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RESPONSES TO FREEZING EXPOSURE OF HATCHLING TURTLES *TRACHEMYS SCRIPTA ELEGANS*: FACTORS INFLUENCING THE DEVELOPMENT OF FREEZE TOLERANCE BY REPTILES

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Summary

Hatchling red-eared turtles *Trachemys* (=*Pseudemys*) *scripta elegans* (Wied) from a Louisiana population display a significant ability to withstand the freezing of extracellular body fluids. All animals survived at least 2 h of freezing at −2.5 or −4°C. At −2.5°C, survival declined to 50% after 6 h of freezing and no animals recovered after 24 h or longer, when mean ice content reached 54.7±1.4% of total body water. At −4°C, all turtles recovered from 4 h of freezing exposure with a mean ice content of 49.6±2.4%, but survival dropped sharply thereafter with no animals recovering after 8 h, when ice content had reached 64.5±0.7%. Survival times were substantially shorter and percentage ice values greater than comparable values for hatchling painted turtles (*Chrysemys picta* (Schneider)) from northern populations subjected to identical freezing exposures. The ability to synthesize cryoprotectants in response to freezing was poorly developed in *T. s. elegans*; maximal accumulation of glucose was only 3.2 μmol g^{-1} wet mass in liver. Lactate content increased two- to threefold in oxygen-sensitive organs (heart and brain) during freezing, but levels of lactate and other putative cryoprotectants were unchanged in other organs. Total free amino acid content rose significantly in liver, muscle and blood during freezing; increased taurine concentration was primarily responsible for the changes in liver and blood. The capacity for freezing survival by *T. s. elegans* hatchlings from southern populations would be of limited use for hibernation in a cold climate, but the metabolic responses to freezing displayed by these animals might be enhanced by northern populations to increase their freeze tolerance.

Introduction

The ability to endure the freezing of extracellular body fluids is an important component of winter cold-hardiness for many ectothermic animals. Freeze

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tolerance has been reported for numerous species of insects, some other invertebrates, and also for several terrestrially hibernating amphibians and reptiles (Storey and Storey, 1988; Storey, 1990). Among vertebrates, the species with well-developed natural freeze tolerance include several frogs [*Rana sylvatica*, *Hyla versicolor*, *H. chrysoscelis*, *Pseudacris triseriata*, *Pseudacris* (formerly *Hyla*) *crucifer*], the Siberian salamander (*Hynobius keyserlingi*), box turtles (*Terrapene carolina*) and hatchlings of the painted turtle (*Chrysemys picta*) (Schmid, 1982; Berman *et al.* 1984; Storey and Storey, 1984, 1985, 1986; Storey *et al.* 1988; Costanzo and Claussen, 1990; Costanzo *et al.* 1990; Churchill and Storey, 1992*a*; K. B. Storey, J. R. Layne, M. M. Cutwa, T. A. Churchill and J. M. Storey, in preparation). All of these species tolerate high amounts of body ice (> 50 % of total body water) and long durations of freezing (days or weeks) at temperatures sometimes encountered in their hibernacula (lows may reach −6 to −8°C). Much more limited freezing survival, which probably has little or no physiological relevance for long-term hibernation, has also been reported for various aquatic frogs (Duméril, 1849; Cameron and Brownlee, 1913; Holmes, 1927), wall lizards (Weigmann, 1929; Claussen *et al.* 1990) and garter snakes (Costanzo *et al.* 1988; Churchill and Storey, 1992*b*). For example, garter snakes can recover after very brief or very mild freezing exposures that keep the percentage of body ice low, but their primary hibernation strategy is to avoid freezing exposure by hibernating in thermally buffered dens under water or deep underground (Macartney *et al.* 1989; Costanzo, 1989).

Previous studies show considerable variation and gradation in the ability of different vertebrate species to endure freezing. Some have perfected the strategy to the point where it is an integral part of natural hibernation, others can tolerate short freezing exposures, and many are killed by even the briefest freezing exposures. To help to determine which physiological and biochemical traits are important for freezing survival by reptiles, we chose to examine the freezing abilities of a turtle species that has little or no natural need for freeze tolerance and yet is closely related to another species with highly developed freeze tolerance.

