveal a great deal of geographically (Cassey et al. 2005; Eeva et al. 2011) and seasonally (Dierenfeld et al. 2002; Hargitai et al. 2006) associated variation in yolk antioxidant concentrations. It has thus been suggested that these patterns are driven by corresponding spatial and temporal disparities

in food supply and consequently antioxidant availability (Török et al. 2007; Catoni, Peters, & Schaefer 2008; Eeva et al. 2011; García-Tarrasón et al. 2014). Finally, adding additional support for the expectation that yolk antioxidant concentrations are environmentally constrained is a recent multi-generational study performed in canaries (*Serinus canaria*). Specifically, Müller et al. (2012) found no evidence for repeatability or heritability of yolk antioxidant compositions, suggesting that any genetic contribution to the maternal transfer of dietary antioxidants to eggs is unlikely. Instead the only factor that was significantly related to yolk antioxidants was dietary availability (Müller et al. 2012). Overall, there is ample evidence showing that yolk antioxidant deposition is environmentally limited, at least in avian systems.

In addition to the environmental availability of dietary antioxidants, two other noted influences of yolk antioxidant composition are maternal condition and paternal attractiveness. In general maternal condition as assessed by body condition (Williamson, Surai, & Graves 2006; Navara et al. 2006), immune status (Saino et al. 2002; Hargitai et al. 2009), or ornamentation (Weis et al. 2011; Zanollo et al. 2013) all have been positively related to concentrations of yolk antioxidants. There are two potential non-mutually exclusive explanations for why this relationship between maternal condition and yolk antioxidants is so prevalent. First, females in good condition may be better able to acquire antioxidants from their environment to deposit into eggs. Alternatively, females in superior body condition are likely able to allocate more of their antioxidants to reproduction as opposed to self-maintenance. Unlike with maternal condition, the relationship between paternal attraction and yolk antioxidant deposition is inconsistent among studies. Some have found that yolk antioxidant concentrations increase along with

mate attractiveness, suggesting that females invest more in their offspring when mated with a higher quality male (Williamson, Surai, & Graves 2006; Zanollo et al. 2013). In contrast, other studies have found no (Safran et al. 2008), or negative associations between mate attractiveness and yolk antioxidants suggesting that females may be compensating for the poor quality of their mate (Saino et al. 2002; Navara et al. 2006). The noted discrepancies among these studies suggest that the influence of mate quality on antioxidant deposition may be species or context specific.

The consequences of variations in yolk antioxidant presence are largely unknown. However, given the fact that maternally provided yolk antioxidants have been observed to have a number of beneficial effects on offspring phenotypes, they are expected to be crucial for offspring fitness. For example, yolk antioxidants provide offspring with protection from oxidative stress faced during hatching and the subsequent period of rapid growth. As a result, individuals hatching from eggs with elevated yolk antioxidants are less susceptible to oxidative damage accumulation in their tissues following hatching (Surai & Speake 1998; Blount et al. 2002b; Surai et al. 2003; Tsai et al. 2008). Second a couple of studies have suggested that yolk antioxidants promote both growth (Biard, Surai, & Møller 2005; Rubolini et al. 2006; Deeming & Pike 2013) and accelerated immune system development in some bird species (Saino et al. 2003; Biard, Surai, & Møller 2005; Biard, Surai, & Møller 2007). Finally, yolk carotenoids have been linked to enhanced carotenoid based signal and plumage development in birds (McGraw, Adkins-Regan, & Parker 2005; Biard, Surai, & Møller 2007; Biard et al. 2009). This effect could have important implications for the future reproductive success of individuals in the many species that use carotenoid based ornaments during mating.

In addition to yolk antioxidants, another maternal effect shown to influence the development of an individual's oxidative stress resistance phenotype is differential prenatal exposure to steroid hormones. Specifically, in the domestic chicken (*Gallus gallus*) the elevation of yolk corticosterone levels resulted in higher levels of oxidative damage markers and an over-representation of short telomeres in chicks (Hausmann et al. 2012). Similarly, several studies have also linked prenatal testosterone exposure to early-life differences in oxidative stress (Tobler & Sandell 2009; Noguera et al. 2011; Tobler et al. 2013; Treidel et al. 2013). However, due to inconsistent results among studies whether the influence of testosterone on offspring oxidative status is beneficial or costly is still debated. For example, suggesting that yolk testosterone may be beneficial to offspring, Noguera et al. (2011) found that elevated yolk testosterone concentrations were associated with decreased lipid peroxidation and increased total antioxidant capacity in yellow-legged gull (*Larus michahellis*) chicks. In contrast, male zebra finch (*T. guttata*) offspring exposed to prenatal testosterone had decreased total antioxidant capacity relative to controls (Tobler & Sandell 2009; Tobler et al. 2013) and poor DNA repair ability in female domestic chicken chicks (Treidel et al. 2013).

After hatching or birth, the quality of the postnatal nutrient environment has also been related to differences in offspring susceptibility to oxidative stress in a number of species. For example, low dietary supplementation with vitamin C to juvenile three-keeled pond turtles (*Chinemys reevesii*) had beneficial effects for individual antioxidant defense systems during hibernation (Zhang, Niu, & Xu 2012). Similarly, in juvenile Senegalese sole (*S. senegalensis*) a higher protein content diet was associated with better resistance to oxidative stress (Castro et al. 2012). Finally, in rats (*Rattus norvegicus*) poor

postnatal nutrition is related to high levels of oxidative damage markers and lower antioxidant capacities (Tarry-Adkins et al. 2008; Tarry-Adkins et al. 2009). Thus, growing up in nutrient enriched postnatal environments also appears to play a positive role in the development of resistance to oxidative stress.

A final environmental influence important for the development of oxidative stress resistance is variation in the frequency and intensity of oxidative stress challenges experienced early in life. It has been recently suggested that oxidative stress resistance is shaped by a hormetic response, characterized by the enhancement of oxidative stress resistance in response to moderate oxidative stress challenges (Constantini 2013). A couple of studies have already provided support for this idea. For instance, one study showed that exposure to lower temperatures during incubation resulted in an increase in catalase activity on day of hatch in chickens (Yalçin et al. 2012). However, more convincingly demonstrating that mild oxidative stress challenges experienced early in life influence oxidative stress phenotypes in adulthood was a recent study by Constantini, Monaghan, and Metcalfe (2012). They found that juvenile exposure to a mild heat stress was associated with an enhanced ability to handle heat stress in adulthood in comparison to control individuals whom experienced no juvenile exposure to heat stress. In contrast, and in accordance with the theory of hormesis, individuals exposed to an extreme heat stress as juveniles, were more susceptible to heat stress as adults in comparison to control individuals (Constantini, Monaghan, & Metcalfe 2012). From this study it can be suggested that moderate oxidative stress challenges are beneficial to oxidative stress resistance while severe challenges are detrimental. Accordingly, in predicting the

potential effects of environmental stressors on oxidative stress phenotypes, it is critical to take the intensity and frequency of stressors into consideration.

Utilizing Reptiles as Model Organisms to Study Oxidative Stress

The work described herein is focused on exploring the development of oxidative stress phenotypes in the model oviparous reptile, the red-eared slider turtle (*Trachemys scripta elegans*). Reptiles are one class of animals for which little work on the link between oxidative stress and life history has been conducted. Being a long-lived organism with seemingly well adapted protective systems against oxidative stress, I believe that reptiles could serve as a great model system for the study of the impact of early life history processes on oxidative stress and aging processes. Reptiles are relatively accessible to study both in natural and laboratory settings. Within the class there exists variation in strategies for dealing with oxidative stress both over developmental stages and between species, for which natural selection might have acted (Willmore & Storey 1997; Bronikowski 2008). Furthermore, as mentioned in Selman et al. (2012), understanding the life histories and specific mechanisms utilized by long lived species, such as reptiles, to mitigate oxidative stress and comparing them to shorter lifespan species may shed light on the role of oxidative stress in the evolution of life histories.

There are several reasons why reptiles are likely a good system for the further study of the role of maternal antioxidants on the development of oxidative stress resistance. Prenatal antioxidants likely play similar important roles in terms of offspring development and phenotype among oviparous reptiles as those seen in avian species. Prior studies looking at the nutrient composition of reptilian eggs have reported yolks with similar constitutions of dietary antioxidants as those among birds (Thompson et al.

1999; Speake et al. 2001; Dierenfeld et al. 2002; Weiss et al. 2011). Consistent with this a preliminary analysis of the yolk antioxidants present in eggs of *T. scripta* showed all of these major antioxidants to be present as well (Treidel, unpublished analysis; Table 1). Since hatchling reptiles appear to have less developed antioxidant systems potentially placing them at a greater risk for oxidative stress (Hermes-Lima et al. 2012), maternally provided antioxidants may be able to compensate.

Finally, studies of the functional importance of prenatal antioxidants in reptiles may shed light on additional functions of antioxidants in offspring development and fitness not observed among avian species. In contrast to birds, reptiles generally provide little or no post-laying care. As a result of this, reptile offspring face a number of additional metabolic and possibly oxidative stress promoting challenges during pre-natal development and the early postnatal period. For example, unattended eggs often experience large temperature fluctuations during development that vary along with nest location (Weisrock & Janzen 1999; Paitz et al. 2010). Given that reptilian embryos are ectothermic temperature changes are likely also accompanied by changes in metabolic rate (Gatten 1974; Litzgus & Hopkins 2003). Accordingly, since ROS production is a byproduct of metabolism, differences in the amplitudes of temperature fluctuations associated with varying metabolic loads during incubation could result in additional oxidative stress in some developing reptile embryos. Furthermore, a number of freshwater turtles rely on yolk nutrients for survival for up to nine months post-hatch, and during overwintering in the nest hatchlings are likely to experience periods of hypoxia or freezing. Recovery from these challenges is associated with a large influx of ROS as embryos shift from anaerobic to aerobic cellular respiration (Storey 1996). Due to the

pervasiveness of frequent early-life oxidative stress in reptiles, they may also be an optimal group to use in an attempt to determine the impact of stressful early life environments on oxidative status and life history. As a final point, in terms of studying oxidative stress during development, limited parental investment in reptiles in turn constrains the potential actions of environmental determinants of oxidative stress resistance phenotypes, making this a good system for focusing on early maternal contributions, as well.

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TABLES

Table 1

Dietary Yolk Antioxidants Found in Red-Eared Slider Turtle Eggs. Average concentrations of all major dietary antioxidants in two *T. scripta* egg yolk pools (n=5 eggs/pool) as detected by HPLC analysis (Craft Industries, North Carolina, USA). Reported concentrations are in µg/g of yolk. Undetectable antioxidants are noted by “n.d.”.

