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ORIGINAL PAPER

Oxidative stress and antioxidant capacity of a terrestrially hibernating hatchling turtle

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Abstract Hatchlings of the painted turtle, *Chrysemys picta*, hibernate terrestrially and can survive subfreezing temperatures by supercooling or by tolerating the freezing of their tissues. Whether supercooled or frozen, an ischemic hypoxia develops because tissue perfusion is limited by low temperature and/or freezing. Oxidative stress can occur if hatchlings lack sufficient antioxidant defenses to minimize or prevent damage by reactive oxygen species. We examined the antioxidant capacity and indices of oxidative damage in hatchling *C. picta* following survivable, 48 h bouts of supercooling (−6°C), freezing (−2.5°C), or hypoxia (4°C). Samples of plasma, brain, and liver were collected after a 24 h period of recovery (4°C) and assayed for Trolox-equivalent antioxidant capacity (TEAC), thiobarbituric acid reactive substances (TBARS), and carbonyl proteins. Antioxidant capacity did not vary among treatments in any of the tissues studied. We found a significant increase in TBARS in plasma, but not in the brain or liver, of frozen/thawed hatchlings as compared to untreated controls. No changes were found in the concentration of TBARS or carbonyl proteins in supercooled or hypoxia-exposed hatchlings. Our results suggest that hatchling *C. picta* have a well-developed antioxidant defense system that minimizes oxidative damage during hibernation.

Keywords *Chrysemys picta* · Cold hardiness · Overwintering · Freeze tolerance · Supercooling · Hypoxia · Oxidative damage

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Abbreviations

ABTS	2,2-Azino-di (3-ethylbenzthiazoline)
DNPH	2,4 Dinitrophenylhydrazine
FP_{eq}	Equilibrium freezing point
ROS	Reactive oxygen species
TEAC	Trolox-equivalent antioxidant capacity
TBARS	Thiobarbituric acid reactive substances

Introduction

The North American painted turtle, *Chrysemys picta*, is renowned among vertebrates for its tolerance to prolonged oxygen deprivation. Laboratory studies have described several biochemical and physiological adaptations that allow adult *C. picta* to hibernate in cold, anoxic water for ~180 days without significant risk of mortality (Herbert and Jackson 1985a, b). This remarkable feat is accomplished by reducing metabolic rate, relying on anaerobic metabolism, and using minerals in the bony carapace to bind H^+ in order to prevent development of a lethal lactacidosis (Jackson 2002). Some turtles are also adapted to prevent oxidative damage that can result from free radicals generated on resumption of aerobic metabolism following anaerobic dives (Perez-Pinzon and Rice 1995; Willmore and Storey 1997a, b).

Hatchlings of *C. picta* are less tolerant to anoxia than adult conspecifics (Reese et al. 2004) and can avoid months of anoxic submergence by hibernating in the shallow natal nest chamber. In the northern regions, hatchlings of *C. picta* are frequently exposed to subfreezing conditions in the nest and can survive either by supercooling (i.e., remaining unfrozen below the freezing point of their tissues) or by tolerating the freezing of their tissues (Costanzo et al. 1995b; Storey 2006). Whether supercooled or frozen,

an ischemic hypoxia or anoxia likely develops because tissue perfusion is limited at low temperature, whether or not freezing occurs. A turtle's heart rate decreases with temperature; therefore, supercooled hatchlings rely increasingly on anaerobic respiration (Costanzo et al. 2001; Packard and Packard 2005a) and, indeed, they accumulate significant concentrations of lactate in their brain, heart, and liver during extended exposure to −6°C (Packard and Packard 2005b). Hatchlings of *C. picta* tolerate extensive freezing, surviving the conversion of 50–60% of body water into ice (Churchill and Storey 1992; Costanzo et al. 1995b, 2006; Storey et al. 1988). As ice propagates throughout the body, the heart rate decreases and cardiac function ultimately stops (Rubinsky et al. 1994). Frozen tissues become ischemic and accumulate considerable lactate because they rely on anaerobic glycolysis to meet the metabolic demands (Churchill and Storey 1992; Dinkelacker et al. 2005; Storey et al. 1988). While terrestrial hibernation does not allow the hatchlings to escape anoxia altogether, the duration of ischemic/anoxic periods are likely to be relatively brief because subfreezing excursions are generally less than 24 h (Costanzo et al. 1995b).

