J Comp Physiol B (2006) 176:697–707
DOI 10.1007/s00360-006-0092-9

ORIGINAL PAPER

Physiological responses to freezing in hatchlings of freeze-tolerant and -intolerant turtles

Jon P. Costanzo · Patrick J. Baker · Richard E. Lee Jr

Received: 6 February 2006 / Revised: 27 April 2006 / Accepted: 9 May 2006 / Published online: 7 June 2006

Abstract Freeze tolerance is a complex cold-hardiness adaptation that has independently evolved in a diverse group of organisms, including several ectothermic vertebrates. Because little is known about the mechanistic basis for freeze tolerance in reptiles, we compared responses to experimental freezing in winter-acclimatized hatchlings representing nine taxa of temperate North American turtles, including ones that tolerated freezing and others that did not. Viability rates of hatchlings frozen to –3°C for 72 h ranged from 0 to 100%. Tolerance to freezing was poor in *Sternotherus odoratus*, *Graptemys geographica* and *Trachemys scripta*, intermediate in *Chelydra serpentina*, and high in *Emydoidea blandingii*, *Chrysemys picta bellii*, *C. p. marginata*, *Malaclemys terrapin*, and *Terrapene ornata*, and generally reflected the winter thermal ecology of each taxon. Plasma activity of lactate dehydrogenase (LDH), a novel in vivo index of freeze/thaw damage, corroborated viability assessments and demonstrated that cryoinjury occurred even in surviving turtles. Irrespective of taxon, cryoinjury tended to be higher in smaller individuals and in those having relatively low water contents; however, bases for these associations were not apparent. Screening for certain organic osmolytes that might promote freezing survival by colligatively reducing ice content and limiting cell dehydration showed that the plasma of unfrozen (control) turtles contained small quantities of glucose (1.3–5.8 mmol l^{-1}) and lactate (0.6–3.2 mmol l^{-1}) and modest amounts of urea (range of mean values for all taxa 8.2–52.3 mmol l^{-1}). Frozen/thawed turtles of all taxa accumulated modest amounts of glucose and lactate that jointly raised the plasma solute concentration by 30–100 mmol l^{-1}. We conclude that organic osmolytes accumulated both before and during freezing may promote survival in species that have evolved a tolerance to freezing, but are not necessarily accumulated for that purpose.

Keywords Cold hardiness · Freeze tolerance · Hatchling turtle · Osmolyte · Cryoprotectant

Communicated by H.V. Carey

J. P. Costanzo (✉) · P. J. Baker · R. E. Lee Jr
Department of Zoology, Miami University,
Oxford, OH 45056, USA
e-mail: costanjp@muohio.edu

Introduction

The radiation of ectothermic animals and their successful colonization of high latitudes necessitated evolution of enhanced tolerance to environmental variability, including extreme cold (Addo-Bediako et al. 2000). Natural freeze tolerance, the inherent ability to survive the freezing of body water under ecologically-relevant thermal and temporal conditions, is an important adaptation in some inhabitants of cold climatic regions. Freeze tolerance is known only in ectotherms but is represented in diverse taxa, including myriad terrestrial and intertidal invertebrates, and several temperate species of amphibians and reptiles (Storey and Storey 1988).

In order to evolve a tolerance to somatic freezing, organisms require mechanisms for coping with various physicochemical stresses that are simultaneously manifested at multiple levels of biological organization. Key among these are cell dehydration and osmotic/

ionic disruption of intracellular macromolecules, organelles, membranes, and other structures (Storey and Storey 1988; Mazur 2004). In vertebrate ectotherms, pervasion of ice throughout the body eventually arrests breathing and cardiac function, rendering tissues ischemic and hypoxic (Churchill and Storey 1991, 1992; Packard and Packard 2004). Consequently, energy stores are depleted and oxidative stress may occur coincident with reoxygenation upon thawing (Storey 2006).

Investigation of the molecular and physiological responses to freezing has revealed that freeze-tolerant organisms employ a suite of coordinated responses that limit or repair injury caused directly or indirectly by the formation and subsequent melting of ice (Storey and Storey 1988). Some of the protective mechanisms in freeze tolerance probably were derived from more fundamental responses to osmotic and hypoxic stress. For many species, these include hypometabolism, upregulation of antioxidant defense systems, and expression of genes involved in homeostasis and somatic repair. In addition, freeze-tolerant organisms commonly accumulate cryoprotectants, organic osmolytes that colligatively reduce ice formation and the attendant cell dehydration, and also preserve the structural integrity of proteins, membranes, and other cell structures (Storey and Storey 1988; Mazur 2004; Yancey 2005).

The collective efforts of investigators working at multiple levels of biological organization have provided a reasonably comprehensive understanding of freeze tolerance, although much of this work has focused on insects and anurans. Freeze tolerance also occurs in reptiles, but the underlying molecular and physiological mechanisms remain incompletely understood (Storey 2006). Studies of reptilian freeze tolerance commonly involve the hatchlings of northern turtles (e.g., Costanzo et al. 1995b; Packard et al. 1999; Dinkelacker et al. 2005) because sympatric species exhibiting diverse hibernation habits are subjected to differential selection pressures to develop extreme cold hardiness. For example, whereas the neonates of some species characteristically vacate their nests and overwinter in thermally-buffered aquatic habitats or deep burrows, others hibernate on land, either within or outside the natal nest, and are exposed to widely-varying temperatures that potentially can fall below the tissue freezing point. These species probably vary in their tolerance to freezing, although comparing survival rates among them is confounded by inter-study variation in experimental conditions.