Antioxidant	Average Concentration (µg/g of yolk)
trans-Lutein	29.165
trans-zeaxanthin	5.635
cis-Lutein/zeaxanthin	5.055
α-cryptoxanthin	0.52
β-cryptoxanthin	0.295
trans-lycopene	n.d.
cis-lycopene	n.d.
α-carotene	0.15
trans-β-carotene	2.12
cis-β-carotene	0.465
α- tocopherol	75.4
β- tocopherol	3.05
γ- tocopherol	5.75
δ- tocopherol	n.d.
Retinol	1.35

CHAPTER II

THE INFLUENCE OF INCUBATION THERMAL ENVIRONMENTS ON

OXIDATIVE DAMAGE AND ANTIOXIDANTS IN RED-EARED

SLIDER TURTLE (*TRACHEMYS SCRIPTA ELEGANS*)

HATCHLINGS

Introduction

Incubation temperatures have important implications for a large number of reptilian phenotypic traits. As an example, many reptiles exhibit temperature-dependent sex determination, where an individual's sex is determined by temperatures experienced during incubation (Ewert & Nelson 1991). Thus, incubation temperature has long been recognized for its direct impact on sex-ratios of reptilian populations. However, many other fitness-related traits are known to be influenced by incubation temperature including body size, growth, locomotor performance, metabolic rates, immune responses, and early survival (Reviewed by Deeming 2004; Booth 2006; DuRant et. al 2013; Bowden, Carter, & Paitz 2014). One additional trait that can be influenced by incubation temperature is oxidative stress.

Oxidative stress is a physiological state during which the generation of highly unstable oxygen molecules called reactive oxygen species (ROS) exceeds antioxidant activity (Monaghan, Metcalfe, & Torres 2009; Finkel & Hollbrook 2000). ROS are a type of free radical characterized by a missing valence shell electron and are often produced

during aerobic respiration. When they interact with any of the major cellular macromolecules (lipids, proteins, and DNA), ROS cause a type of cellular damage referred to as oxidative damage. To protect cells from oxidative damage, organisms have both enzymatic and dietary antioxidants that act to neutralize ROS. The combined activities of one's enzymatic and dietary antioxidants can be measured and is referred to as a total antioxidant capacity (TAC) (Monaghan et al. 2009).

Incubation temperature may be a primary environmental determinate of an individual's susceptibility to oxidative stress. ROS are predominately generated during aerobic cellular respiration (Finkel & Hollbrook 2000). Temperature changes are often associated with alterations in metabolic rates and consequently changes in ROS production. Accordingly, extreme temperature exposure has been related to both changes in antioxidant activity and oxidative damage accumulation in a number of vertebrate and invertebrate species (Parihar et al. 1996; Heise et al. 2006; Mujahid & Furuse 2009; An et al. 2010; Ibarz et al. 2010; Lalouette et al. 2011; Ballen et al. 2012; Castro et al. 2012; Rosa et al. 2012). While the amplitude and duration of temperature change necessary to elicit oxidative stress varies across species, temperature-induced oxidative stress appears to be widespread. Critically, as ectotherms whose metabolic rate is continuously affected by temperature, reptiles may be particularly susceptible to temperature induced oxidative stress during development. For example, in the ectothermic squid, the European squid (*Loligo vulgaris*), increases in incubation temperatures as small as two degrees centigrade are sufficient to elicit oxidative stress (Rosa et al. 2012). However, to date no one has evaluated the role of incubation temperature on oxidative stress in a reptile.

Prior work has demonstrated that early-life exposure to thermal challenges can have a long-term impact on oxidative stress resistance and life history. For example, in a recent study by Constantini, Monaghan, and Metcalfe (2012) exposure to a mild heat stress as a juvenile was associated with an enhanced ability to handle heat stress in adulthood in comparison to control individuals whom experienced no juvenile exposure to heat stress. In contrast, individuals exposed to an extreme heat stress as juveniles, were more susceptible to heat stress as adults in comparison to control individuals (Constantini, Monaghan, & Metcalfe 2012). Similarly, among both vertebrates and invertebrates early exposure to a mild heat or cold shock is associated with increased longevity (Caratero et al. 1997; Shama et al. 1998; Yahav & McMurty 2001; Cypser & Johnson 2002; Hercus et al. 2003; Le Bourg 2005; Olsen et al. 2006; Scannapieco et al. 2007; Lopez-Martinez & Hahn 2012; Lopez-Martinez & Hahn 2014). It has been suggested that changes in oxidative stress susceptibility underlies the beneficial effect of thermal stress on lifespan in these studies (Constantini 2014). Taken together, moderate oxidative stress challenges experienced by young individuals may be beneficial to oxidative stress resistance, while severe challenges are likely detrimental. Importantly, the potential beneficial effect of early-life exposure to stressors is noted to be highly context-dependent and based on the magnitude, duration, and timing of the thermal challenge (Lagisz et al. 2013).

The work discussed above has been limited to investigating the effects of thermal challenges post-embryonically, on young individuals. Yet, individuals often encounter thermal challenges prenatally during embryonic development, particularly among many oviparous species (DuRant et al. 2013). In general, during embryonic development

individuals are passive recipients of their thermal environments with little ability for behavioral thermoregulation. Ontogenic studies suggest that the complete maturation of antioxidant systems is not achieved until young adulthood and enzymatic antioxidant activity is often lacking until the very end of embryonic development (Khan & Black 2003; Barata et al. 2005; Fontagné et al. 2008; Rizzo et al. 2007). Consequently, during the prenatal period individuals may be more susceptible to oxidative stress in comparison to other life history stages. Furthermore, prenatal thermal challenges may coincide with metabolic and antioxidant system development. Thus, we speculate that permanent organizational effects of prenatal oxidative stress on metabolic or antioxidant programming may result. Accordingly, there remains the possibility that oxidative stress experienced prenatally will have an even greater impact on offspring oxidative stress resistance phenotypes than those observed post-embryonically.

A few recent studies have been conducted to investigate the impact of temperature on oxidative stress during development (Rosa et al. 2012; Yalçın et al. 2012; Loyau et al. 2014). However, the observed effects of temperature were variable. Specifically, exposure to cold temperatures periodically during incubation resulted in an increase in catalase activity on day of hatch in the domestic chicken, *Gallus gallus* (Yalçın et al. 2012; Loyau et al. 2014). In contrast, in a study by Rosa et al. (2012), exposure to elevated incubation temperatures had no influence on the antioxidant status of the European squid, *L. vulgaris*. However, warm temperatures resulted in an increased amount of oxidative damage and reduced survival (Rosa et al. 2012). These inconsistencies in results may simply be related to species-specific differences in responses. Thus, to fully understand the role of the thermal developmental environment

on oxidative stress phenotypes, work in a variety of systems is necessary. We investigated the effect of incubation temperature on total antioxidant capacity (TAC) and oxidative damage in a model oviparous reptile the red-eared slider turtle (*Trachemys scripta*) hatchlings.

Distinct from the organisms previously utilized to investigate the impact of incubation temperature on oxidative stress phenotypes, large thermal variations represents an ecologically and physiologically relevant metabolic challenge faced during embryonic development of oviparous reptiles. Unlike many avian species, most reptiles do not provide any post-laying parental care. As a direct consequence of this, embryos are exposed to a broad range of daily and seasonal temperature fluctuations during development (Weisrock & Janzen 1999; Paitz et al. 2010a; Micheli-Campbell et al. 2012). Even within single nesting sites, large differences in how embryos experience thermal flux result from dissimilarities in nest location and nest depth (Weisrock & Janzen 1999; Shine & Elphick 2001; Booth 2006; Warner & Shine 2008; Paitz et al. 2010a; Micheli-Campbell et al. 2012). Importantly, in comparison to constant temperature incubation, the experience of incubation temperature fluctuations have notably different effects on offspring phenotypes (Schwarzkopf & Brooks 1985; Shine & Harlow 1996; Demuth 2001; Shine & Elphick 2001; Webb et al. 2001; Ashmore & Janzen 2003; Du & Ji 2006; Les et al. 2007; Du & Feng 2008; Lin et al. 2008; Patterson & Blouin-Demers 2008; Du et al. 2009; Les et al. 2009; Paitz et al. 2010a; Paitz et al. 2010b; Micheli-Campbell et al. 2012; Li et al. 2013; Refsnider 2013; Horne et al. 2014). In terms of oxidative stress, temperature fluctuations and metabolic shifts may also represent an additional metabolic challenge experienced by embryos independent of

mean temperature (Ruel & Ayers 1999). Thus, developing under conditions of temperature fluctuations either large in frequency or magnitude is likely more oxidatively challenging compared to incubation in thermally stable conditions.

Here, we performed three distinct experiments to evaluate hatchling response to different aspects of incubation conditions. We asked; 1) Do *T. scripta* hatchlings incubated in fluctuating temperature regimes experience different levels of oxidative stress than hatchlings incubated at a constant temperature? (Constant versus Fluctuating), 2) Do increases or decreases in the frequency of incubation temperature fluctuations affect levels of oxidative stress experienced by *T. scripta* hatchlings, due to different frequencies of metabolic shifts? (Fluctuation Frequency), and 3) What is the effect of mean temperature on oxidative damage and TAC in *T. scripta* hatchlings? (Mean Temperature).

We hypothesized that during the development of ectothermic reptiles, both high temperatures (Booth & Astill 2001) and temperature fluctuations (Ruel & Ayers 1999) experienced during incubation 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 mean temperature or frequency of temperature fluctuations would result in increases in oxidative stress. In all experiments, we expected that the experience of oxidative stress during embryonic development would result either in a reduction of TAC or increased levels of oxidative damage in hatchling tissues.

Material and Methods

Egg Collection and Incubation

Experiment 1- Constant versus fluctuating incubation. Eggs were collected from red-eared slider turtles during the 2013 summer nesting season at Banner Marsh State Fish and Wildlife Area (Illinois, USA). Six early season (June 4, 2103- June 9, 2013) and five late season (June 16, 2013-June 19, 2013) gravid females were caught in baited hoop traps. Females were measured and brought back to lab where an oxytocin injection was used to induce oviposition (Ernst & Lovich 2009). We collected a total of 11 clutches of *T. scripta* eggs (n=153), which ranged in size from 10-20 eggs. Prior to incubation treatment assignment, all eggs were weighed to the nearest 0.01 gram and marked to indicate clutch and individual. One or two eggs from each clutch was removed and immediately frozen at -20°C for yolk antioxidant analysis in a future study. Eggs were collected under Illinois Department of Natural Resources permit NH13.2084.