Reactive oxygen species (ROS) are a class of oxygen-containing molecules that include hydrogen peroxide (H_2O_2), ions, and free radicals. ROS, such as the superoxide anion (O_2^-) and H_2O_2, are normal byproducts of aerobic metabolism and are important intracellular signaling molecules (Rice et al. 2002). However, ROS increase during environmental hypoxia (Duranteau et al. 1998) and ischemic anoxia (Vanden Hoek et al. 1996), and their concentration varies with the intensity of the stress. Under these conditions, the production of ROS (e.g., O_2^-) occurs primarily in the mitochondria (Duranteau et al. 1998). As the oxygen availability to cells decreases, mitochondria generate increasing amounts of oxidants (Duranteau et al. 1998). Oxidative stress occurs when the production of ROS exceeds the capacity of enzymatic and non-enzymatic antioxidants to regulate concentrations of these compounds.

Tissue damage can occur when excess ROS, formed during the early stages of oxidative stress, are converted to the highly reactive hydroxyl radical (·OH). Free radicals readily react with and damage polyunsaturated fatty acids, structural proteins, enzymes, and nucleic acids (Li and Jackson 2002). The duration of ischemic exposure is correlated with an increase in cell death (Vanden Hoek et al. 1996), and localized tissue damage that occurs during ischemia expands proportionately upon reperfusion (Vetterlein et al. 2003). For this reason, cold-hardy organisms must be adapted to prevent oxidative damage following either supercooling or freezing (Storey 2006; Voituron et al. 2006).

The purpose of this study was to determine whether hatchlings of *C. picta* are susceptible to oxidative damage following exposure to subfreezing temperature. To this end, we examined the total antioxidant capacity and quantified markers of oxidative damage in blood plasma, brain, and liver following periods of supercooling and freezing. For comparison, we also examined the physiological responses of hatchling turtles after exposure to environmental hypoxia, a much more commonly used stressor.

Materials and methods

Gravid females of *C. picta* were collected at Beem Lake in Hyannis, Nebraska and injected with synthetic oxytocin to induce oviposition (Ewert and Legler 1978). Eggs were incubated on a substratum of moist vermiculite (1.0 g water g^{-1} dry vermiculite) at 28°C and the resulting hatchlings were cold acclimated following the protocol outlined by Costanzo et al. (2000). Briefly, the hatchlings were held in plastic boxes containing moist vermiculite in a darkened environmental chamber (model I-35X; Percival, Boone, IA) at 20°C during September, but the temperature was lowered to 15°C on 1st October and then to 10°C on 1st November. The hatchlings were kept at 4°C from 1st December until used in experimental procedures.

Turtles ($n = 8$), taken directly from their containers in midwinter, were sampled to provide baseline (control) values for antioxidant capacity and markers of oxidative stress. These values were compared to hatchlings ($n = 8$) sampled 24 h after a 48-h bout of supercooling, freezing, or hypoxia as described below. Although previous research suggests that these treatments are readily survived by cold-acclimated *C. picta* (Costanzo et al. 2001; Dinkelacker et al. 2005), a reference group ($n = 5$) was monitored during an extended period of recovery from each of these experimental treatments.

Supercooling

A solution that is unfrozen below its equilibrium freezing point (FP_{eq}) is considered to be supercooled. When exposed to subfreezing temperatures, many organisms can supercool several degrees before ice crystals begin to form in their tissues; however, they must avoid contact with ice and other ice-nucleating agents (Lee and Costanzo 1998). Hatchlings of *C. picta* ($FP_{eq} = -0.6$°C) were kept supercooled for 48 h following a protocol described by Costanzo et al. (1998). On the day before testing, the turtles were removed from their holding boxes, gently cleaned to remove any adhering vermiculite, and held overnight at 4°C in a dry, loosely-covered plastic cup. This procedure eliminates foreign matter and permits evaporation of any surface moisture that might seed the freezing of their tissues. Each hatchling was placed vertically inside a clean, dry, plastic 50 ml centri-

fuge tube. A copper–constantan thermocouple, placed in contact with the plastron, was used to record the temperature at 30 s intervals on a data logger (Omega 3D752, Stamford, CT, USA). The space above the hatchling was filled with insulative plastic foam. The tubes were immersed in a refrigerated ethanol bath (Model RTE 140 Neslab; Portsmouth, NH) and, after all turtles reached equilibrium at −0.6°C, the bath was programmed to cool (0.5°C h^{-1}) to a minimum temperature of −6.0°C. The hatchlings were held at this temperature for 24 h and then warmed (0.5°C h^{-1}) to −0.6°C. The tubes were transferred to a temperature-controlled environmental chamber (4°C) where the hatchlings were allowed to recover for 24 h before being assayed.