In the present investigation we sought clues to the mechanistic basis of reptilian freeze tolerance by comparing responses to experimental freezing/thawing among hatchlings of temperate species that potentially vary in their capacity to withstand freezing. We studied the western painted turtle (*Chrysemys picta bellii*), midland painted turtle (*C. p. marginata*), northern diamondback terrapin (*Malaclemys terrapin terrapin*), northern map turtle (*Graptemys geographica*) and red-eared slider (*Trachemys scripta elegans*), taxa that can successfully overwinter within the natal nest (Cagle 1944; Baker et al. 2003, 2006; Costanzo et al. 2004). We also studied the ornate box turtle (*Terrapene ornata*) which hibernates in soil beneath the nest chamber, possibly within reach of frost (Costanzo et al. 1995b), as well as Blanding's turtle (*Emydoidea blandingii*), whose winter habitat is as yet unknown but could include terrestrial sites (see Dinkelacker et al. 2003 and references therein). We included the common musk turtle (*Sternotherus odoratus*), a species that probably hibernates under water (Nagle et al. 1998), and the common snapping turtle (*Chelydra serpentina*), which usually, though not invariably (Obbard and Brooks 1981), emerges from the nest soon after hatching and moves to ponds or streams for hibernation.

One aim of our study was to confirm that species differ in their innate capacity to withstand freezing. Secondly, because the cold-hardiness strategies adopted by vertebrate ectotherms could be influenced by somatic characteristics such as body size, hydro-osmotic balance, and energetic reserves (reviewed by Costanzo and Lee 1995), we tested associations between several such traits and the ability to withstand freezing. Finally, we examined the potential for certain organic osmolytes, or cryoprotectants, to promote freeze tolerance in hatchling turtles. Earlier work suggests these animals can accumulate organic osmolytes during winter acclimatization (Costanzo et al. 2000) or during freezing and/or thawing (Churchill and Storey 1992; Hemmings and Storey 2000; Dinkelacker et al. 2003; others), but the relative importance of these solutes in reptilian freeze tolerance remains unclear (Storey 2006).

Materials and methods

Animals and acclimatization

We collected eggs from gravid female turtles in Nebraska (*Chelydra serpentina, Chrysemys p. bellii, Emydoidea blandingii*, and *Terrapene ornata*), Indiana (*Chrysemys picta marginata, Graptemys geographica, Sternotherus odoratus*, and *Trachemys s. elegans*), and New Jersey (*Malaclemys t. terrapin*) via oxytocin

administration (Ewert and Legler 1978). Eggs were grouped by clutch and incubated at 28°C on moist vermiculite (1.0 g water g^{-1} dry vermiculite) in the laboratory. Hatchlings, which emerged from their eggs in late summer, were kept on moist vermiculite in plastic boxes inside a darkened incubator (Percival I–35X, Boone, IA, USA). Ambient temperature was held at 20°C during September, lowered to 15°C on 1 October, and then reduced to 10°C on 1 November. Hatchlings were kept at 4°C from 1 December until they were used in the experiments 8–12 weeks later. This acclimation regimen closely mimicked seasonal changes in turtle nest temperature at the Nebraska collection site (see Costanzo et al. 1998).

Freeze tolerance

We used a standardized freezing/thawing protocol to test for taxonomic variation in freeze tolerance capacity. Hatchlings (n = 7–8 per species) were prepared for freezing trials by weighing them to 0.01 g, wetting them with cold water, and individually placing them vertically, with the head up, in the bottom of a plastic tube (33 × 105 mm). Because wet hatchlings are readily inoculated by physical contact with ice (Attaway et al. 1998), we effectively controlled the freezing event by gently tamping a few ice chips around each turtle. In order to monitor temperature, each turtle was outfitted with a type T thermocouple (Omega, Stamford, CT USA) whose tip was placed against the plastron. A small sponge inserted into the tube above the turtle moderated the rate at which it cooled.

Tubes containing the turtles were immersed in a refrigerated ethanol bath (Neslab RTE 140, Portsmouth, NH, USA). During cooling, body temperature was recorded by a data logger (Omega RD3752, Stamford, CT, USA) at 30-s intervals. Ice nucleation, evidenced by the appearance of a freezing exotherm in the temperature recording, generally occurred between –0.6 and –1.2°C. We allowed turtles to attain thermoequilibrium at –1.2°C before cooling them at 0.05°C h^{-1} to the ultimate temperature, –3.0°C. Turtles were held frozen at –3.0°C for 36 h (total time frozen 72 h) and then transferred to an ice bath where they slowly thawed. They were removed from their tubes 24 h later, placed on a moist paper towel inside individual plastic cups, and held in darkness at 4°C for 7 days. Our experimental freeze/thaw regimen was intended to be sufficiently rigorous as to distinguish between freeze-tolerant and freeze-intolerant species whilst also simulating actual thermal conditions experienced by terrestrial hibernators (Costanzo et al. 1995b, 2004; Baker et al. 2003, 2006).

Viability assessments

Turtles were considered to have survived freezing if they exhibited normal neurobehavioral reflexes during the 7-day, post-thaw period. Each one was examined daily by gently prodding its limbs with a blunt probe. Individuals retracting at least one limb within 2 s of receiving the stimulus were scored as being ‘‘alive’’. Because survival tests often reveal only the extreme consequences of cold exposure (see Sinclair and Roberts 2005), we also evaluated each turtle using a more sensitive index of cryoinjury. Following the final (day 7) examination, turtles were decapitated and blood was drawn into heparinized microcapillary tubes from the severed neck vessels and, in some cases, also from the heart after quickly removing the plastron. Blood was immediately centrifuged (2,000g, 5 min) and the plasma was stored at –20°C. Within 2 days, plasma samples were thawed and assayed (Sigma TOX-7, St. Louis, MO, USA) for the activity of total lactate dehydrogenase (LDH), a cytoplasmic protein whose appearance in the general circulation served to index plasma membrane damage. Baseline LDH activity was measured in plasma collected from control animals (n = 7–8 per species) sampled directly from their boxes.