Eggs (n=132) within each clutch were then randomly assigned to either a constant temperature incubation (n=69) (constant) or fluctuating temperature incubation group (n=63) (flux). Constant eggs were incubated at a constant temperature of 29.5°C, while flux eggs were incubated at temperatures sinusoidally fluctuating $\pm 3^{\circ}\text{C}$ around a mean of 28.7 °C (Figure 1a). This fluctuating regime has a constant temperature equivalent (CTE) of 29.4 °C. CTE was calculated using the curvilinear mathematical model developed by Georges et al. (2005). 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). Since the CTE of our flux treatment is essentially the same as the constant incubation temperature, treatment effects can be attributed to temperature fluctuation experience and not necessarily simply due to exposure to higher temperatures and developmental rates (Georges et al. 2005). Additionally, these treatments expose eggs to temperatures well within the range of temperatures experienced in natural nests of this species (Amanda Wilson Carter, Unpublished Data) and are suitable for development (Les, Paitz, & Bowden 2009). Eggs were incubated while partially buried in moist vermiculite (-150 kPa) in plastic incubation boxes. Boxes were weighed periodically and water loss was replaced to maintain hydric conditions throughout incubation. Also, to minimize the effects of temperature inconsistencies within the incubators, box position was rotated every five days.

Upon pipping, hatchlings were housed individually and maintained in a constant 29.5°C incubator. Day of pip was determined as the date on which the hatchling first breached the egg shell. One day prior to euthanasia, hatchling mass to the nearest 0.01g and plastron and carapace lengths to the nearest 0.01mm were recorded for all individuals. Between post-pip day 8-16 hatchlings (n=130) were sacrificed by an intrapleuroperitoneal injection of pentobarbital sodium solution (60 mg/kg). Liver tissue samples were harvested within 15 minutes after injection, snap 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 and 17-2013).

Experiment 2- Effect of temperature fluctuation frequency. In order to assess the effects of different frequencies of temperature fluctuations on hatchling oxidative damage accumulation and TAC we collected an additional 22 clutches between May 29, 2013 and June 7, 2013 at Banner Marsh State Fish and Wildlife Area (Illinois, USA). Eggs were obtained either from excavation of nests laid within six hours prior to collection (n=5) or via oxytocin injection (Ernst & Lovich 2009) of gravid trap caught females (n=17). Prior to incubation treatment assignment, all eggs (n=268) were weighed to the nearest 0.01 gram and marked to indicate clutch and individual. Eggs were collected under Illinois Department of Natural Resources permit NH13.2084.

T. scripta eggs were randomly assigned to one of three fluctuating incubation regimes: 1) 28.7 ± 3 °C sinusoidal fluctuations every 12 hours (Hyper) (n=91), 2) 28.7 ± 3 °C sinusoidal fluctuations every 24 hours (Normal) (n=87), or 3) 28.7 ± 3 °C sinusoidal fluctuations every 48 hours (Hypo) (n=90) (Figure 1b; Bowden et al. 2014). All three of these fluctuating treatments have a CTE of 29.4°C since the total amount of time spent at any given temperature over the entire course of incubation is consistent (Georges et al. 2005). By manipulating fluctuation frequency in this manner, we were able to determine how individuals respond to deviations in environmental thermal instability and systematically control the number of metabolic changes experienced throughout incubation (Bowden et al. 2014). Furthermore, these treatments exposed individuals to warmer or cooler temperature for different continuous durations, without affecting the overall mean and range of temperatures experienced (Bowden et al. 2014). Thus, from this experiment we were able to establish which is more important for hatchling oxidative stress phenotypes; the number of metabolic changes or continuous time spent at a given

temperature. Eggs were incubated in plastic incubation boxes while partially buried in moist vermiculite (-150 kPa). Every five days box position within incubators was rotated and water loss was replaced to maintain humidity near 100% during incubation.

Following pipping, hatchlings were individually housed and maintained at room temperature. Ten days post-pip, hatchling mass to the nearest 0.01g and plastron and carapace lengths to the nearest 0.01mm were recorded. As part of a related study, all hatchlings underwent behavioral testing and were sacrificed between the ages of 69-102 days post-pip. For the subset of individuals (n=108) involved in this study liver tissue was harvested within 15 minutes post-mortem. Collected liver samples represented 13 out of the 22 collected clutches and each incubation treatment group (Hypo, Normal, and Hyper) included 36 individuals. Upon collection, tissue samples were immediately frozen in liquid nitrogen and stored at -80°C prior to oxidative damage and antioxidant analysis. This work was approved by the Illinois State University Institutional Animal Care and Use Committee (Protocols 09-2013 and 16-2013).

Experiment 3- Effect of mean incubation temperature. In order to determine the importance of mean temperature during a fluctuating incubation regime, we also collected red-eared slider turtle clutches during the summer 2014 nesting season at Banner Marsh State Fish and Wildlife Area (Illinois, USA). Clutches were obtained either via excavation of freshly laid nests or from gravid females caught in baited hoop traps. Trap caught females were all brought back to lab where an oxytocin injection was used to induce oviposition (Ernst & Lovich 2009). For this study a total of 21 clutches (n=230 eggs) were collected. Of these clutches, 10 were collected early in the nesting

season (May 29, 2014- June 6, 2014) and the remaining 11 clutches were collected later in the season (June 17, 2014- June 21, 2014). Prior to incubation treatment assignment, all eggs were weighed to the nearest 0.01 gram and marked to indicate clutch and individual. One or two eggs from each clutch was removed and immediately frozen at -20°C for yolk hormone and antioxidant analysis in another study. Eggs were collected under Illinois Department of Natural Resources permit NH14.2084.

Eggs within each clutch were then randomly assigned to one of three fluctuating temperature incubation regimes: 1) 26.5 ± 2 °C sinusoidal fluctuations every 24 hours (Low) (n=76), 2) 27.1 ± 2 °C sinusoidal fluctuations every 24 hours (Medium) (n=78), or 3) 27.7 ± 2 °C sinusoidal fluctuations every 24 hours (High) (n=76) (Figure 1c). The CTE of these treatments was 26.9 °C, 27.5 °C, and 28.0 °C respectively (Georges et al. 2005). As in prior experiments, eggs were incubated while partially buried in moist vermiculite (-150 kPa) in plastic incubation boxes. Boxes were monitored throughout incubation for maintenance of hydric conditions and periodically rotated within incubators.

Following hatching, hatchlings were individually housed and maintained at constant room temperature and dark light conditions. Ten days post-pip hatchling mass to the nearest 0.01g and plastron and carapace lengths to the nearest 0.01mm were recorded. All hatchlings underwent behavioral testing prior to being sacrificed between the ages of 37 and 44 days post-pip, as part of a related study. Within 15 minutes post-mortem hatchling liver tissue (n=224) was harvested, immediately frozen in liquid nitrogen, and stored at -80°C prior to oxidative damage and antioxidant analysis. This work was

approved by the Illinois State University Institutional Animal Care and Use Committee (Protocols 09-2013 and 08-2014).

Antioxidant and Oxidative Damage Analyses

Antioxidant analysis. Measurement of total antioxidant capacity (TAC) of all hatchling liver tissue samples was used to determine the combined activity of both endogenous and dietary antioxidants. TAC was determined spectrophotometrically using a modified cupric reducing antioxidant capacity (CUPRAC) method. The CUPRAC method estimates TAC based on ability tissue samples to convert copper(II)-neocuproine to copper(I)-neocuproine (Özyürek et al. 2008). We chose to use the CUPRAC assay for 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, Güçlü, & Apak 2011).

Here we performed the CUPRAC assay as described by Özyürek et al. (2008) with the following modifications. Briefly, liver samples were homogenized at a 1:40 (weight:volume) dilution of 2% Methyl- β -Cyclodextrin (M- β -CD) dissolved in an 9:1 (volume:volume) acetone-water mixture to simultaneously extract and dissolve hydrophobic and hydrophilic antioxidants. Homogenates were centrifuged at room temperature for 10 minutes at 10,000 RCF and the supernatant was removed for use in the assay. Reactions were set-up in a 96-well plate format and consisted of 75 μ L of

10mM copper(II) chloride solution prepared in water, 75 μ L of 7.5mM neocuproine solution prepared in 96% ethanol, 75 μ L 8M Urea Buffer (pH 7.0) prepared in a standard Tris buffer (86 mM tris, 90 mM glycine, and 4mM citrate, pH 8.0), and 55 μ L of 2% M- β -CD prepared in an 9:1 (volume: volume) acetone-water mixture . All reagents were added in the order listed prior to the addition of tissue samples. Finally, 20 μ L of tissue homogenate was added, bringing the total volume of each reaction to 300 μ L. Reactions were incubated for 30 minutes at room temperature, after which absorbance was measured at 405 nm (BioTek ELx 800). TAC was calculated in micromolar Trolox equivalents per milligram of wet tissue weight (μ M Trolox Equivalents/mgww) via absorbance comparison to a standard curve that ranged from 100 μ M- 12.5 μ M Trolox. The 1mM 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 (volume:volume) acetone-water solution. All samples were run in duplicate. A single assay was performed for each of the three experiments (see Figure 1) and had intra-assay CVs of 6%, 8%, and 9% respectively.

Oxidative damage analysis. As our primary measure of oxidative damage, lipid hydroperoxides were spectrophotometrically quantified using the Ferrous Oxidation-Xylenol Orange Method (FOXO) (Hermes-Lima, Willmore, & Storey 1995). Lipid hydroperoxides are long-term by-products of oxidative damage to lipids that both threaten cell membrane stability and composition (Monaghan et al. 2009). 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 since 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, Willmore, and Storey (1995). Lipids were extracted from tissue via homogenization in ice cold methanol at a 1:40 weight: volume ratio. Samples were centrifuged at room temperature for 10 minutes at 10,000 RCF and the supernatant was saved. Assay reactions composed of 250 μ L of 1mM FeSO₄, 50 μ L of 500 mM H₂SO₄, 100 μ L of 1 mM xylenol orange, 580 μ L of H₂O, and 20 μ L of tissue extract, were set up in 1.5mL Eppendorf tubes. All solutions were prepared just prior to use and added in the order listed. Reagent blanks were prepared by replacing the 20 μ L aliquot of tissue extract with water. Following addition of tissue extract all reactions were vortexed and allowed to incubate at room temperature for five hours. Absorbance at 580nm was subsequently measured for all samples and blanks. Next, 5 μ L of 1mM cumene hydroperoxide was added to every reaction and 580nm absorbance was re-measured following a 60 minute incubation at room temperature. Lipid hydroperoxides are expressed in nanomolar cumene hydroperoxide equivalents (CHE) per milligram of wet tissue weight (nmol CHE/mgww) and were calculated using the following equation; Lipid hydroperoxides= ((A580 nm sample-blank)/ (A580 nm 5nmol CHE-blank))*5 nmol CHE *(1000/20)*41 (Hermes-Lima et al. 1995). All samples were run in duplicate. Inter-assay CVs for the Constant versus Fluctuation (Figure 1a), Fluctuation Frequency (Figure 1b), and Mean Temperature (Figure 1c) experiments were 7%, 6%, and 5% respectively. All intra-assay CVs of replicates were below 10%.