Freezing

We used a protocol (Costanzo et al. 2006) designed to induce nucleation at a temperature near the FP_{eq} and allow a slow, controlled freezing of the tissues, as these conditions promote freezing survival (Storey et al. 1988). The hatchlings were prepared for the trials by brushing away any adherent vermiculite, wetting them with cold water, and placing them vertically (head up) in a plastic 50 ml centrifuge tube. A copper–constantan thermocouple placed against the plastron was used to record the body temperature at 30 s intervals. A few ice chips were added to the tube and gently tamped around the turtle. Plastic foam was placed in the tube, above the hatchling, to retain the latent heat of fusion and moderate the rate of freezing.

The tubes containing the turtles were immersed in a refrigerated ethanol bath maintained at −1.2°C. Once the turtles attained thermoequilibrium at −1.2°C, the bath was programmed to cool (0.1°C h^{-1}) to the target temperature, −2.5°C. The frozen hatchlings were held at this temperature for 24 h and then warmed (0.2°C h^{-1}) to −0.5°C. The total time of tissue freezing was 48 h. The tubes were transferred to a temperature-controlled environmental chamber (4°C), where the hatchlings were allowed to thaw and recover for 24 h before being assayed.

Hypoxia

The hatchlings were exposed to a 48 h period of hypoxia at 4°C. They were held, in darkness, in a 188 ml plastic chamber containing moist sponges to maintain high humidity and inhibit dehydration. The chamber was flushed with N_2 gas for 90 s and sealed. After 48 h, a 50 ml sample of chamber air was drawn into a Gastight® syringe (Hamilton Co., Reno, NV, USA). The air sample was injected into a calibrated oxygen analyzer (Model S-3A/II, Ametek, Pittsburgh, PA, USA); the reading (0.5% O_2) confirmed that the turtles had been exposed to hypoxic conditions. The hatchlings were then removed from the chamber, transferred to ventilated plastic containers, and allowed to recover for 24 h at 4°C before being assayed.

Tissue preparation

The hatchlings were killed by severing the spinal cord with a sharp pair of scissors and blood was collected from the severed neck vessels in heparinized microcapillary tubes. The tubes were centrifuged (4,000×*g*, 5 min) to separate the plasma from the packed cells. The plasma was frozen and stored at −80°C until assayed. The brain (ca. 40 mg) and a portion of the right lobe of the liver (ca. 65 mg) were surgically removed, placed in separate, pre-weighed, 1.5 ml Eppendorf tubes and ground with a plastic pestle. The tissues were homogenized in sufficient ice-cold buffer to yield a final tissue concentration of ca. 65 mg ml^{-1}. A 25 μl aliquot of the homogenate was reserved on ice for TBARS assay. The remainder was centrifuged (2800×*g*, 10 min; 4°C) and portions of the supernatant were reserved for antioxidant and carbonyl assays. These aliquots were stored at −80°C until used in the assays.

Lactate measurement

To confirm that all experimental treatments induced hypoxia/anoxia, the aliquots of plasma collected from experimental and control animals were assayed for lactate, the major end product of anaerobic metabolism in turtles. Plasma lactate was assayed using an enzymatic, colorimetric procedure (Sigma no. 735, St Louis, MO, USA).

Determining antioxidant capacity

There is a growing diversity in the methods to evaluate total antioxidant capacity (Schlesier et al. 2002.). We chose a commercially available assay (Antioxidant Assay, Cayman Chemical, Ann Arbor, MI, USA) because we wanted to examine the sum of antioxidant defenses in one simple procedure. This colorimetric assay, based on the methods of Miller et al. (1993), was used to quantify the net effect of aqueous and lipid-soluble antioxidants in plasma and tissue extracts. The antioxidant capacity was determined using 5 μl of plasma diluted (1:5) in assay buffer or 25 μl of supernatant from homogenized brain or liver tissue. The sample, 25 μl metmyoglobin, and 325 μl of chromogen [ABTS (2,2-Azino-di [3-ethylbenzthiazoline])] were mixed in a semi-micro cuvette and the contents were used to blank the absorbance ($\lambda = 750$ nm) in a spectrophotomer (Beckman DU 530; Beckman Instruments, Fullerton, CA, USA). Free radicals were generated, via the Fenton reaction, by adding 100 μl of H_2O_2 to the cuvette. The absorbance increased as the reaction progressed because a deep green