Morphometrics and somatic characteristics

Euthanized, bled turtles were measured to determine carapace length (to 0.1 mm) and body mass (to 0.01 g), and then rapidly dissected to permit removal of the residual yolk sac, which, along with the remaining carcass, was weighed (to 0.1 mg) and thoroughly dried in a 65°C-oven. The dried components were weighed separately and the original water content of each was determined from the mass lost during drying. We assumed that body water content determined for each species represented the physiologically regulated state because all turtles were kept in a humid environment. It was unnecessary to correct these data for taxonomic variation in relative shell size because concentrations of water in the immature turtle shell and the remaining carcass are similar (Dinkelacker et al. 2005).

Organic osmolytes

Aliquots of the blood plasma collected from frozen/thawed turtles were assayed for glucose (Sigma 510) and lactate (Sigma 735), metabolites that may increase during freezing and/or thawing in some species. Plasma from controls was assayed for these metabolites, as well as urea (Sigma 640), a principal organic osmolyte in cold-acclimated hatchlings (Costanzo et al. 2000).

When sufficient plasma was available, we measured osmolality on 7 μl plasma samples using a vapor pressure osmometer (Wescor 5520, Logan, UT, USA) and NaCl standards.

Statistical inferences

Means ± SE are presented except as otherwise noted. Generally, data were compared using analysis of variance (ANOVA) followed by Tukey's HSD multiple contrast. LDH data were not normally distributed and thus were compared using nonparametric tests. Spearman rank correlations were used to test associations between indices of freezing tolerance (survival rates for each species or individual values of plasma LDH activity) and selected morphometric/physiological variables. Percentage data were transformed (arcsine of square-root) before analysis. Significance was accepted at $P \leq 0.05$.

Results

Freezing survival and cryoinjury

Our method for controlling ice nucleation in experimental freezing trials was highly effective, as 71 of the 72 hatchlings representing nine taxa froze near –0.6°C, the approximate equilibrium freezing/melting point of body fluids. One *C. p. marginata* remained unfrozen after cooling to –1.2°C and was eliminated from the study.

Survival rates and dynamics of recovery from freezing/thawing are summarized in Table 1. None of the hatchlings of two species, *S. odoratus* and *G. geographica*, and only one *T. scripta*, exhibited reflexive responses or spontaneous movement after thawing.

Table 1 Survival and dynamics of recovery from experimental freezing in hatchling turtles

Taxon	Number of turtles tested	Number of turtles in sample meeting viability criteria after		
		1 day	3 days	7 days
S. odoratus	7	0	0	0
G. geographica	8	0	0	0
T. scripta	8	0	0	1
C. serpentina	8	2	2	4
E. blandingii	8	4	6	6
C. p. bellii	8	4	6	6
C. p. marginata	8	4	4	6
M. terrapin	8	4	8	7
T. ornata	8	8	8	8

Four of the eight *C. serpentina* ultimately met the survival criterion, albeit two required the full recovery period. In the remaining taxa, most or all individuals survived freezing/thawing, usually exhibiting normal neurobehavioral functions by the third day after thawing (Table 1).

LDH activity in plasma of control animals generally was low but varied significantly (Kruskal Wallis, $P = 0.019$) among taxa; median values ranged from 0.9 IU ml^{-1} in *T. ornata* to 9.5 IU ml^{-1} in *C. p. bellii*. Enzyme activity in plasma of frozen/thawed turtles was up to 33-fold higher (Mann–Whitney *U* test, $P < 0.006$ for all taxa) than in control counterparts and varied (Kruskal Wallis, $P < 0.0001$) markedly among species. Median values, which ranged from 5.9 IU ml^{-1} in *T. ornata* to 247.0 IU ml^{-1} in *S. odoratus*, generally were higher in taxa that poorly tolerated freezing (Fig. 1). Within species, individuals failing to meet the recovery criterion accumulated more LDH than ones that recovered (Fig. 2). However, this distinction was not prominent in *C. serpentina* and *E. blandingii*, which had exceptionally low levels of LDH activity.

Somatic correlates of freeze tolerance

Carapace length varied significantly among species ($P < 0.0001$), ranging from 22.4 ± 0.4 mm in *S. odoratus* to 33.9 ± 0.4 mm in *E. blandingii*. In addition, body mass varied by a factor of ~3.5 and body water

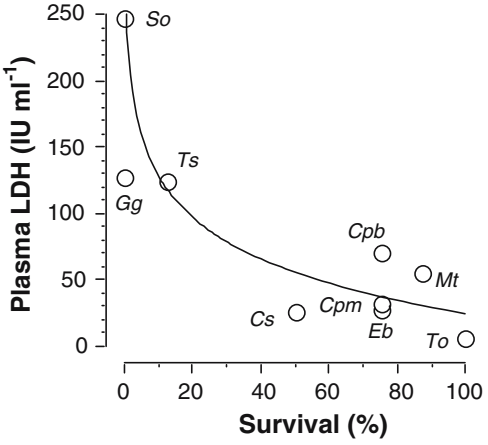

Fig. 1 Relationship between cryoinjury, as indexed by plasma LDH activity, and viability after experimental freezing/thawing in hatchlings representing nine taxa of turtles: So, *Sternotherus odoratus*; Gg, *Graptemys geographica*; Ts, *Trachemys scripta*; Cs, *Chelydra serpentina*; Eb, *Emydoidea blandingii*; Cpb, *Chrysemys picta bellii;* Cpm, *Chrysemys p. marginata*; Mt, *Malaclemys terrapin*; To, *Terrapene ornata*. Coordinates are median LDH activity (n = 5–8 animals per group) and percent survival (n = 7–8 animals per group). *Line* represents the logarithmic equation, $y = -104.0\ \mathrm{LOG}(x) + 232.4$ ($r^2 = 0.665$)