Pond turtles of the *Chrysemys* and *Trachemys* (=*Pseudemys*) genera are widely distributed throughout North America. Adults hibernate under water and have extremely well-developed anoxia tolerance, making them the premier facultative anaerobes among vertebrates (Ultsch, 1985). Hatchlings of both share the strategy of delayed emergence from the nest; when eggs hatch late in the season, the young remain within the terrestrial nest cavity over the winter months and emerge in the spring when conditions are optimal for rapid juvenile growth (Gibbons and Nelson, 1978). In the north, this strategy has necessitated the development of freeze tolerance by *C. picta* hatchlings, both by the midland (*C. p. marginata*) and by the western (*C. p. bellii*) subspecies (Storey *et al.* 1988; Churchill and Storey, 1992*a*). Both the placement of nests on exposed banks and their shallow depth (less than 10 cm) are such that temperatures within the nest cavities frequently fall to subzero values (Breitenbach *et al.* 1984; Storey *et al.* 1988; Packard *et al.* 1989). In the south, however, hatchlings of the red-eared pond slider *Trachemys scripta*

elegans do not experience subzero temperatures while hibernating in the nest. Our study analyzes the extent of freezing survival by *T. s. elegans* hatchlings from a Louisiana population, comparing and contrasting the freezing limits, the amount of ice accumulated and the biochemical responses to freezing of these animals with the equivalent parameters displayed by *C. picta* hatchlings.

Materials and methods

Animals and chemicals

Hatchling red-eared pond sliders *T. s. elegans* were purchased in September 1989 from a commercial breeder in Louisiana. The animals were placed in plastic boxes containing damp sphagnum moss and were held without feeding in an incubator at 5°C (range±0.5°C) for up to 3 months before use. Mean hatchling mass was 6.84±0.13 g; animals were of indeterminate sex. All biochemicals were purchased from Sigma Chemical Co., St Louis, MO, or Boehringer Mannheim, Montreal, PQ.

Freezing survival and percentage ice

To monitor cooling and freezing of the turtles, individual turtles were placed on a pad of paper towelling, centred over a thermistor that was in contact with the plastron. A band of masking tape was used to secure the turtle and thermistor in place. Thermistors were connected to a YSI telethermometer with output to a linear recorder. Turtles were then placed in an incubator at either −2.5 or −4°C (±0.1°C) and were allowed to cool over 30–40 min until the exotherm resulting from ice nucleation within the body fluids was recorded. The length of freezing was timed from the exotherm, with sampling at intervals up to 3 days at −2.5°C or 8 h at −4°C for analysis of percentage ice and survival. If turtles supercooled to −2.5°C without freezing, the incubator temperature was further lowered to −3.5°C and cooling was continued until an exotherm was recorded. Within 5 min of the appearance of an exotherm, incubator temperature was raised to −2.5°C and maintained at −2.5°C for the duration of the experiment.

After a timed interval of freezing, turtles were thawed in a calorimeter, to determine the percentage of body water as ice, and were then returned to the 5°C incubator for assessment of survival after 24 h. If the turtles were alive after 24 h, survival was again checked after 2 weeks. Calorimetry was performed as outlined by Lee and Lewis (1985) in an insulated vessel containing 30 ml of water at 21°C and using experimentally determined factors: body water content=79.5±0.8 % of total mass, specific heat of the dry mass=0.336±0.019 cal g^{-1} $degree^{-1}$, and F factor for the calorimeter=1.03±0.01. Survival was assessed by several physical responses including breathing, retraction of head or legs when touched, and walking.

Metabolic responses to freezing

Cooling and freezing were carried out as described above but turtles were

sampled over a period of up to 4 h at −4 °C; a survivable stress. After freezing, animals were rapidly removed from the incubator, killed by decapitation, and organs were quickly excised and frozen in liquid nitrogen. Tissues were transferred to −80 °C for storage until processed. Control turtles were treated similarly to the experimental animals, with the thermistor attached and cooling in the incubator, but these animals were removed and killed when plastron surface temperature reached 0 °C.

Perchloric acid extracts of turtle organs were prepared as outlined by Storey and Storey (1984), except that Tris buffer, instead of imidazole, was included in the neutralization solution. Samples of the neutralized extracts were immediately removed and assayed for pyruvate and phosphoenolpyruvate (PEP); the remainder of the extracts was frozen for subsequent use. Metabolite levels were measured using either fluorometric or spectrophotometric coupled enzyme assays (Lowry and Passonneau, 1972; Bergmeyer, 1984) as described previously (Storey and Storey, 1984; Kelly and Storey, 1988). For analysis of free amino acid levels, frozen tissue samples were homogenized in 0.5 % w/v sulphosalicylic acid and then centrifuged at 6000 *g* in an IEC benchtop centrifuge. Amino acids were analyzed in samples of the supernatant using a Waters HPLC after precolumn derivatization with orthophthalaldehyde.

Data and statistics

Data are presented as means±S.E.M. Statistical testing used the two-tailed Student's *t*-test.

Results

When cooled slowly in an incubator with an air temperature of −4 °C, *T. s. elegans* hatchlings showed a moderate ability to supercool. The mean (±S.E.M.) whole-animal supercooling point was −3.2±0.08 °C (N=11). Upon nucleation, plastron surface temperature rebounded to −1.13±0.04 °C.