For a subset of samples (n=40) from experiment 1 (Figure 1a; Constant versus Fluctuation) long term oxidative damage was also determined using telomere length analysis. Telomeres, which are the true ends of chromosomes, are a portion of DNA particularly susceptible to the accumulation of oxidative damage. Oxidative damage to telomeres cannot be repaired and instead results in shortening of telomeres. Thus, through use of the Telomere Restriction Fragment (TRF) assay, increased long term exposure to oxidative damage can be detected by the presence of higher proportions of shorter telomeres (Houben et al. 2008).

The TRF assay was conducted as described by Haussmann et al. (2012). Genomic DNA was extracted from liver tissues collected from four individuals (two constant and two fluctuation) per clutch (n=10) using a Gentra Puregene Tissue kit. Prior to TRF analysis the suitability of samples was assessed by running 10ng of genomic DNA on 1% agarose gel for 1 hour at 100V. Next, 9µg of genomic DNA was digested at 37°C overnight by mixture of 27 U of RSA I, 30 U of Hinf I, and 30 U of Hae III. Samples were separated using pulsed field gel electrophoresis (3 V/cm, 0.5-7s switch time, 14°C) for 21 hours on a 0.8% agarose gel. An overnight in-gel hybridization was performed at 37°C with a P32 labeled oligo (CCCTAA)₄ to label telomere bands. Gels were placed on phosphorscreens and visualized on a Storm 540 Variable Mode Imager (Amersham Biosciences, Buckinghamshire, UK). Densitometry was subsequently performed using ImageQuant and Image J software. Via comparison to a molecular marker (1 kb DNA Extension Ladder; Invitrogen, CA, USA) we determined median and telomere lengths of each lane. Observed telomere distributions were heavily skewed left and fell largely above the largest band (40 KB) in our molecular marker. The median is more resistant to

the influences of outliers and extrapolation. Thus, we chose to analyze the median as opposed to mean telomere lengths.

Statistical Analysis

All statistical analyses were performed using SAS statistical software (SAS 9.3). 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 due to 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 as well, since these two variables were dependent on one another. We used the Satterhwaite method to calculate the proper degrees of freedom. All data is presented as mean ($\pm$ SE).

For each experiment we independently tested for clutch, treatment, and seasonal effects on general hatchling characteristics using mixed effects ANOVAs (PROC GLM). Characteristics analyzed included incubation length (number days from the start of incubation to piping), hatchling mass, and hatchling plastron length. When necessary, data was transformed to meet assumptions of normality and homogeneity of variances.

Three separate analyses for each of our experiments were then performed to determine the effects of incubation conditions on measures of oxidative damage and TAC in hatchlings. For the first experiment (Constant versus Fluctuation; Figure 1a), we performed a mixed-effect MANOVA (PROC GLM). Prior to analysis TAC was transformed using a reciprocal transformation. Clutch, season, and treatment effects on median telomere length distributions were analyzed using a mixed-effect ANOVA

(PROC GLM). For the analysis of the Fluctuation Frequency experiment (Figure 1b), measurements of TAC could not be transformed to meet the assumptions of the MANOVA. Thus, we analyzed effects on 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 the ARTool (v. 1.5.1; Wobbrok et al. 2011). The aligned ranked transformation procedure can be applied for the non-parametric analysis of multi-factorial designs (Higgins & Tastoush 1990; Higgins & Tastoush 1994; Wobbrok et al. 2011). Specifically, data is aligned, ranked, and analyzed using an ANOVA (PROC GLM) procedure. During the alignment process contributions of other all effects are removed prior to ranking (Higgins & Tastoush 1990; Higgins & Tasoush 1994). Thus, separate ranks and ANOVA's were conducted to test for clutch, treatment, and the clutch by treatment interaction effects on hatchling TAC. Finally, to test for the influence of mean incubation temperature (Figure 1c) on hatchling measures of oxidative stress we conducted a MANOVA (PROC GLM) with oxidative damage and TAC as response variables. To meet assumptions of normality and homogeneity of variances, measurements of oxidative damage were transformed prior to analysis using a cubed transformation. For the MANOVAs we used Pillais'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. 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.

Results

Effects of Incubation Conditions on Hatchling Phenotypes

Experiment 1- Constant versus fluctuating incubation. When subjected to either a constant or fluctuating temperature regime, incubation duration ($F_{1, 9.4367} = 316.08$, $p < 0.0001$) was significantly affected by treatment, but hatchling mass ($F_{1, 9.7551} = 0.06$, $P = 0.8126$) and size ($F_{1, 9.545} = 1.27$, $P = 0.2868$) were not. Temperature fluctuations of 28.7 ± 3 °C every 24 hours resulted in a significant increase in incubation length in comparison to a constant temperature incubation of 29.5 °C. Exposure to temperature fluctuations was associated with approximately a four-day extension in incubation duration. Specifically, the average ($\pm$ SE) incubation length of the constant and fluctuating temperature incubation was 51.73 ± 0.17 days and 55.98 ± 0.17 days respectively. Hatchlings from early and late season clutches did not differ in measures of incubation duration ($F_{1, 9.0763} = 1.32$, $P = 0.2799$), mass ($F_{1, 9.0144} = 0.83$, $P = 0.3850$), or plastron length ($F_{1, 9.0349} = 0.66$, $P = 0.4381$). However, clutch identity was highly significantly related to incubation length ($F_{9,9} = 5.60$, $P = 0.0086$), hatchling mass ($F_{9,9} = 50.77$, $P < 0.0001$), and hatchling size ($F_{9,9} = 15.21$, $P = 0.0002$).

Experiment 2- Effect of temperature fluctuation frequency. Temperature fluctuation frequencies also had a significant effect on incubation length ($F_{2, 29.632} = 4.83$, $p = 0.0152$). Based on pairwise comparisons, a low frequency fluctuation (every 48 hours) was associated with about a one-day extension in incubation duration in comparison to

normal fluctuations (every 24 hours). There was however, no effect of fluctuation frequency on hatchling mass ($F_{2, 34.514} = 0.57$, $P = 0.5720$) or plastron length ($F_{2, 29.045} = 0.60$, $P = 0.5547$) at ten days post-hatch. Additionally, clutch was significantly related to all measured hatchling characteristics including incubation length ($F_{12,24.926} = 7.58$, $P < 0.0001$), mass ($F_{12,25.704} = 31.98$, $P < 0.0001$), and size ($F_{12,24.831} = 5.58$, $P = 0.0002$).

Experiment 3- Effect of mean incubation temperature. Finally, there was a significant effect of mean incubation temperature on incubation length ($F_{2,42.016} = 580.97$, $p < 0.0001$), as lower mean incubation temperatures were associated with extended incubation durations. Average ($\pm$ SE) incubation lengths for a high (27.7°C), medium (27.1°C), and low (26.5°C) mean incubation temperature were 61.12 ± 0.13 , 63.64 ± 0.13 , and 67.24 ± 0.13 days respectively. Mean incubation temperature also had a marginally significant effect on hatchling mass ($F_{2,42.25} = 3.02$, $p = 0.0593$), with the trend of hatchlings experiencing higher incubation temperatures being lighter in mass. Average ($\pm$ SE) hatchling mass from high (27.7°C), medium (27.1°C), and low (26.5°C) mean incubation temperature were $7.36 \pm 0.12\text{g}$, $7.52 \pm 0.14\text{g}$, and $7.57 \pm 0.13\text{g}$, respectively. In contrast to incubation period and hatchling mass, there was no significant effect of incubation treatment on hatchling plastron length ($F_{2,41.642} = 0.11$, $p = 0.8980$). There were no significant seasonal differences in incubation length ($F_{1, 19.129} = 1.98$, $p = 0.1759$), mass ($F_{1, 19.051} = 0.71$, $p = 0.4096$), or plastron length ($F_{1, 19.078} = 0.65$, $p = 0.4302$) of hatchlings coming either from early-laid or later-laid clutches. Finally clutch once again was significantly related to incubation duration ($F_{19, 38.527} = 13.30$, $p < 0.0001$), hatchling mass ($F_{19, 38.557} = 35.41$, $p < 0.0001$), and hatchling size ($F_{19, 38.479} = 20.00$, $p < 0.0001$).

Effects of Incubation Conditions on Hatchling Levels of Oxidative Damage and TAC

Experiment 1- Constant versus fluctuating incubation. Incubation temperature fluctuations did not have a significant effect on measures of *T. scripta* hatchling oxidative status ($F_{2,8} = 2.38$, $p = 0.1544$; Table 2). Average ($\pm$ SE) measures of oxidative damage and TAC of hatchlings subjected to a constant temperature incubation were 5.55 ± 0.06 nmol CHE/ mgww and 34.17 ± 0.67 μ M Trolox Equivalents/mgww, respectively (Figure 2). Similarly, in hatchlings experiencing incubation temperature fluctuations mean oxidative damage was 5.44 ± 0.06 nmol CHE/ mgww and mean TAC was 33.03 ± 0.63 μ M Trolox Equivalents/mgww (Figure 2). In the subset of individuals ($n = 40$) for whom we had measured telomere lengths, incubation treatment did not significantly affect median telomere lengths either ($F_{1,8} = 0.01$, $p = 0.9427$). Average median telomere lengths of hatchlings subjected to constant or fluctuating temperature incubation were 276.29 ± 14.48 KB and 274.77 ± 14.48 KB, respectively (Figure 3). Together the lack of treatment effects observed, suggests that normal diurnal temperature fluctuations do not elicit oxidative stress in *T. scripta*.

Additionally, there was no significant interaction between treatment and season on measures of oxidative damage and TAC ($F_{2,8} = 1.63$, $p = 0.2543$; Table 2) or median telomere lengths ($F_{1,8} = 0.88$, $p = 0.3750$). Similarly, the interaction between clutch and treatment was not significantly associated with either oxidative damage ($F_{9,108} = 0.96$, $p = 0.4772$) or TAC ($F_{9,108} = 1.31$, $p = 0.2415$) and was estimated to explain 0% and 4% of the variation in these measures respectively. Finally, there was no significant interaction between treatment and clutch ($F_{8,20} = 1.70$, $p = 0.1609$) on hatchling telomeres.