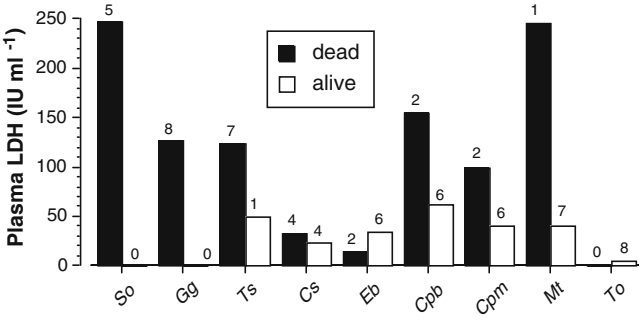

Fig. 2 Cryoinjury as indexed by plasma LDH activity in hatchling turtles (taxa indicated as in Fig. 1) that succumbed to or survived experimental freezing/thawing. *Bars* represent median (or single) values; the number of animals in each group is shown above each *bar*

content ranged from 74.9 ± 0.5% in *M. terrapin* to 81.2 ± 0.6% in *C. serpentina* (Table 2). However, less than 2% of the carcass was comprised of residual yolk, the mass of which varied little (Table 2).

Tolerance to experimental freezing, represented by the percent survival value for each taxon, was not correlated with the corresponding mean value of body mass ($P = 0.22$), carcass dry mass ($P = 0.11$), carapace length ($P = 0.41$), water content ($P = 0.37$), or residual yolk ($P = 0.75$). However, pooling data for all frozen/thawed turtles showed that LDH leakage, a more sensitive indicator of cryoinjury, increased with decreasing body mass ($\rho = -0.62$, $P < 0.0001$), carcass dry mass ($\rho = -0.46$, $P < 0.001$), and carapace length ($\rho = -0.52$, $P < 0.0001$); thus, smaller turtles incurred greater damage. We found a marginally significant ($\rho = -0.27$, $P = 0.044$), inverse correlation between plasma LDH and body water content. Residual yolk mass was not measured in frozen/thawed turtles, so we could not determine its relationship to cryoinjury.

Organic osmolytes

Plasma glucose levels in control turtles were low and typical of unstressed, cold-acclimated hatchling turtles (Table 3; Baker et al. 2003; Dinkelacker et al. 2005). Mean values for species that fared poorly in freeze-tolerance trials (*G. geographica*, *T. scripta*, and *C. serpentina*) ranged 1.3–2.2 mmol l^{-1} and were nominally lower than means (range 2.7–5.8 mmol l^{-1}) for the more freeze-tolerant species. All species accumulated glucose with freezing and, in some taxa, mean plasma concentrations exceeded 40 mmol l^{-1}, increasing 5- to 20-fold over corresponding controls. Relatively minor accumulations were found in *C. serpentina* and *E. blandingii*.

Plasma lactate concentrations in control animals were low (range of mean values 0.6–3.2 mmol l^{-1}; Table 3). In contrast, lactate levels in frozen/thawed turtles were ~10- to 30-fold higher and mean concentrations in some species exceeded 50 mmol l^{-1}. The species that best tolerated freezing, *T. ornata*, accumulated the least lactate.

Glucose and lactate accumulating in frozen/thawed turtles jointly raised the plasma solute concentration by an average of 56 mmol l^{-1} (30–100 mmol l^{-1} in individual taxa; Table 3). This rise closely matched the measured increment in plasma osmolality, 59 mosmol kg^{-1}, indicating that no other major osmolyte was accumulated with freezing. Osmolality values for individual frozen/thawed turtles were strongly correlated ($\rho = 0.51$, $P < 0.0001$) with plasma lactate concentration, but only weakly correlated ($P = 0.063$) with glucose; therefore, lactate accumulation had the greater effect on the osmotic increase.

Plasma of control turtles contained substantial amounts of urea (30–50 mmol l^{-1} in most taxa; Table 4).

Table 2 Taxonomic variation in somatic characteristics of hatchling turtles

	Body mass (g)	Body water (% fresh mass)	Residual yolk (% dry carcass mass)
S. odoratus	2.6 ± 0.1^{a} (7)	76.9 ± 0.8^{abd} (7)	– (0)
G. geographica	5.7 ± 0.1^{b} (18)	76.2 ± 0.7^{ad} (11)	0.90 ± 0.27^{ab} (5)
T. scripta	6.7 ± 0.2^{c} (15)	77.0 ± 0.7^{abd} (13)	0.70 ± 0.18^{ab} (6)
C. serpentina	8.4 ± 0.2^{d} (16)	81.2 ± 0.6^{c} (14)	0.63 ± 0.30^{ab} (8)
E. blandingii	6.8 ± 0.2^{c} (15)	78.7 ± 0.6^{ab} (13)	1.06 ± 0.93^{ab} (6)
C. p. bellii	3.6 ± 0.2^{ae} (17)	78.2 ± 0.4^{ab} (15)	1.62 ± 0.43^{ab} (9)
C. p. marginata	4.3 ± 0.1^{e} (17)	77.3 ± 0.6^{abd} (15)	0.28 ± 0.10^{a} (9)
M. terrapin	6.3 ± 0.2^{bc} (14)	74.9 ± 0.5^{d} (15)	2.23 ± 0.91^{b} (7)
T. ornata	9.0 ± 0.4^{d} (17)	78.9 ± 0.6^{b} (17)	0.48 ± 0.14^{ab} (9)