color developed in the solution as free radicals oxidized ABTS; however, antioxidants in the sample inhibited the reaction and retarded color development. The final absorbance was read after 5 min and compared to a standard curve prepared from various concentrations of Trolox, a water-soluble tocopherol (Vitamin E) analogue. Antioxidant capacity was expressed in millimolar Trolox equivalents per milliliter of plasma or per milligram of soluble protein in the tissue homogenate or supernatant.

Indices of oxidative damage

Lipid peroxidation by free radicals results in the formation of several end products, including malondialdehyde, that react with thiobarbituric acid. Although the measurement of thiobarbituric acid-reactive substances (TBARS) can produce artifacts (Halliwell and Gutteridge 1999), their presence is commonly used as an indicator of oxidative damage (e.g., Willmore and Storey 1997b; Valdivia et al. 2006). The concentration of TBARS was determined in samples of plasma and homogenized tissue extract using an assay kit (OXItek; ZeptoMetrix Corp., Buffalo, NY, USA). The assay was conducted using 12.5 µl of plasma diluted (1:1) with buffer or 25 µl crude tissue homogenate (~65 mg tissue ml^{-1}). The sample was combined with 25 µl of the supplied SDS solution and 625 µl TBA reagent in a 2.0 ml microcentrifuge tube and incubated (95°C) for 1 h in a thermostatically controlled water bath (Precision Scientific, Chicago, IL, USA). After cooling the samples to room temperature, 675 µl of butanol was added to each tube. The tubes were vortexed thoroughly and then centrifuged (1,000 × g, 5 min; room temperature). Two 220 µl aliquots from the butanol layer were transferred to a 96-well plate and the absorbance ($\lambda = 532$ nm) was read on a SpectraMax Plus[384] spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The duplicate values were averaged and TBARS concentration (nmol ml^{-1}) was calculated from a standard curve prepared from various concentrations (0–50 nmol ml^{-1}) of malondialdehyde. TBARS concentration was expressed per milliliter of plasma or per milligram of soluble protein in the crude homogenate.

Carbonyl proteins, markers of oxidative damage to proteins, form during oxidative stress when free radicals modify amino acid side chains. We measured carbonyl proteins in supernatant from homogenized brain or liver tissue by reacting samples with 2, 4 dinitrophenylhydrazine (DNPH) to generate dinitrophenylhydrazones (Reznick and Packer 1994). For this assay, a 400-µl aliquot of supernatant was divided equally between two 2.0-ml microcentrifuge tubes. Next, 800 µl of DNPH solution (10 mmol DNPH in 2 M HCl) was added to one tube and 800 µl of 2 M HCl to the other. Both tubes were incubated at room temperature in darkness for 1 h. Proteins were precipitated with 1 ml 20% TCA and the resulting pellet was rinsed thrice with 1-ml volumes of an ethanol:ethyl acetate (1:1) solution to remove excess DNPH. The final precipitate was solubilized in 500 µl of 6 M guanidine hydrochloride. Carbonyl content of DNPH-reacted and corresponding HCl samples was determined in duplicate. The difference between the average absorbance ($\lambda = 370$ nm) of DNPH-reacted and unreacted (HCl only) samples was used to calculate carbonyl protein concentration (nmol ml^{-1}). Carbonyl proteins were expressed as nmol per milligram of protein in the sample.

Standardization of values

Brain and liver antioxidant capacity and concentrations of the oxidative damage products, TBARS and carbonyl proteins, were standardized by expressing them per milligram of protein. Protein concentrations in the prepared tissue homogenates were determined by the Bradford method (Bio-Rad, Hercules, CA, USA) using BSA standards (0–1.0 mg ml^{-1}).

Statistical analyses

Values are reported as means ± SEM. Mean values for treatment groups were compared by Kruskal–Wallis test followed by Dunn's multiple comparisons test. Mann–Whitney U tests were used to compare antioxidant capacity and markers of oxidative damage between brain and liver for each experimental group and untreated controls. Significance was accepted when $P < 0.05$.