Experiment 2- Effect of temperature fluctuation frequency. Temperature fluctuation frequency did not have a significant effect on levels of lipid hydroperoxides in hatchling livers ($F_{2, 37.018}=0.65$, $p=0.5$; Table 3). The average amount of lipid peroxidation in hatchlings experiencing hyper (every 12 hours), normal (every 24 hours), and hypo (every 48 hours) temperature fluctuation frequencies were 5.94 ± 0.075 nmol CHE/ mgww, 5.97 ± 0.078 nmol CHE/ mgww, and 6.05 ± 0.073 nmol CHE/ mgww respectively (Figure 4). In contrast to measures of oxidative damage, incubation temperature fluctuation frequency did have a significant effect on hatchling TAC ($F_{2, 101}=8.30$, $p=0.0005$; Table 3). Average TAC from hatchlings in the hyper (every 12 hours), normal (every 24 hours), and hypo (every 48 hours) treatment groups were 46.23 ± 1.15 μ M Trolox Equivalents/mgww, 46.38 ± 1.34 μ M Trolox Equivalents/mgww, and 42.83 ± 1.37 μ M Trolox Equivalents/mgww (Figure 4). Pairwise comparisons showed that TAC following low frequency (every 48 hours) temperature fluctuation is significantly reduced in comparison to normal ($p=0.0020$) and hyper ($p=0.0018$) frequency incubations. There are, however, no differences in the TAC of hatchlings experiencing temperature fluctuations either every 12 or 24 hours ($p=0.9997$). Finally, there was no significant interaction effect between fluctuation frequency treatment and clutch on either oxidative damage ($F_{24,69}=0.89$, $p=0.6122$; Table 3) or TAC ($F_{24, 65}=0.94$, $p=0.5520$; Table 3) in hatchlings.

Experiment 3- Effect of mean incubation temperature. Hatchling oxidative status was significantly related to mean incubation temperature ($F_{4, 76}=4.23$, $p=0.0038$; Table 4). Based on the standardized canonical coefficients TAC contributed more to the MANOVA effect of temperature treatment than did accumulated oxidative damage and

the two measures were positively associated with one another (Table 4). Accordingly, follow-up analyses via multiple pair-wise comparisons revealed that there were no significant differences in levels of lipid peroxidation. Average amounts of oxidative damage for a high (27.7 °C), medium (27.1 °C), and low (26.5 °C) mean incubation temperature were 5.63 ± 0.13 nmol CHE/ mgww, 5.64 ± 0.14 nmol CHE/ mgww, and 5.79 ± 0.12 nmol CHE/ mgww (Figure 5). However, the low mean temperature incubation was associated with a significant increase in hatchling TAC in comparison to the medium ($p=0.0032$) and high ($p=0.0007$) mean temperature treatment groups. There was no significant difference in TAC following incubation at medium mean temperature in comparison to the high mean temperature ($p=0.8322$). Specifically mean TAC of hatchlings exposed to a lower average temperature during incubation was 55.20 ± 0.49 μ M Trolox Equivalents/mgww (Figure 5). In contrast, average TAC of the medium and high temperature groups was 52.76 ± 0.49 μ M Trolox Equivalents/mgww and 52.35 ± 0.51 μ M Trolox Equivalents/mgww, respectively (Figure 5). Consistent with the other experiments there was however no significant clutch by treatment interaction on either oxidative damage ($F_{38, 151} = 0.81$, $p=0.7798$) or TAC ($F_{38, 151} = 0.98$, $p= 0.5156$). Finally, the treatment by season interaction was also not significant ($F_{4, 76} = 1.54$, $p= 0.1978$; Table 4).

Discussion

This was the first investigation to measure levels of oxidative damage and TAC in hatchlings of the model oviparous reptile *T. scripta*. In all experiments conducted, there was no effect of incubation temperature or temperature fluctuations on the accumulation

of lipid hydroperoxides post-hatch. Furthermore, median telomere length, a long term measure of oxidative stress, was not affected by incubation temperature fluctuations. These results suggest that *T. scripta* hatchlings have antioxidant systems that effectively prevent oxidative damage during incubation. This conclusion is consistent with prior work conducted to characterize the antioxidant system of adult Red-eared sliders (Willmore & Storey 1997; Willmore & Storey 2005). Despite having a much lower aerobic metabolic rate, similar levels of enzymatic antioxidants in adult *T. scripta* to those measured in mammalian tissues have been reported (Willmore & Storey 1997; Willmore & Storey 2005). Furthermore, in comparison to other reptiles and amphibians, red-eared sliders had two to seven fold higher enzymatic antioxidant activity levels (Willmore & Storey 1997). These findings may be initially surprising as the lower metabolic rate should be associated with less daily ROS generation in turtles. However, it appears that through investing in the maintenance of a proficient antioxidant system to minimize oxidative damage in their cells, turtles are able to live long, reproduce throughout adulthood and grow for an indeterminate period (Wilbur 1975). These constitutively active antioxidant systems are sufficient to prevent oxidative stress in adult red-eared sliders (Willmore & Storey 1997). For example, during the oxidative stress challenge of recovery from anoxia, despite few changes in enzymatic antioxidant activities, no differences in oxidative damage in *T. scripta* tissues were detected (Willmore & Storey 1997). Taken together, the work here adds support to the idea that turtles consistently allocate a large proportion of resources to cellular maintenance throughout their lifetimes.

Also based on our results, and in contrast to what we expected, incubation thermal fluctuations 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 oxidative damage or TAC following a constant or fluctuating incubation 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 any differences in the oxidative status of hatchlings either. As mentioned previously, under natural incubation conditions oviparous reptile embryos are normally exposed to incubation temperature fluctuations (Weisrock & Janzen 1999; Shine & Elphick 2001; Booth 2006; Warner & Shine 2008; Paitz et al. 2010a; Micheli-Campbell et al. 2012). Consequently, *T. scripta* may be adapted to cope with temperature changes during embryonic development.

There are several possible proximate mechanisms not explored here that *T. scripta* embryos may utilize to avoid oxidative stress associated with temperature fluctuations during incubation. For one, incubation temperature fluctuations may promote the thermal acclimation of metabolism. Acclimation of metabolic rates would be reflected either by the maintenance of a constant metabolic rate despite temperature changes or a decrease in the Q_{10} of metabolic rate (Du & Shine 2015). Q_{10} is simply the rate of change in metabolism in response to a ten degree increase in temperature. Accordingly, a reduction of Q_{10} indicates that changes in metabolism are less sensitive to temperature alterations. Metabolic depression in response to temperature fluctuations has been observed in ectothermic insects (Williams et al. 2012; Bozinovic et al. 2013). While not ubiquitous

(Booth 1998; Du et al. 2010), acclimation of metabolic rates in response to thermal challenges during incubation is widespread among avian and reptilian embryos (Du et al. 2010; Du & Shine 2015). To date there are no reports about whether or not *T. scripta* embryos specifically are capable of thermal acclimation. However, metabolic rate depression is employed by adult red-eared sliders during thermal and anoxic challenges (Storey & Storey 2004; Storey 2006; Galli & Richards 2012). Alternatively, if red-eared slider embryos do not exhibit any metabolic thermal acclimation, a stable metabolic rate during temperature fluctuations might be also achieved through behavioral thermoregulation. Recent work has shown that Chinese soft-shelled turtle (*Pelodiscus sinensis*) embryos exploit small scale temperature gradients within eggs to behaviorally thermoregulate during incubation (Du et al. 2011; Zhou et al. 2013). Regardless, the avoidance of temperature associated metabolic changes either by metabolic depression or behavioral thermoregulation may consequently enable embryos to also avoid oxidative stress during incubation temperature fluctuations.

Independent of thermoregulation or temperature acclimation, changes in embryonic mitochondrial dynamics may also be involved in the avoidance of oxidative stress during thermal changes. Recently, Du & Shine (2015) reported that incubation of turtle embryos at different temperatures is associated with differences in mitochondrial density, respiration, and the activity of cytochrome oxidase (involved in the electron transport chain) and lactate dehydrogenase (involved in anaerobic respiration). Such alterations in mitochondrial characteristics potentially directly influence ROS production rates and reduce the risk of oxidative stress. Similarly, expression of uncoupling proteins during cold exposure (Loyau et al. 2014) may also occur. 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 & Hollbrook 2000). Whether any of these mitochondrial modifications are induced by incubation thermal fluctuations in *T. scripta* remains to be determined.

While 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 *T. scripta* TAC development. For instance, low frequency temperature fluctuations were associated with decreases in TAC of hatchlings. Via the manipulation of temperature fluctuation frequencies we were able to hold the range and average temperatures experienced by hatchlings during development constant. However, the length of times for 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 bouts that were two and four times longer than hatchlings in the normal and high fluctuation frequency groups, respectively. We suspect that the extended exposure to higher temperatures were also associated with longer periods of elevated metabolic rates (Gatten 1974). These high metabolic rates may, in turn, have resulted in increased sustained ROS production and depletion of antioxidants for protection. Consistent with this interpretation, higher average incubation temperatures were associated with lower TAC of hatchlings. The observed increase in TAC of hatchlings from the low mean incubation treatment group may have resulted from the stimulation of enzymatic antioxidant (i.e. catalase) activity during cold

temperature exposure (Yalçın et al. 2012; Loyau et al. 2014). Alternatively, 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 increase in TAC following low temperature incubation may simply be reflective of a greater deposition of maternally derived yolk antioxidants into hatchling liver tissue. The assay we utilized to measure TAC did not distinguish between the activities of dietary and enzymatic antioxidants. Thus, we cannot determine which of these non-mutually exclusive alternative explanations is responsible for our observed effects of temperature on TAC. Further work including the analysis of multiple tissue types is also needed in order to determine if our observed results are a tissue-specific effect.

Despite significant treatment effects of low frequency temperature fluctuations and low temperature on TAC there were no consequences of these differences for hatchling levels of oxidative damage. However, there are potentially unexplored long term consequences of the observed differences in TAC for hatchling life history and fitness. In our population of red-eared sliders, following hatching in early or late August, hatchlings remain in their natal nest and do not emerge until the following spring (Ernst & Lovich 2009). During this period of overwintering, hatchlings survive only on the resources provided by their residual yolk. Importantly, during the winter months hatchlings are likely to encounter many metabolic and oxidative stress promoting challenges such as recovery from freezing and anoxic environments (Storey 1996; Baker, Costanzo, & Lee 2007; Costanzo, Lee, & Ultsch 2008). Individuals with lower TAC due to the incubation thermal 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 of *T. scripta* hatchlings. Furthermore, if observed incubation temperature associated TAC persists into adulthood it may play a role in mediating future life history trade-offs and survival (Alonzo-Alvarez et al. 2006; Bize et al. 2008; Kim et al. 2009). Future longitudinal studies focused on determining if there are any long term consequences of differences in hatchling TAC are necessary to test these predictions.