Means ± SE (*n*)

Data for control and frozen/thawed animals were pooled, except for residual yolk, which was measured for control animals only

Within each column, means with different superscripted letters were statistically distinguishable ($P < 0.05$; ANOVA/Tukey–Kramer)

Table 3 Changes in organic osmolyte concentrations and plasma osmolality with freezing/thawing in hatchling turtles

Taxon	Glucose (mmol l^{-1})		Lactate (mmol l^{-1})		Osmolality (mosmol kg^{-1})	
	Control	Frozen/thawed	Control	Frozen/thawed	Control	Frozen/thawed
S. odoratus	– (0)	25.7 ± 8.9 (5)	– (0)	52.3 ± 2.3 (5)	– (0)	347 ± 36 (2)
G. geographica	2.2 ± 0.1 (10)	44.9 ± 5.0* (8)	1.6 ± 0.3 (10)	57.5 ± 2.3* (8)	320 ± 4 (10)	393 ± 15* (6)
T. scripta	2.2 ± 0.2 (7)	43.5 ± 4.0* (8)	3.2 ± 1.3 (7)	56.4 ± 4.5* (7)	319 ± 7 (7)	384 ± 17* (7)
C. serpentina	1.3 ± 0.3 (8)	5.2 ± 1.1* (8)	1.1 ± 0.2 (8)	26.6 ± 2.7* (8)	307 ± 23 (8)	368 ± 46 (8)
E. blandingii	3.5 ± 1.2 (7)	15.9 ± 2.7* (8)	2.0 ± 0.5 (7)	43.4 ± 2.2* (8)	307 ± 10 (6)	327 ± 17 (8)
C. p. bellii	3.8 ± 0.7 (8)	46.7 ± 5.7* (8)	3.1 ± 0.7 (8)	54.4 ± 4.6* (8)	336 ± 9 (7)	413 ± 13* (6)
C. p. marginata	2.7 ± 0.5 (8)	42.2 ± 6.1* (8)	2.0 ± 0.3 (8)	44.3 ± 7.9* (8)	338 ± 9 (8)	410 ± 16* (7)
M. terrapin	4.0 ± 0.5 (7)	30.8 ± 4.7* (8)	0.6 ± 0.1 (7)	42.1 ± 7.3* (8)	368 ± 13 (7)	416 ± 19* (7)
T. ornata	5.8 ± 0.4 (5)	46.5 ± 3.1* (8)	1.0 ± 0.1 (5)	11.5 ± 1.5* (8)	250 ± 4 (5)	306 ± 10* (8)

Means ± SE (*n*)

*Indicates values for frozen/thawed animals differing significantly ($P < 0.05$; Student's *t* test) from values for corresponding controls

Uremia was strongly correlated ($P < 0.0001$) with osmolality and accounted for ~10% of the total plasma osmotic potential. Frozen/thawed turtles were not assayed for urea; however, it is unlikely that urea is synthesized or excreted during freezing and therefore these animals probably contained as much urea as the unfrozen controls (see Costanzo and Lee 2005). Thus, in frozen/thawed turtles, glucose, lactate, and urea collectively accounted for 19–35% of the total plasma osmotic pressure and, in some taxa, urea represented over one-half of the pool of major organic osmolytes (Table 4).

We found no correlation ($P = 0.56$) between freezing survival rate and mean glucose level in frozen/thawed turtles. However, taxa having lower prefreeze levels of glucose fared worse ($\rho = 0.90$, $P = 0.018$) in freeze tolerance trials. We also found lower survival rates among taxa that accumulated relatively large amounts of lactate with freezing/thawing ($\rho = 0.71$, $P = 0.046$); accordingly, among individual turtles lactemia was positively correlated ($\rho = 0.70$, $P < 0.0001$) with LDH leakage. In frozen/thawed turtles, LDH leakage also increased with increasing levels of plasma glucose ($\rho = 0.32$, $P = 0.008$) and osmolality ($\rho = 0.34$, $P = 0.001$).

Post-hoc analyses of data pooled across taxa revealed some noteworthy associations among certain morphological and physiological characteristics. For example, body size, as represented by dry carcass mass, was inversely related to lactemia in both control ($\rho = -0.42$, $P = 0.004$) and frozen/thawed ($\rho = -0.38$, $P = 0.005$) turtles. Turtles having relatively low concentrations of water in tissues tended to have higher plasma osmolality (control $\rho = -0.41$, $P = 0.005$; frozen/thawed $\rho = -0.35$, $P = 0.017$) and, after freezing/thawing, higher levels of plasma lactate ($\rho = -0.29$, $P = 0.035$) and glucose ($\rho = -0.51$, $P < 0.001$).

Table 4 Significance of urea as an osmolyte in (unfrozen) control and frozen/thawed hatchling turtles

Taxon	Plasma urea (mmol l^{-1}), Control	Urea's contribution to major organic osmolyte pool (%)[a]		Organic osmolytes, contribution to total osmotic activity (%)[b]	
		Control	Frozen/thawed	Control	Frozen/thawed
G. geographica	34.0 ± 2.2 (10)	89.9	24.9	11.8	34.7
T. scripta	31.6 ± 2.6 (7)	85.4	24.0	11.6	34.2
C. serpentina	38.7 ± 12.7 (8)	94.2	54.9	13.4	19.2
E. blandingii	19.2 ± 7.2 (6)	77.7	24.5	8.0	24.0
C. p. bellii	40.3 ± 6.2 (7)	85.4	28.5	14.0	34.2
C. p. marginata	46.0 ± 5.4 (8)	90.7	34.7	15.0	32.3
M. terrapin	52.7 ± 4.7 (7)	92.0	42.0	15.6	30.2
T. ornata	8.2 ± 2.8 (5)	54.7	12.4	6.0	21.6