Results

The experimental treatments induced a measure of physiological stress, but were survived by all subjects. After 24 h of recovery at 4°C, the hatchlings from all experimental treatments exhibited normal muscle tone and retracted their limbs in response to handling. Moreover, hatchlings in the reference group for each treatment eventually recovered normal neurobehavioral function as manifested by the righting response. Mean plasma lactate concentrations of supercooled (6.0 ± 0.7 mmol l^{-1}, $n = 8$), frozen/thawed (16.4 ± 1.1 mmol l^{-1}, $n = 7$), and hypoxia-treated (10.0 ± 1.9 mmol l^{-1}, $n = 8$) hatchlings were elevated ($P < 0.001$) relative to untreated controls (0.9 ± 0.4 mmol l^{-1}, $n = 8$), confirming that our experimental conditions induced anaerobiosis.

The antioxidant capacity of plasma from experimentally treated hatchlings was not different from that of untreated controls ($P = 0.92$; Table 1). Results for TBARS were similar except that we found a significant difference ($P = 0.04$)

Table 1 Antioxidant capacity of plasma, brain tissue, and liver tissue from hatchlings of *Chrysemys picta*

	Control	Supercooled	Frozen/thawed	Hypoxia	*H*	*P*
Plasma	0.65 ± 0.05	0.70 ± 0.09	0.61 ± 0.06	0.68 ± 0.07	0.50	0.92
Brain	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.004	0.02 ± 0.003	7.10	0.07
Liver	0.13 ± 0.02	0.14 ± 0.03	0.13 ± 0.02	0.15 ± 0.03	1.21	0.75

Means ± SE; $n = 7–8$. Plasma values are in mmol Trolox ml^{-1}; brain and liver tissue values are in mmol Trolox mg $protein^{-1}$

in frozen/thawed hatchlings relative to hypoxia-treated ones (Table 2). We did not have sufficient sample volume to measure carbonyl proteins in the plasma of any animals.

The brain tissue was well protected against free-radical damage following experimental treatments (Tables 2, 3). The antioxidant capacity in brain tissue from treated hatchlings did not differ from that in untreated controls ($P = 0.07$, Table 1). Although not significantly different from controls, the antioxidant capacity was lowest in the brain tissue from hatchlings in the hypoxia treatment group. We found no change in oxidative damage to brain tissue as evidenced by concentration of TBARS ($P = 0.49$, Table 2) or carbonyl proteins ($P = 0.12$; Table 3). Carbonyl protein concentrations in brain tissue were nominally lower in hatchlings subjected to supercooling or freezing, but these values were not significantly different from untreated controls.

Experimental treatments had no effect on antioxidant capacity of liver tissue ($P = 0.75$; Table 1). We found no change in TBARS ($P = 0.24$, Table 2) or carbonyl proteins ($P = 0.51$, Table 3) in liver tissue following experimental treatment. In control turtles, the antioxidant capacity of liver was nearly four times higher ($U = 4.0$; $P = 0.003$) than brain tissue, and this parameter was consistently higher ($P < 0.05$) in liver relative to brain in all groups of experimentally treated animals. TBARS concentrations in liver and brain tissues were similar ($P > 0.05$) in all group comparisons, except that concentrations in liver of supercooled turtles were significantly higher ($U = 12.5$; $P = 0.04$). Carbonyl protein concentrations in liver tissue were significantly lower than those in brain tissue from hypoxia-treated ($U = 13.0$; $P = 0.05$) and control turtles ($U = 10.0$; $P = 0.02$); however, we found no differences ($P > 0.05$) between these tissues of animals from supercooled and frozen/thawed groups.

Table 3 Carbonyl proteins in brain and liver tissue from hatchling *Chrysemys picta*

	Control	Supercooled	Frozen/thawed	Hypoxia	*H*	*P*
Brain	3.7 ± 0.4	2.6 ± 0.5	2.2 ± 0.4	3.8 ± 0.7	5.93	0.12
Liver	2.2 ± 0.2	2.3 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.29	0.51