All of the experiments we conducted utilized incubation thermal regimes that were both mild in comparison to thermal profiles experienced naturally (Weisrock & Janzen 1999; Paitz et al. 2010a; Micheli-Campbell et al. 2012) and remained within the optimal development range (ODR) for *T. scripta* (23°C -31°C) (Les et al. 2009). The ODR encompasses the range of temperatures during which the rate of embryonic development increases linearly with temperature (Georges et al. 2005). This continuous relationship between temperature and developmental rate is reflected here by the significant effect of incubation temperature treatment on incubation length observed in all our experiments. When temperatures drop below the lower thermal limit (LTL) of the ODR, the developmental rate drops to physiological zero and ceases. When temperatures exceed the upper thermal limit (UTL) of the ODR, the developmental rate reaches an asymptote and is at a maximum (Georges et al. 2005; Les et al. 2009; Bowden et al. 2014). In the wild, the range of daily temperatures experienced during incubation is both highly variable and unpredictable (Weisrock & Janzen 1999; Paitz et al. 2010a; Micheli-Campbell et al. 2012). For example, in the freshwater Mary River turtle (*Elusor*

macrurus) the daily range of nest temperatures were reported to vary from 2°C to 22°C during incubation (Micheli-Campbell et al. 2012). Similarly, this past year, the average range of daily temperatures experienced throughout the incubation period was 5.6°C for *T. scripta* nests at our field site (Banner Marsh, Illinois, USA) and the minimum and maximum nest temperatures recorded were 17.5°C and 33.0°C (Amanda W. Carter, unpublished data). Consequently during incubation, periods of brief exposure to both extreme high and low temperatures outside of the ODR occur (Weisrock & Janzen 1999; Demuth 2001; Les et al. 2009; Paitz et al. 2010a; Micheli-Campbell et al. 2012). This exposure to extreme temperatures during incubation may have different consequences for hatchling oxidative stress than those observed here (Les et al. 2009) and should be assessed either by conducting field incubation studies or more closely mimicking field incubation thermal patterns in the lab.

We can speculate that when subjected to extreme heat, excessive oxidative damage may be avoided due the activity of heat shock proteins. Heat shock proteins (HSP) are a general class of chaperone proteins produced during any stress response to promote protein stabilization. HSP expression has been demonstrated to be involved in the protection against cellular damage during oxidative stress (Finkel & Hollbrook 2000). A recent study by Gao et al. (2014) demonstrated that reptilian embryos increase HSP production during exposure to high temperatures. Specifically, in the Chinese soft-shelled turtle (*Pelodiscus sinensis*) a linear increase in HSP70 production occurred when ambient temperature exceeded the ODR by five to ten degree Celsius (Gao et al. 2014). Based on this finding and given that all the incubation temperatures used in this study were within the ODR of *T. scripta*, it is unlikely that HSP activity played a role in the results observed

here. Additionally, this finding suggests that while cellular protection at high temperatures can be accomplished, embryos may be less equipped to handle and more threatened by colder temperatures below the ODR. Consistent with this, *T. scripta* embryos had a reduced survival probability following a constant temperature incubation at the LTL (23°C) but not following a constant temperature incubation at the UTL (31°C) (Les et al. 2009).

In summary, our results demonstrate that during incubation temperature fluctuations red-eared slider hatchlings are well protected from oxidative stress and do not accumulate excessive lipid peroxidation in their livers. This effect is potentially reflective of a life-long combined adaptive advantage of high antioxidant activities and metabolic plasticity. Despite this, the development of *T. scripta* TAC does appear to be influenced by the duration of continuous exposure and the absolute magnitude of incubation temperature. While no consequences of variability in TAC is evident in hatchlings, if persistent these differences could have long-term effects on red-eared slider life history strategies and fitness.

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TABLES

Table 2

Effect of Constant and Fluctuating Incubation Temperature on Oxidative Stress.
MANOVA table of the effects of clutch, season, and constant (29.5 °C) or fluctuating (28.7 ± 3 °C) incubation temperature on *T. scripta* hatchling measures of oxidative damage and TAC. All significant effects are in bold.

Source	Standardized Canonical Coefficients				
	Pillai's Trace (F)	df	P-value	Damage	TAC
Clutch	9.05	18,18	<0.0001	1.67063334	0.60377406
Treatment	2.38	2,8	0.1544	1.39081961	0.94563154
Season	0.57	2,8	0.5860	0.01584268	0.44144248
Season*Treatment	1.63	2,8	0.2543	0.14056485	-1.0924733

Table 3

Effect of Fluctuating Temperature Frequency on Oxidative Stress. ANOVA tables of the effects of incubation temperature fluctuation frequency (48, 24, or 12 hour sinusoidal fluctuations) and clutch on hatchling lipid hydroperoxide accumulation and TAC. All significant effects are in bold.

	Oxidative Damage			TAC		
Source	F-value	Df	P-value	F-value	Df	P-value
Clutch	14.16	12, 26.1	<0.0001	11.19	12,91	<0.0001
Treatment	0.65	2, 37.018	0.5258	8.30	2,101	0.0005
Clutch*treatment	0.84	24, 69	0.6708	0.94	24,65	0.5520

Table 4

Effect of Mean Incubation Temperature on Oxidative Stress. MANOVA table of the effects of clutch, season, and various mean incubation temperatures (27.7 °C, 27.1 °C, or 26.5 °C) on *T. scripta* hatchling measures of oxidative damage and TAC. All significant effects are in bold

Source	Standardized Canonical Coefficients				
	Pillai's Trace (F)	df	P	Damage	TAC
Clutch	16	38,76	<.0001	1.0212239	1.19213519
Treatment	4.23	4,76	0.0038	0.5614358	1.48840626
Season	2.34	2,18	0.1254	0.4204202	-0.26507178
Season*Treatment	1.54	4,76	0.1978	0.9014117	1.28693589

FIGURES

Figure 1

Experimental Design and Incubation Manipulations for the Three Studies Conducted.

a) A split-clutch design was used to randomly assign eggs (n=130) from 11 clutches to incubate at either a constant (29.5 °C) or fluctuating (28.7 ± 3 °C) temperature treatment group. b) A split-clutch design was used to randomly assign eggs (n=268) from 22 clutches to either a hyper (28.7 ± 3 °C sinusoidal fluctuations every 12 hours), normal (28.7 ± 3 °C sinusoidal fluctuations every 24 hours), or hypo (28.7 ± 3 °C sinusoidal fluctuations every 48 hours) c) A split-clutch design was used to randomly assign eggs (n=214) from 21 clutches to fluctuation incubation regimes with varying average temperatures (27.7 ± 2 °C, 27.1 ± 2 °C, or 26.9 ± 2 °C)

Figure 2

Effect of a Constant or Fluctuating Incubation Temperature on Hatchling Oxidative Damage and TAC. Average $\pm$ S.E.M liver lipid hydroperoxides and TAC in *T. scripta* hatchlings following either a constant or fluctuating incubation temperature regime. There are no significant differences in either measure between groups.

Figure 3

Effect of a Constant or Fluctuating Incubation Temperature on Hatchling Median Telomere Length. Average $\pm$ S.E.M median telomere lengths in a subset of *T. scripta* hatchlings (n=40) following either a constant or fluctuating incubation temperature regime. There are no significant differences in between treatment groups.

Figure 4

Effect of Incubation Temperature Fluctuation Frequency on Hatchling Oxidative Damage and TAC. Average $\pm$ S.E.M liver lipid hydroperoxides and TAC in *T. scripta* hatchlings following incubation temperature regimes that varied in fluctuation frequency. While there are no significant differences in oxidative damage accumulation, TAC is significantly reduced followed by a low frequency incubation temperature fluctuations (48 hour cycle).

Figure 5

Effect of Mean Incubation Temperature on Hatchling Oxidative Damage and TAC.

Average $\pm$ S.E.M liver lipid hydroperoxides and TAC in *T. scripta* hatchlings following incubation temperature regimes that varied in mean temperature. While there are no significant differences in oxidative damage accumulation, TAC is significantly increased following incubation with a low (26.9 °C) average temperature.

CHAPTER III

THE INFLUENCE OF CLUTCH IDENTITY AND NESTING SEASON ON
OXIDATIVE DAMAGE AND ANTIOXIDANTS IN RED-EARED
SLIDER (*TRACHEMYS SCRIPTA ELEGANS*) HATCHLINGS

Introduction

An individual's phenotype is determined by direct contributions of one's 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 offspring phenotype and fitness (Lindström 1999). However, relatively little is known about the role that genetic and environmental contributions play in the development of oxidative stress resistance (Metcalf & Alonso-Alvarez 2010; Selman et al. 2012; Constantini 2013).

Oxidative stress has long been recognized for its involvement in signal transduction and aging (Harman 1956; Beckman & Ames 1998). More recently, it has been given considerable attention by ecologists and evolutionary biologists, due to its potential role as a mediator of life history strategies and trade-offs (Dowling & Simmons 2009; Monaghan, Metcalfe, & Torres 2009; Constantini et al. 2010; Selman et al. 2012).

Importantly, oxidative stress experienced early in life has been shown to have long-term consequences for offspring fitness and life-history traits. Work done in a number of avian species has demonstrated that nestling oxidative damage levels are predictive of growth rates (Hall et al. 2004; Foote et al. 2011; Caprioli et al. 2013), survival probability (Losdat et al. 2012), and future reproductive success (Noguera, Kim, & Velando 2012). Thus, by studying the factors that influence the development of oxidative stress phenotypes we may be able to provide insight into the evolution of life-history strategies.

Here, in three independent experiments we first aimed to determine if clutch identity and nesting season are related to measures of oxidative damage and total antioxidant capacity (TAC) in hatchlings of a model oviparous reptile, red-eared slider turtles (*Trachemys scripta*). Next, we further investigated clutch effects using a correlative approach to determine if either clutch size or maternal age were associated with hatchling oxidative status. While there are many potential correlates of oxidative stress, we focused on nesting season, clutch size, and maternal age, due to their potential links to concentrations of yolk dietary antioxidants. Maternally deposited yolk dietary antioxidants are known to play a crucial role in protection from oxidative stress both embryonically and during the early postnatal period of oviparous species (Surai, Speake, & Sparks 2001). While presently unexplored in reptiles, among avian species, variations in yolk antioxidant concentrations have a direct influence on offspring redox physiology; individuals hatching from eggs with elevated yolk antioxidants have reduced oxidative damage markers in their tissues (Surai & Speake 1998; Blount et al. 2002; Surai et al. 2003; Tsai et al. 2008). Reptilian eggs have similar compositions of yolk antioxidants to those observed in avian systems (Thompson et al. 1999; Speake, Surai & Gore 2001;

Dierenfeld et al. 2002; Weiss et al. 2011; Treidel unpublished analysis, Table 1), and it is likely they play similar protective roles in reptiles as well. Thus, we expect that traits positively associated with yolk antioxidants will also be both negatively and positively correlated to *T. scripta* hatchling oxidative damage and TAC, respectively.