For plasma urea, means ± SE (*n*); other values are group averages

Urea levels were measured only in controls but were assumed to be similar in frozen/thawed animals (see text)

[a]Plasma urea concentration (in mmol l^{-1}) divided by the sum of the concentrations of urea, glucose, and lactate

[b]Sum of the concentrations (in mmol l^{-1}) of urea, glucose, and lactate divided by the measured plasma osmolality

Discussion

Given that freeze-tolerant organisms possess mechanisms that minimize and efficiently repair freeze/thaw damage (Storey 2006), variation in extent of cryoinjury and recovery rate should indicate the degree to which various species have adapted to these stresses. Nervous tissues are particularly sensitive to freezing stress (Cameron 1930) and, accordingly, among reptiles cryoinjury manifested as neurobehavioral dysfunction (e.g., lethargy, impassiveness to tactile stimulation, absence of the righting response) varies with severity of the freezing exposure (Costanzo et al. 1995a; Burke et al. 2002; Dinkelacker et al. 2003). Because our experimental protocol caused all turtles to slowly freeze and thaw under uniform thermal conditions, interspecific variation in survival rates and indices of cryoinjury probably reflects inherent capacities to cope with freezing/thawing stress. Outcomes of our freezing trials ranged from an absence of vital signs (*S. odoratus*, *G. geographica*) to a rapid restoration of neurobehavioral functions in all individuals (*T. ornata*). In many instances, viability was not evident until several days after thawing (up to 1 week in some *C. serpentina*; Table 1), perhaps because the nervous system is particularly susceptible to freeze/thaw stress and requires more time for recovery. Assessing viability too soon after thawing (i.e., within 24 h; Packard and Packard 1993, 2003; Packard et al. 1999) could lead investigators to erroneous conclusions about the capacity to tolerate somatic freezing.

Our present results confirm that freezing survival in hatchling turtles varies taxonomically (Costanzo et al. 1995b; Dinkelacker et al. 2005) and is not a universal trait among chelonians, as has been suggested (Packard et al. 1999). Except for *S. odoratus* (Kinosternidae) and *C. serpentina* (Chelydridae), the taxa we studied belong to the Emydidae; thus, our results can offer few insights into the phylogeny of freeze tolerance. Dinkelacker et al. (2005) observed that capacity for freeze tolerance associated with hibernaculum choice and freezing risk in hatchling turtles. Similarly, in the present study, freezing survival was higher in species known (*C. picta*, *M. terrapin*, and *T. ornata*) or suspected (*E. blandingii*) to hibernate where frost occurs as compared to species that have a limited northern distribution (*T. scripta*), a high resistance to inoculative freezing under natural environmental conditions (*G. geographica*; see Baker et al. 2003), and a habit of overwintering under water (*S. odoratus* and *C. serpentina*). The implication of this relationship (see Sinclair et al. 2004) is that, among northern turtles, capacity to tolerate tissue freezing has been improved through natural selection for winter survival.

We used plasma LDH activity as a marker of cryoinjury because freezing can perturb membrane composition and fluidity, permitting leakage of cytosolic contents into the extracellular space. The LDH leakage assay is widely used to quantify in vitro damage to cells and organs subjected to experimental stress, including hypothermia and freezing (e.g., Storey and Mommsen 1994; Costanzo and Lee 2005), but to our knowledge was not heretofore used in intact animals. Circulating levels of this enzyme presumably reflect interplay among its production and clearance rates, cell membrane permeability, and vascular patency, and, because the turtles necessarily were sampled late in the recovery period, we do not know whether plasma levels of this enzyme (and other metabolites) varied during freezing exposure or thawing. Nevertheless, plasma LDH activity correlated with increased freezing mortality (Fig. 1), attesting that this metric is a reasonably good index of cryoinjury. Significant LDH leakage occurred even in surviving turtles; hence, freezing/thawing can cause extensive, albeit survivable, cellular damage. In some frozen/thawed animals, hemolysis contributed to the LDH measured in plasma. However, elevated LDH was also found in non-hemolyzed samples, attesting that LDH leaked from cells in addition to erythrocytes. Generally, LDH leakage in the surviving turtles was less extensive than in the individuals that succumbed (Fig. 2). Apparently, this was not the case with *C. serpentina* and *E. blandingii*, perhaps because cryoinjury involved relatively few organs, organs that contained little LDH, or tissues in which perfusion had ceased. These anomalies notwithstanding, our results indicate that protein leakage assays may provide a quantitative index for evaluating tolerance to freeze/thaw stress.

Somatic correlates of freeze tolerance

Relationships between life-history traits and cold-hardiness strategies of ectothermic animals have received scant attention in the literature. However, in principle, certain morphological attributes can influence whether freeze avoidance or freeze tolerance is adopted. For example, ice-nucleation theory predicts that supercooling is enhanced in small organisms and this pattern is found both within and among diverse taxa (Costanzo and Lee 1995; Lee and Costanzo 1998). By inference, freeze tolerance ought to be the more prominent strategy among relatively large organisms that routinely face subzero cold. Obviously, having a large body is advantageous if, by cooling relatively slowly, ice growth is controlled and less body water freezes before rewarming. However, this effect was not a fac-

tor in the present study because all turtles were uniformly cooled under conditions conducive to attaining an equilibrium ice content (Churchill and Storey 1992). Our finding that LDH leakage was less severe among larger hatchlings (whether represented by body mass, dry carcass mass, or carapace length) supports the contention that larger species are predisposed to evolving freeze tolerance and further suggests that body size is a heritable trait upon which natural selection can operate in the evolution of cold hardiness.