Means ± SE; $n = 7–8$. Values are in nmol mg $protein^{-1}$

Discussion

Supercooling and freeze tolerance are generally considered dichotomous strategies for survival at subzero temperatures (Lee and Costanzo 1998). However, hatchlings of *C. picta* and the European common lizard (*Lacerta vivipara*) are rare examples of vertebrate species whose cold-hardiness strategy permits them to survive subzero exposures in either the supercooled or frozen state (Costanzo et al. 1995a, b; Voituron et al. 2006). Our finding that plasma lactate concentration increases in response to supercooling, freezing/thawing, and hypoxia suggests that adaptations that support prolonged anaerobic metabolism may be an important determinant of winter survival in hatchling *C. picta* (Costanzo et al. 2001; Dinkelacker et al. 2005). We examined the antioxidant capacity and measures of oxidative damage of hatchling *C. picta* because the potential for oxidative stress following subzero exposure exists regardless of the survival strategy.

All oxygen-breathing animals rely on antioxidant defenses to prevent oxidative damage to cellular components; however, antioxidant systems are complex and diverse in their constitution (Halliwell and Gutteridge 1999; Hermes-Lima and Zenteno-Savin 2002). Most

Table 2 Thiobarbituric acid reactive substances (TBARS) in plasma, brain tissue, and liver tissue from hatchling *Chrysemys picta*

	Control	Supercooled	Frozen/thawed	Hypoxia	*H*	*P*
Plasma	7.8 ± 0.2	8.0 ± 1.0	10.0 ± 0.7	6.8 ± 0.5*	8.59	0.04
Brain	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	2.40	0.49
Liver	0.9 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	4.20	0.24

Means ± SE; $n = 6–8$. Plasma values are in nmol ml^{-1}; brain and liver tissue values are in nmol mg $protein^{-1}$

* Significantly different from frozen/thawed

anoxia-tolerant organisms increase specific antioxidant enzymes during anoxic exposure in preparation for ROS generation during aerobic recovery (Hermes-Lima et al. 1998). Alternatively, some adult aquatic turtles maintain high constitutive activities of antioxidant enzymes, such as superoxide dismutase, catalase, and alkyl hydroperoxide reductase, to neutralize any O_2^- and H_2O_2 generated following anaerobic dives (Willmore and Storey 1997b). Hatchlings of *C. picta* appear to improve their antioxidant defenses following freezing and anoxia by increasing the activity of catalase in liver (Dinkelacker et al. 2005) and upregulating genes that code for glutathione peroxidase, glutathione S-transferase, and peroxiredoxin in liver and heart (Storey 2006); however, the contribution of non-enzymatic radical scavengers must be considered when evaluating antioxidant capacity. For instance, potent radical scavengers such as ascorbic acid (Vitamin C) and uric acid have been shown to increase in adult aquatic turtles during cold-acclimation (Rapatz and Musacchia 1957; Perez-Pinzon and Rice 1995). Further, the role of carotenoids and tocopherols must be considered because these lipid-soluble molecules protect polyunsaturated fatty acids in cell membranes (DiMascio et al. 1991).

Previous studies on anoxia-tolerant and freeze-tolerant animals have largely focused on the activity of antioxidant enzymes and/or the oxidation state of glutathione (Hermes-Lima and Storey 1993, 1996; Joanisse and Storey 1996a, b; Voituron et al. 2006; Willmore and Storey 1997a, b). This study, in which we measured the total antioxidant capacity, apparently was the first to examine the net effect of experimental treatment on antioxidant defenses of anoxia-tolerant animals, accounting for changes in both enzymatic and non-enzymatic antioxidants. Individual components of the antioxidant defense system may have increased or decreased during treatment and recovery; however, our data suggest that total antioxidant capacity, as measured by the TEAC assay, did not change appreciably in the plasma, brain, and liver following experimental stress.

The antioxidant capacity of adult aquatic turtles is considered to be exceptional among ectothermic vertebrates (Rice et al. 2002; Willmore and Storey 1997b). Mean plasma antioxidant capacity of untreated hatchling turtles (0.65 ± 0.05 mmol Trolox l^{-1}) is low relative to human plasma (1.46 mmol Trolox l^{-1}) as reported by Miller et al. (1993); however, little is known about the antioxidant capacity of hatchlings of *C. picta* relative to other animals. For comparative purposes, we assayed plasma from untreated hatchlings of *C. picta*, leopard frogs (*Rana pipiens*), wood frogs (*R. sylvatica*), laboratory mice (*Mus musculus*), and adult red-eared sliders (*Trachemys scripta*) that were killed for unrelated laboratory projects. The antioxidant capacity of hatchling *C. picta* plasma was nominally, but not significantly, higher than that of an anoxia-tolerant frog (*R. pipiens*), a freeze-tolerant frog (*R. sylvatica*), and the mammal *M. musculus* (Table 4). Hatchling *C. picta* had significantly higher antioxidant capacity than adult *T. scripta*, a model organism for hypoxia/anoxia tolerance studies. While this finding was unexpected, adult *C. picta* are considerably more tolerant of anoxia than adult *T. scripta* (Ultsch 2006). Further experimentation to compare the antioxidant capacity of hatchling *C. picta* to conspecific adults is planned to address whether the observed differences in antioxidant capacity are ontogenetic or taxonomic.