Based on this assumption, and observed patterns of yolk antioxidants in other species, we made several predictions about the expected associations between season, clutch size, and maternal age and oxidative damage and TAC in hatchling red-eared slider turtles. First we predicted that season would significantly affect oxidative damage and TAC in hatchlings. *T. scripta* in our study population lay two clutches per season. We expect early season clutches to be richer in dietary antioxidants than late season clutches for several reasons. For one, female production of their first clutch begins in August and is not completed until the following May (Ernst & Lovich 2009). Maternal deposition of dietary antioxidants appears to be directly constrained by their environmental availability during egg production (Surai, Speake, & Sparks 2001). Taken together, the extended production of first clutches potentially allows for greater accumulation of maternal antioxidants as individuals will likely also have ample access to dietary antioxidant. In contrast, second or late season clutches are produced very rapidly following completion of first clutches (Ernst & Lovich 2009). This may in turn limit the temporal accessibility of dietary antioxidants for deposition into eggs. Additionally, in the closely related freshwater turtle the painted turtle (*Chrysemys picta*) there are known differences in the seasonal allocation of resources to eggs (Harms et al. 2005). Specifically, early season clutches have a larger yolk mass and lipid concentrations than late season clutches (Harms et al. 2005). Yolk mass has been positively related to yolk antioxidant

concentrations (Safran et al. 2008). Thus, seasonal variability in antioxidant allocation is likely occurring as well. In accordance, with the expected seasonal differences in yolk antioxidants, we predicted that hatchlings from clutches laid early in the nesting season will have lower levels of oxidative damage and higher TAC than hatchlings from clutches collected later in the nesting season.

We also anticipated that clutch size would be positively related to oxidative damage and negatively associated with TAC. This may be a consequence of the fact that eggs from larger clutches are expected to have lower concentrations of antioxidants than those of smaller clutches. Since all *T. scripta* eggs within a clutch are produced simultaneously resulting in little intra-clutch variation in egg size and constituents (Ernst & Lovich 2009), antioxidants may have to be divided more extensively during the production of larger clutches. This is a pattern which has been observed in lizards (Weiss et al. 2011). Alternatively, if production of larger clutches is more energetically taxing, females may need to utilize a greater amount of available antioxidants for self-maintenance as opposed to reproduction resulting in the predicted pattern.

Finally, maternal allocation of resources has been demonstrated to also vary with age (Harms et al. 2005). Older moms are observed to lay larger eggs (Bowden et al. 2004; Harms et al. 2005; Wilkinson & Gibbons 2005), with yolks containing both increased amounts of lipids and proteins (Harms et al. 2005). As mentioned before yolk mass is positively related to yolk antioxidants (Safran et al. 2008) and so the larger eggs from older moms likely also contain more antioxidants. Furthermore, life history theory predicts that investment in reproduction should increase with age (Ricklefs & Wikelski

2002). Accordingly, older moms may invest a greater amount of dietary antioxidants into their eggs for offspring protection as opposed to self-maintenance. Red-eared slider turtles exhibit indeterminate growth during their lifespans (Wilbur 1975). Consequently we can use maternal plastron length as a proxy for maternal age, with older individuals having longer plastrons. Thus, we also expected that in *T. scripta* hatchlings, maternal plastron length will be negatively related to oxidative damage and positively correlated with TAC.

Materials and Methods

Egg Collection and Incubation

Clutches of *T. scripta* eggs were collected during the 2013 and 2014 summer nesting seasons from either freshly laid nests or field trapped gravid females at Banner Marsh State Fish and Wildlife Area (Illinois, USA). Plastron length (mm) of all gravid females was recorded and individuals were transported to ISU, where an oxytocin injection was used to induce oviposition (Ernst & Lovich 2009). Over the course of the two years we collected a total of 45 clutches. These clutches were utilized in one of three independent studies conducted to investigate the influence of thermal environments of oxidative stress.

The first experiment (Constant versus Fluctuation) was conducted to compare the effect of fluctuating incubation temperatures on hatchling oxidative stress to constant temperature incubation and included six early season and five late season clutches collected in 2013. Eggs within each clutch were randomly assigned to either a constant temperature incubation (29.5 °C) or fluctuating temperature incubation group (28.7 ± 3

°C) (Figure 1a). The constant temperature equivalent (CTE) of the fluctuating treatment was 29.4 °C (Georges et al. 2005).

The second experiment (Fluctuation Frequency) included 13 early season clutches that were collected during the summer of 2013. This experiment was designed to investigate the effects of temperature fluctuation frequency on hatchling oxidative damage and TAC. Eggs from clutches involved in this study were randomly assigned to one of three fluctuating temperature incubation regimes; $28.7 \pm 3^\circ\text{C}$ sinusoidal fluctuations every 12 (Hyper), 24 (Normal), or 48 hours (Hypo) (Figure 1b).

The third experiment (Mean Temperature) was performed in order to assess the effects of mean incubation temperature on *T. scripta* oxidative damage and TAC. For this study during the summer 2014 nesting season a total of 21 clutches were collected, ten from early in the season and 11 later. A split-clutch design was again used to randomly assign eggs to one of three incubation treatment group. Treatments were characterized by daily sinusoidal fluctuations that cycled two degrees Celsius above and below a mean temperature of 26.5 °C (Low), 27.1 °C (Medium), or 27.7 °C (High) (Figure 1c). The CTE of these treatments was 26.9 °C, 27.5 °C, and 28.0 °C respectively (Georges et al. 2005).

For all experiments, eggs were incubated at ISU, while partially buried in moist vermiculite (-150 kPa) in plastic incubation boxes. Boxes were weighed weekly and lost water was replaced to maintain constant hydric conditions throughout incubation. Upon hatching, all offspring were euthanized, and liver tissue samples were collected for subsequent analysis of TAC and short and long term oxidative damage markers.

Antioxidant and Oxidative Damage Analyses

Measurement of offspring total antioxidant capacity (TAC) from the collected liver tissues was used to determine an individual's combined activity of both endogenous and dietary antioxidants. TAC was determined spectrophotometrically using a modified cupric reducing antioxidant capacity (CUPRAC) method. The CUPRAC method estimates TAC based on the ability tissue samples to inhibit oxidation by the chemical copper(II)-neocuproine and was performed as described by Özyürek et al. (2008).

Oxidative damage accumulated by all individuals during embryonic development and hatching to lipids in their liver tissue was also quantified spectrophotometrically via detection of lipid hydroperoxides. Lipid hydroperoxides are a byproduct of oxidative damage to lipids of cells (Monaghan et al. 2009). Specifically, lipid hydroperoxides were measured using the Ferrous Oxidation-Xylenol Orange Method (FOXO) as described by Hermes-Lima, Willmore, and Storey (1995). Additionally, for a subset of hatchlings (n=40) from our first experiment (constant versus fluctuation; Figure 1a) as a proxy for long term oxidative damage accumulation (Houben et al. 2008), we measured telomere lengths as described in Haussmann et al. (2012). Briefly, DNA from collected liver tissue samples was isolated using a Gentra Puregene Tissue kit (Qiagen, USA). The isolated DNA was subsequently digested (New England Biolabs, USA) and run on pulsed field gel electrophoresis. Following a Southern-hybridization and labeling of telomere bands, the median telomere length of each lane was subsequently determined using image analysis (Haussmann et al. 2012). For more specific details about all assays performed please refer to Chapter II of this thesis.

Statistical Analysis

All statistical analyses were performed using SAS statistical software (SAS 9.3) and data is presented as mean ($\pm$ SE). Analysis of clutch and season effects was performed for each of our three experiments separately as described previously (see Chapter II). Briefly, to analyze the results from our first (constant versus fluctuation; Figure 1a) and third (mean temperature; Figure 1c) experiments we used a mixed-effects multivariate analysis of variance (MANOVA) (PROC GLM) to determine the significance of treatment, clutch, season, and interaction effects on hatchling levels of oxidative stress. In our general linear models oxidative damage and TAC were the response variables and were transformed when necessary to meet assumptions of normality and homogeneity of variances. Treatment and season were considered fixed effects, while clutch and any interaction including clutch was considered a random effect. We also nested clutch within season as these two variables are not independent of one another. Pillai's Trace was used to derive our F-statistics and the standardized canonical coefficients were used to interpret relative contributions of our response variables to significant effects. Measures of hatchling TAC in our second experiment (Fluctuation Frequency; Figure 1b) could not be successfully transformed to meet the assumptions for parametric analysis. Thus, we applied an aligned-ranked transformation using the ARTool (v. 1.5.1; Wobbrok et al. 2011) prior to analysis. Treatment and clutch effects on hatchling measures of oxidative damage were subsequently analyzed separately by conducting two mixed effect Two-way ANOVAs (PROC GLM). Finally, to assess the effect of treatment, season, and clutch on hatchling measures of median telomere length, we ran a mixed effects ANOVA (PROC GLM). All significant fixed effects were

followed-up using pairwise comparisons with a Tukey correction and significant random effects were estimated by determining the variance components via PROC MIXED.

In order to determine if clutch size and maternal age are predictive of oxidative damage and TAC, we also performed a step-wise multiple regression (PROC REG) analysis of the data. To do this, we first conducted a MANOVA analysis for each of the three experiments with only treatment, season, and the treatment by season interaction included as fixed effects and compiled the residual measures of oxidative damage and TAC. The residuals of this analysis represent the amount of oxidative damage or TAC that could not be explained by incubation treatment or season. What remains are influences of clutch and random variation among individuals. Using these residuals we next ran two step-wise multiple regressions with clutch size and maternal plastron length as the independent variables to predict oxidative damage and TAC separately.

Results

Effects of Clutch and Season on Hatchling Oxidative Damage and TAC

Experiment 1- Constant versus fluctuating incubation. There were no seasonal differences in hatchling measures of oxidative damage or TAC ($F_{2,8} = 0.57$, $p=0.5860$; Table 2). Specifically, hatchlings from early season clutches had an average of 5.56 ± 0.26 nmol CHE/ mgww of oxidative damage and 34.81 ± 0.59 μ M Trolox Equivalents/mgww of TAC in their livers (Figure 6). Mean levels of oxidative damage and TAC in hatchlings from late season clutches were similar at 5.42 ± 0.28 nmol CHE/ mgww and 32.45 ± 0.69 μ M Trolox Equivalents/mgww, respectively (Figure 6). Similarly, in a subset of individuals, there was also no observed difference in mean

median telomere lengths of hatchlings from early (258.20 ± 29.13 KB) or late (292.86 ± 29.13 KB) season clutches ($F_{1,8} = 0.71$, $p = 0.4245$) (Figure 7).

There was however, a significant clutch effect on oxidative damage and TAC ($F_{18,18} = 9.05$, $p < 0.0001$; Table 2). Based on the Standardized Canonical coefficients, oxidative damage made a stronger contribution to the significant effect of clutch in comparison to TAC. Accordingly, clutch identity explained 65% of random variation in oxidative damage, but only 36% of random variation in TAC (Table 5). Within clutch measures of oxidative damage and TAC were positively related to one another such that clutches with higher levels of oxidative damage also had higher levels of TAC. Clutch identity was also significantly associated with telomeres ($F_{8,8} = 4.05$, $p = 0.0323$) and accounted for approximately 49% of random variation in median telomere length.