Hydration levels varied markedly among the turtle taxa we studied. Because a lower hydration state limits the absolute quantity of ice forming (all else being equal) and, thus, the potential for mechanical damage to the microvasculature, we anticipated that freeze-tolerance would be better developed in species that maintained lower tissue water contents. However, we found no association between body water content and freezing survival rate among turtle taxa and, to the contrary, individuals that maintained lower hydration levels had higher plasma LDH activity, perhaps because they incurred more cryoinjury.

Hatchlings of species that commonly overwinter on land tend to have larger supplies of yolk and storage lipids than species whose hatchlings hibernate aquatically (e.g., Rowe et al. 1995; Nagle et al. 1998). Not only is residual yolk important to the energetics of aphagic, overwintering hatchlings, but it also might contribute to their cold hardiness (Bleakney 1963). Freezing increases the vascularity and shrinks the internalized yolk sac, presumably as yolk materials are mobilized to other tissues (Hemmings and Storey 2000). In the present study, we found no association between residual yolk mass and capacity for freeze tolerance, although this result may not be definitive because little yolk remained when the turtles were examined.

Physiological responses to freezing/thawing

The crystallization of extracellular water can devastate the structural and functional integrity of biological tissues. In order to survive even a single, mild freezing episode, freeze-tolerant organisms must withstand myriad stresses simultaneously manifested at multiple levels of biological organization. These stresses derive from the formation and melting of ice in extracellular compartments, osmotic withdrawal of water from cells, and attendant osmotic and ionotropic effects on membranes, macromolecules, and cell homeostasis (Storey and Storey 1988; Mazur 2004). Freeze-tolerant organisms employ mechanisms that not only minimize this upheaval, but also aid in post-thaw recovery by repairing cells and tissues, restoring energy balance, and contending with a profusion of reactive oxygen species.

Our premise in this project was that comparing physiological responses to freezing/thawing in freeze-tolerant and freeze-intolerant species may shed new light on the mechanistic basis for freeze tolerance in reptiles. We were particularly interested in the dynamics of organic osmolytes, which are known to play a central role in freezing adaptation in diverse organisms (Storey and Storey 1988; Yancey 2005). Cryoprotectants colligatively reduce the amount of body water that freezes and, if taken up by cells, also reduce cellular dehydration. Additionally, they help preserve the integrity of macromolecules, membranes, and other cellular structures, and can fuel metabolism in frozen and/or recently-thawed tissues. Because our turtles were sampled during the recovery period, osmolyte levels measured in these animals do not necessarily match concentrations present during freezing or thawing. On the other hand, by deferring the sampling we were able to associate osmolyte levels with the viability status of individuals. Using this design also reduced the likelihood of overlooking post-thaw responses aiding recovery processes.

Freeze-tolerant anurans are distinguished from freeze-intolerant ones in part by their capacity to rapidly accumulate potentially large amounts of glucose and/or glycerol after freezing commences (Storey and Storey 1988). The case with freeze-tolerant turtles apparently differs because glucose levels rise only modestly (Churchill and Storey 1992) or not at all (Hemmings and Storey 2000), and glycerol is virtually undetectable (Storey et al. 1988; Churchill and Storey 1992; Costanzo et al. 2000, 2004). In the present study, we found blood glucose levels in frozen/thawed turtles generally 5- to 20-fold higher than in unfrozen controls. Our finding that glucose accumulated in freeze-intolerant species as well as freeze-tolerant ones suggests that the mobilization is a fundamental response to stress, rather than an adaptation specific to freeze tolerance.

Lactate rapidly accumulates to high levels in the blood and organs of freezing-exposed hatchling turtles (Storey et al. 1988; Churchill and Storey 1991, 1992; Dinkelacker et al. 2003, 2005; Packard and Packard 2004; Table 3), probably because, as compared to adults, their buffering capacity is poorly developed (Reese et al. 2004; Dinkelacker et al. 2005). The early initiation and rapidity of this response suggested to some authors (Churchill and Storey 1991; Hemmings and Storey 2000) that the rise, at least initially, is not

triggered by ischemic hypoxia, but rather is protective (Loomis et al. 1989). We cannot discern the time course or proximal cause for lactate accumulation in our turtles, but finding that lactate increased with freezing exposure in all taxa suggests the response is not a specific adaptation to freezing survival.

Although lactate accumulation invariably occurs with experimental freezing, a recent study (Costanzo et al. 2004) showed that blood lactate levels in hatchling *C. p. marginata* and *C. p. bellii* hibernating in natural nests remained low (2–6 mmol l^{-1}) throughout winter, even though some of the animals had frozen. One possible explanation for this discrepancy is that freezing-exposed turtles had metabolized the lactate load before their blood was sampled. However, this scenario seems doubtful because blood lactate remains elevated for at least 7 days following thawing (Table 2) and Costanzo et al. (2004) analyzed their samples, including recently frozen turtles, no more than 24–48 h after they were collected. Resolution of this disparity will require additional study of hatchlings overwintering in nature.

Investigation of the putative importance of cryoprotectants in reptilian freeze tolerance has focused mainly on glucose and lactate, although in sufficient quantity any permeable solute will colligatively moderate ice formation and limit cell shrinkage. Investigators have screened the blood and other tissues of freezing-exposed turtles for amino acids (Storey et al. 1988; Churchill and Storey 1992; Costanzo et al. 2000), sugars such as sorbitol, fructose, and mannose (Churchill and Storey 1992; Costanzo et al. 2000), and the metabolic end products, alanine and succinate (Churchill and Storey 1991), but found these compounds present only in minute amounts. In our study, glucose and lactate synthesis jointly increased plasma solute concentration by a margin closely approximating the measured increment in osmolality (up to 77 mosmol kg^{-1} in *C. p. bellii*), indicating that no other major osmolyte was mobilized with freezing.