Because terrestrially hibernating hatchlings of *C. picta* are presumably sheltered from protracted periods of anoxia that conspecific adults routinely survive in aquatic hibernacula, the need for high constitutive antioxidant defenses is not immediately apparent. However, at low temperatures, the ventilatory pattern of hatchling *C. picta* is characterized by periods of apnea greater than 0.5 h (Larson 2004). A high constitutive antioxidant capacity is an adaptation to apneic breathing patterns commonly found in diving animals and other species that experience drastic changes in oxygen availability (Wilhelm et al. 2002; Hermes-Lima and Zenteno-Savin 2002).

Despite the extraordinary antioxidant capacity of *C. picta*, oxidative stress may nevertheless present a problem for these hatchlings during ecologically relevant cold exposures. Hatchlings that were frozen/thawed sustained oxidative damage to plasma lipids (TBARS). Lipid peroxidation is facilitated by hemoglobin, a catalyst for generating hydroxyl radicals (Sadrzadeh et al. 1984), which can leak from damaged erythrocytes following freeze/thaw stress (Costanzo et al. 1991). Free iron may participate in ROS-producing Fenton reactions that can lead to oxidative damage; however, during freezing/thawing, hatchlings of *C. picta* upregulate genes for iron-binding proteins (e.g., ferritin) that apparently minimize iron-mediated ROS

Table 4 Antioxidant capacity of plasma from untreated hatchling painted turtles in comparison to some other species

Species	*n*	Antioxidant capacity (mmol Trolox ml^{-1})
Painted turtle (*Chrysemys picta*)	5	0.67 ± 0.06^{a}
Red-eared slider (*Trachemys scripta*)*	5	0.33 ± 0.08^{b}
Leopard frog (*Rana pipiens*)*	3	$0.40 \pm 0.11^{a,b}$
Wood frog (*R. sylvatica*)**	5	$0.45 \pm 0.06^{a,b}$
Mouse (*Mus musculus*)***	5	$0.52 \pm 0.02^{a,b}$

Means ± SE. Values not sharing a letter are statistically different ($P < 0.05$)

*Adult males, held in aquaria at 5°C prior to testing

**Adult males, held in darkened boxes on damp sphagnum moss at 5°C prior to testing

***Adult unsexed, held on dry bedding, with controlled lighting and temperature (12 light:12 dark at 21°C) prior to testing

formation (Storey 2006). Earlier studies on the antioxidant defenses of freeze-tolerant vertebrates did not examine plasma for markers of oxidative damage (Hermes-Lima and Storey 1993; Joanisse and Storey 1996a; Voituron et al. 2006), but our findings suggest that tissues of the cardiovascular system may be susceptible to free radical attack following freezing/thawing. Additional assays for lipid peroxidation (e.g., isoprostanes) can be used to confirm these results.

The antioxidant capacity of brain tissue was not affected by the experimental stressors used in this study. Albeit lower than in liver tissue, the antioxidant defenses in brain were sufficient to prevent accumulation of oxidative damage products during recovery from stress. The difference in antioxidant capacity between these tissues may reflect lower radical production in the central nervous system due to a suite of physiological adaptations that promote anoxia tolerance in turtles. Whereas ROS generation increases in hypoxic mitochondria from mammalian cardiomycetes (Duranteau et al. 1998), a recent work suggests that a rapid decrease in ROS production occurs in turtle brain during anoxic submergence (Pamenter et al. 2007). These authors hypothesized that lower ROS production could serve as a signal, regulating the adaptations for long-term neuroprotection during anoxia. Chief among these is a profound metabolic depression achieved through the reduction of ATP turnover, limited protein synthesis, and a decrease in ion channel activity (Jackson 2002).