Experiment 2- Effect of temperature fluctuation frequency. All clutches collected for this experiment were from the early season. Thus we were only able to characterize the influence of clutch in this dataset. We found that there was a significant clutch effect on lipid oxidative damage ($F_{12,26.1} = 14.16$, $p < 0.0001$; Table 3) that explained 66.6% (Table 5) of the total variation in damage among individuals. Clutch identity was also significantly related to hatchling TAC ($F_{12,91} = 11.19$, $p < 0.0001$; Table 3) A follow-up analysis of the clutch effect revealed that 39% of the variation in TAC measurements was related to familial identity and occurred between clutches as opposed to among individuals (Table 5).

Experiment 3- Effect of mean incubation temperature. In addition to temperature treatment, there was a significant effect of clutch on the oxidative status of hatchlings ($F_{38,76} = 16.00$, $p = <0.0001$; Table 4). Both oxidative damage and TAC contributed to the significant clutch effect with a similar and strong degree, based on the standardized canonical coefficients (Table 4). Accordingly clutch explained 58% of random variation in levels of lipid peroxidation and 64% of random variation in TAC (Table 5). Furthermore, within clutches the amount of oxidative damage and TAC were directly related to one another such that hatchlings from clutches with higher amounts of oxidative damage also tended to have a higher TAC.

In contrast to clutch, nesting season was not significantly related to hatchling oxidative status ($F_{2,18} = 2.34$, $p = 0.1254$; Table 4), as there were no seasonal differences in hatchling oxidative damage or TAC. Hatchlings from clutches laid early in the nesting season had an average of 5.43 ± 0.11 nmol CHE/ mgww of oxidative damage and 54.28 ± 1.75 μ M Trolox Equivalents/mgww of TAC (Figure 8). Similarly the mean levels of oxidative damage and TAC in hatchling from clutches laid late in the nesting season were 5.93 ± 0.099 nmol CHE/ mgww and 52.59 ± 1.70 μ M Trolox Equivalents/mgww (Figure 8).

Effects of Clutch Size and Maternal Age on Hatchling Oxidative Damage and TAC

Based on a regression analysis, maternal age ($F_{1,358} = 22.83$, $p = <0.0001$) but not clutch size was significantly associated with hatchling measures of oxidative damage and explained approximately 6% of residual variation in lipid peroxidation (Figure 9). Specifically, maternal plastron length was negatively related to oxidative damage. Thus,

individuals from clutches produced by older mothers tended to have a lower accumulation of lipid hydroperoxides (Figure 9). In contrast, neither clutch size nor maternal plastron length was predictive of TAC.

Discussion

We found that there were strong and consistent clutch effects on *T. scripta* hatchling oxidative status. In all three experiments, over half of the observed random variation in oxidative damage and at least thirty-five percent of the random variation in TAC occurred between as opposed to within clutches. This result suggests that despite exposure to identical incubation environments, some clutches may be predisposed to be more susceptible to early life oxidative stress than others. Also, while not unexpected these observed robust clutch effects suggest that there are potentially strong genetic or other maternal effects influencing early life oxidative stress phenotypes in *T. scripta*.

In an attempt to gain further insight into the large clutch effects, we performed a multiple step-wise regression to identify possible contributing hatchling characteristics predictive of oxidative damage and TAC. Surprisingly, there was only a significant negative relationship between maternal plastron length and hatchling measures of oxidative damage. Due to age related differences in resource allocation to eggs (Harms et al. 2005) we had expected that clutches produced by older individuals would have more dietary yolk antioxidants in comparison to clutches produced by younger individuals. The expected presence of a negative correlation between maternal plastron length and oxidative damage, but the unexpected no correlation between maternal plastron length and TAC, may have arisen if there was differential utilization of yolk antioxidants during

incubation. If antioxidant utilization occurs during incubation only when availability is high, then we would expect utilization in clutches laid by older mothers but not younger mothers. This in turn could result in reduced oxidative damage but equal TAC of clutches from older moms relative to individuals from younger mothers, consistent with what we observed. Importantly, in the closely related painted turtle (*Chrysemys picta*) maternal age was also positively associated with hatchling survivorship (Paitz et al. 2007). Paitz et al. (2007) proposed that survival benefits are potentially conferred to offspring via increased investment in the production of higher quality eggs with age. Based on our results we propose that one component of higher quality eggs are yolk antioxidants, which may in turn reduce oxidative damage and increase survival probability. Consistent with this conclusion, the ability of great tit (*Parus major*) nestlings to resist oxidative stress is similarly predictive of short-term survival (Lodstat et al. 2012).

Other than differences in yolk antioxidant concentrations or utilization between clutches of older and younger moms, the observed negative association between hatchling oxidative damage, but not TAC and maternal age, might also be a consequence of genetically linked differences in oxidative stress susceptibility. Consistent with age related patterns of telomere lengths in other long-lived species (Haussmann et al. 2003; Flanary & Kletetschka 2005; Olsson et al. 2010), in a sample of adult red-eared slider females, plastron length was positively related to median telomere lengths (Treidel, unpublished data). This relationship is not likely indicative of telomere extension with age but likely results from differences in survival to old age and individual telomere shortening rates (Monghan & Haussmann 2006). Thus, the presence of longer telomeres among mothers with longer plastron length suggests that these individuals are more

resistant to oxidative stress. Importantly, if increased oxidative stress resistance among older individuals is underlain by differences in free radical production rates, this in turn is an advantage that females could be passing on to their offspring. For instance, among painted dragon lizards (*Ctenophorus pictus*), independent of yolk volume, free radical generation rate is highly heritable (Olsson et al. 2008; Olsson et al. 2009). Congruous with our results familial related differences in free radical production could influence oxidative damage accumulation independent of TAC. However, future work including measurements of maternal and offspring ROS production rates in *T. scripta* is needed to test this prediction. Finally, also in agreement with our observed trends, cross fostering studies performed in avian species have suggested that early-life oxidative stress resistance is highly heritable (Constantini & Dell’Omo 2006; Kim et al. 2010), while variations in antioxidant defenses are accounted for mainly by environmental conditions (Constantini & Dell’Omo 2006).

In contrast to maternal plastron length, we observed no relationship between either season or clutch size and hatchling oxidative status. We had predicted increased oxidative damage accumulation and lower TAC both among individuals from late season and larger clutches. This prediction was based on the above discussed expectation that yolk antioxidants would be reduced among both groups. However, neither oxidative damage nor TAC of red-eared slider hatchlings was affected by season or clutch size, suggesting that dietary antioxidant availability may not be limited in our population. Alternatively, recently the yolk precursor vitellogenin has been proposed to act as an antioxidant (Nakamura et al. 1999; Murphy et al. 2003; Seehuus et al. 2006) utilized by offspring for protection from prenatal and early-life oxidative stress (Olsson et al. 2009;

Ballen et al. 2012). Circulating concentrations of vitellogenin are positively associated with levels of reproductive investment among oviparous species (Garska et al. 1982; Han et al. 2009). In *T. scripta* both production of a second clutch and larger clutches are likely reflective of increased investment in reproduction. Consequently, increased vitellogenin exposure in late season and larger clutches may be able to compensate for potential yolk antioxidant deficits and could thus account for the lack of differences related to season or clutch size in *T. scripta* hatchling oxidative status observed here. While we are currently unable to distinguish between these two alternative interpretations, clarification can be achieved via quantification of yolk antioxidant presence in our collected clutches.

In conclusion, we provide clear evidence that familial identity plays a central role in early-life measures of oxidative damage and TAC in red-eared slider hatchlings. Through a correlative analysis we found that maternal age was predictive of and significantly associated with hatchling oxidative damage accumulation. However, while statistically significant, this association was still only able to explain a small fraction of the observed variation in measures of lipid peroxidation. We were also able to rule out potential associations between nesting season or clutch size and oxidative stress in *T. scripta* hatchlings from our population. Taken together, there are likely both additional maternal and genetic effects contributing to this strong clutch effect not accounted for here that warrant further investigation. For example, direct measurements of yolk antioxidant levels may provide substantial clarity in explaining the observed variability in TAC. In addition, other components of *T. scripta* eggs such as yolk hormone concentrations may be influential; prenatal exposure to the hormones corticosterone and testosterone were shown to alter early-life susceptibility to oxidative stress in avian

species (Tobler & Sandell 2009; Noguera et al. 2011; Haussmann et al. 2012; Tobler et al. 2013; Treidel et al. 2013). Along with the potential investigation of maternal effects, controlled laboratory studies should be conducted in order to gain better insight into the genetic underpinnings of oxidative stress phenotypes. Finally, resistance to oxidative stress likely plays a key role in the determination of life-history strategies and potentially direct fitness related consequences for survival and reproductive success (Monaghan et al. 2009). Therefore, gaining a better understanding of the environmental and genetic agents that shape one's early-life susceptibility to oxidative stress will likely not only enhance our understanding of life-history evolution but could also be useful for the development of nutrition and conservation plans aimed at maximizing the survival and reproductive success of endangered reptiles.

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TABLES

Table 5

Effect of Clutch on Hatchling Oxidative Damage and TAC. Estimated percentage of variation in liver lipid peroxidation and TAC attributed to differences among clutches in each of the three conducted experiments.

Experiment	Oxidative Damage	TAC
Constant vs. Flux	65%	35%
Fluctuation Frequency	66%	39%
Temperature	58%	64%

FIGURES

Figure 6

Effect of the 2013 Nesting Season on Hatchling Oxidative Damage and TAC. Average $\pm$ S.E.M liver lipid hydroperoxides and TAC in *T. scripta* hatchlings from clutches collected either early or late in the summer 2013 nesting season. There are no significant differences in either measure between groups.

Figure 7

Effect of the 2013 Nesting Season on Hatchling Median Telomere Length. Average $\pm$ S.E.M median telomere lengths in a subset of *T. scripta* hatchlings (n=40) from early and late season clutches. There are no significant differences in long-term accumulation of oxidative damage with nesting season.

Figure 8

Effect of the 2014 Nesting Season on Hatchling Oxidative Damage and TAC. Average $\pm$ S.E.M liver lipid hydroperoxides and TAC in *T. scripta* hatchlings from clutches collected either early or late in the summer 2014 nesting season. While, there are no significant differences in either measure between groups early season hatchlings did tend to have a higher TAC and less accumulation of oxidative damage in their liver.

Figure 9

Relationship Between Maternal Plastron Length and Oxidative Damage Accumulation in Hatchlings. Linear regression and trend line of the relationship between maternal plastron length and hatchling accumulation of oxidative damage. *T. scripta* hatchlings from clutches laid by older mothers with longer plastrons had significantly lower levels of lipid peroxidation detected in their liver.