Osmolytes accumulated preparatory to winter promote cold hardiness in many invertebrates (Lee 1991) and in at least some anurans (Layne and Jones 2001; Costanzo and Lee 2005), but the question of whether or not reptiles exhibit an anticipatory response has received little study. Costanzo et al. (2000) reported that seasonal development of cold hardiness in hatchling *C. p. bellii* was associated with an osmotic increase caused primarily by retention of urea (to 80 mmol l^{-1} blood plasma), the principle nitrogenous waste product in most chelonians. High levels of blood urea were found in *C. p. bellii* and several other taxa (albeit not *T. ornata*) in the present study. These turtles do not necessarily remain hyperuremic all winter (Costanzo et al. 2004), but those that do stand to benefit from this solute's cryoprotective properties (Costanzo and Lee 2005). Indeed, urea accumulation could underlie the observed increase in freeze tolerance associated with winter acclimatization (Churchill and Storey 1992; Packard and Packard 2003; Costanzo et al. 2004).

Significance of the pool of organic osmolytes in freezing survival is underscored by our finding that these solutes collectively accounted for up to one-third of the total osmotic activity in frozen/thawed hatchlings (Table 4). Lacking them, substantially more ice would form, leading to greater mechanical stress and cellular dehydration. Turtles would sooner, and at higher temperatures, attain critical ice contents, which, for many freeze-tolerant animals, coincides with the freezing of ~66% of the body water (Storey and Storey 1988). For example, estimates of the percentage of body water freezing at –3°C, based on values of plasma osmolality for our frozen/thawed hatchlings (Table 2) and assuming 20% of body water is osmotically inactive (Eq. 2 in Claussen and Costanzo 1990), ranged from 54.2 ± 1.2% (*M. terrapin*) to 61.1 ± 0.6% (*T. ornata*). Lacking glucose, lactate and urea, plasma osmolality would be ~29% lower and ice content at –3°C would reach 62–65%, very near the critical level. Thus, these osmolytes may play an important cryoprotective role in freeze-tolerant species.

An essential quality of any cryoprotective agent, and that of cytoprotectants in general, is compatibility with cellular processes (Storey and Storey 1988; Mazur 2004; Yancey 2005). Glucose and urea probably are not perturbing at concentrations occurring in hatchling turtles (see Costanzo and Lee 2005), but the concept of lactate as a putative cryoprotectant requires special consideration. Packard and Packard (2004) suggested that freezing mortality in hatchling turtles results from excessive lactate accumulation, failure of the buffering system, and the attendant acid–base disturbance. Whether or not elevated lactate constrains freezing survival is unknown, although Dinkelacker et al. (2005), who studied hatchlings of seven turtle species, found no clear relationship between freeze-tolerance capacity and blood lactate levels in frozen/thawed animals. To the contrary, in the present study, species with relatively high blood lactate levels after freezing/thawing had lower freezing survival rates and, among all individuals, lactemia was strongly correlated with LDH leakage. Disparity between our results and the findings of Dinkelacker et al. (2005) probably derives from the comparatively colder and longer freezing exposure given our animals, as well as their twofold higher lactate concentrations. Unfortunately, we can-

not discern whether the observed association between elevated lactate and freezing injury simply reflects differential lactate clearances among injured and healthy turtles, or whether it stems from adverse effects of the osmolyte (e.g., acidification) or the stress (e.g., hypoxia) triggering its accumulation. If the latter case is true, then freezing survival would be promoted in species that better tolerate or mitigate elevated lactate, or else forestall its accumulation by virtue of a lower metabolism and/or larger endogenous oxygen reserves. Considering that oxygen limitation is a principle determinant of thermal tolerance in ectothermic vertebrates (Pörtner 2002), and that tissue ischemia underlies some of the gene-expression responses to freezing (Storey 2006), anoxia tolerance probably is an important exaptation in natural freeze tolerance.

Perspective

Our results confirm that hatchlings of some, but not all, northern turtle species can survive the freezing of their body fluids. Certain life history traits, such as body size, may predispose a given species to evolving freeze tolerance as a cold-hardiness strategy. Hatchlings of freeze-tolerant turtles can recover from freezing/thawing without the need to accumulate large quantities of colligative cryoprotectants. This also is the case with other freeze-tolerant reptiles, including certain adult turtles, snakes, and lizards, and may be a characteristic of the class (Storey 2006). Modest levels of organic osmolytes, accumulated before and during freezing/thawing, probably enhance survival in species that have evolved freeze tolerance as a cold-hardiness strategy, but species that succumb to freezing also can accumulate these compounds, attesting that natural freeze tolerance is a complex, multi-faceted adaptation deriving from a coordinated suite of protective responses. Additional clues to the physiological basis underpinning reptilian freeze tolerance might be garnered by addressing the possible roles of bound water, innate dehydration tolerance, metabolic depression, and antioxidant defenses.

Acknowledgments We thank P. Myer and the Connealy and Davis families for granting us permission to collect turtles on their lands. We also thank S. Dinkelacker for providing some of the hatchlings, and M. Elnitsky and T. Muir for commenting on the manuscript. Collecting permits were obtained from the Nebraska Game and Parks Commission, Indiana Department of Natural Resources, and New Jersey Division of Fish and Wildlife. Experimental procedures were approved by the Animal Care and Use Committee of Miami University. This work was funded by grants from the National Science Foundation (IBN 98017087 and IOB 0416750) to JPC and by the Miami University Summer Workshop (PJB).

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