Liver antioxidant capacity remained relatively high following exposure to experimental stressors. Superior antioxidant capacity in liver tissue is likely due to tissue-specific activity of antioxidant enzymes. In adult aquatic turtles, activities of catalase, superoxide dismutase, alkyl hydroperoxidase, and glutathione peroxidase are much higher in liver than in brain (Willmore and Storey 1997b). In our study, the antioxidant defenses of the liver apparently prevented oxidative damage following recovery from supercooling, freezing, and hypoxia. Similarly, Willmore and Storey (1997b) found negligible free-radical damage to lipids in the liver, muscle, and kidney from adult *T. scripta* during a 20-h anoxia exposure and after a 24-h aerobic recovery period. When values are expressed per gram wet weight of liver, TBARS from our control hatchling *C. picta* (64.4 ± 4.5 nmol g wet weight^{-1}; $n = 8$) are comparable to control values for adult *T. scripta* (71.0 ± 14.1 nmol g wet weight^{-1}, $n = 12$), but much lower than values (121.3 ± 20.8 nmol g wet weight^{-1}, $n = 16$) for liver samples from adult green sea turtles (*Chelonia mydas agassizii*) that had drowned in gill nets (Valdivia et al. 2006). Although this comparison is only valid if liver composition (particularly water content) is reasonably similar among species, it suggests that damage products can accumulate in turtles under extremely stressful conditions, especially if preventative mechanisms are not well developed.

It is possible that we failed to detect an increase in oxidative damage in hatchlings of *C. picta*, because repair processes effectively removed damage products before we assayed the tissues. In the freeze-tolerant wood frog (*R. sylvatica*), for example, carbonyl proteins are elevated in muscle and liver tissue during 24-h freezing exposure, but these proteins are apparently cleared within 4 h of thawing (Woods and Storey 2006). Oxidatively modified proteins are degraded by the 20s proteasome, the principal component of the multicatalytic proteinase complex. Proteasome activity increased in liver tissue of adult turtles after 24 h recovery from anoxia exposure (Willmore and Storey 1996), but the implications of this finding for hatchling *C. picta* are unknown. Sampling during freezing and at short time intervals during recovery would clarify this point; however, it is clear that carbonyl proteins are not elevated in hatchling *C. picta* 24 h after thawing.

Another potential limitation of our study is that the 48 h treatment period may not have been sufficiently long to tax antioxidant defenses and induce oxidative damage. Hatchling *C. picta* can survive >11 days of freezing at -2.5°C (Churchill and Storey 1992), >20 days of supercooling at -6°C (Packard and Packard 2005a, b), and ~40 days of anoxia at 3–4°C (Dinkelacker et al. 2005; Reese et al. 2004). Indeed, hatchlings of *C. picta* that were held in a nitrogen environment for 16 days maintained high liver catalase activity, suggesting that the antioxidant defenses were still potent (Dinkelacker et al. 2005). Extended freezing and supercooling can be problematic for cold-hardy organisms because, in the absence of a patent circulatory system, their cells rely on limited energy stores to produce ATP (Layne et al. 1998). Furthermore, the potentially harmful waste products of anaerobic metabolism (e.g. lactate) accumulate over time and death may result from the decrease in intracellular pH (Costanzo et al. 2001, 2006; Packard and Packard 2005a, b). Future research on the effect of temperature and duration of these stresses on oxidative damage would be instructive.

Conclusions

Evidences for a strong antioxidant capacity of hatchling *C. picta* and minimal oxidative damage following 48 h anoxia/ischemia suggest that this species is well protected against oxidative stress during hibernation. These findings are consistent with claims that antioxidant defenses are important for animals that can utilize supercooling during hibernation (Joanisse and Storey 1996b; Rice et al. 2002; Voituron et al. 2006). Our results also accord with earlier studies on anoxia- and freeze-tolerant reptiles and amphibians (Hermes-Lima and Storey 1993, 1996; Joanisse and Storey 1996a; Voituron et al. 2006). Antioxidant defenses are an essential

component of anoxia tolerance, which in turn is a prerequisite for animals that utilize supercooling or freeze-tolerance as a winter survival strategy (Costanzo et al. 2001, 2006). The ability to neutralize free radicals, therefore, might be considered an exaptation for preventing oxidative damage following bouts of supercooling and freezing.

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