To test the freeze tolerance of *T. s. elegans* hatchlings, animals were held, after nucleation had occurred, at either −2.5 or −4 °C. Turtles readily survived 2 h of freezing at −2.5 °C with a low ice content, 22.9±2.4 %, of total body water (Fig. 1). However, as body ice content rose above 40 %, survival dropped; 50 % of animals recovered after 6 h of freezing (mean ice content 41.5±4.8 %) but only 25 % survived 12 h of freezing. None of the turtles revived after longer periods of freezing. Maximal ice content in turtles frozen for 3 days at −2.5 °C was 54.7±1.4 % (N=12).

At −4 °C, 100 % of turtles survived up to 4 h of freezing with mean ice contents of 49.6±2.4 % of body water (Fig. 2). However, only 25 % of animals recovered after 5 h of freezing and none revived after 8 h of freezing. Not unexpectedly, ice content was higher in turtles frozen at −4 °C, 64.5±0.7 % after 8 h, than the maximum at −2.5 °C. Fig. 2 also shows that plastron surface temperature remained elevated and fairly constant (−1.3 to −1.4 °C) over the first 2 h of

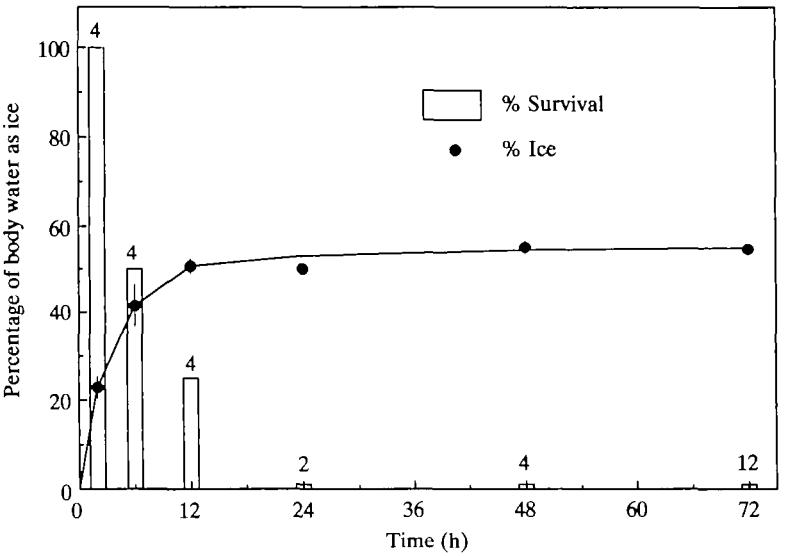

Fig. 1. Freezing survival and percentage of body water as ice for *Trachemys scripta elegans* hatchlings exposed for up to 3 days to −2.5°C. Bars show percentage survival and symbols show mean percentage ice±S.E.M. Values for *N* are indicated above the bars. Where error bars are not shown, these are within the dimensions of the symbol. All turtles supercooled, and the length of freezing exposure was timed from the appearance of an exotherm caused by ice nucleation.

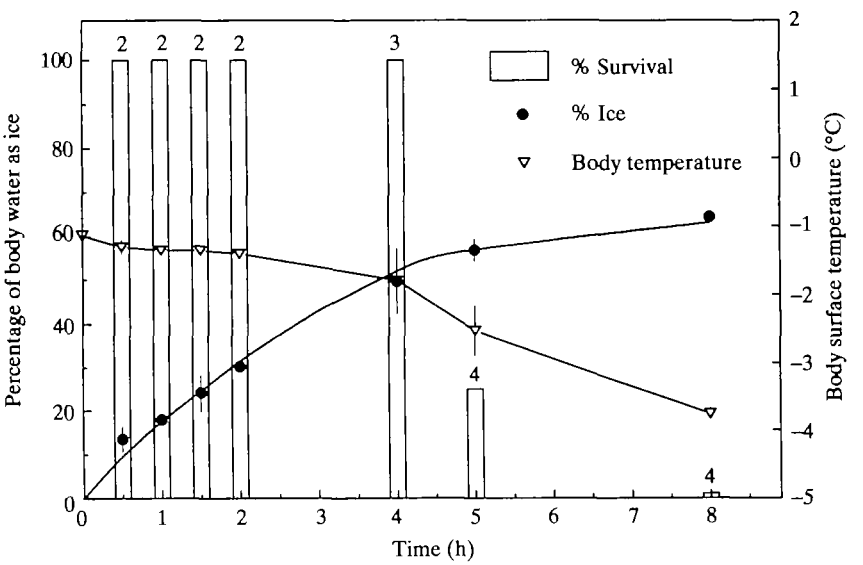

Fig. 2. Freezing survival and percentage of body water as ice for *T. s. elegans* hatchlings exposed for up to 8 h to −4.0°C. Also shown is mean plastron surface temperature±S.E.M., beginning with the rebound temperature recorded immediately after ice nucleation. Other details are as in Fig. 1.

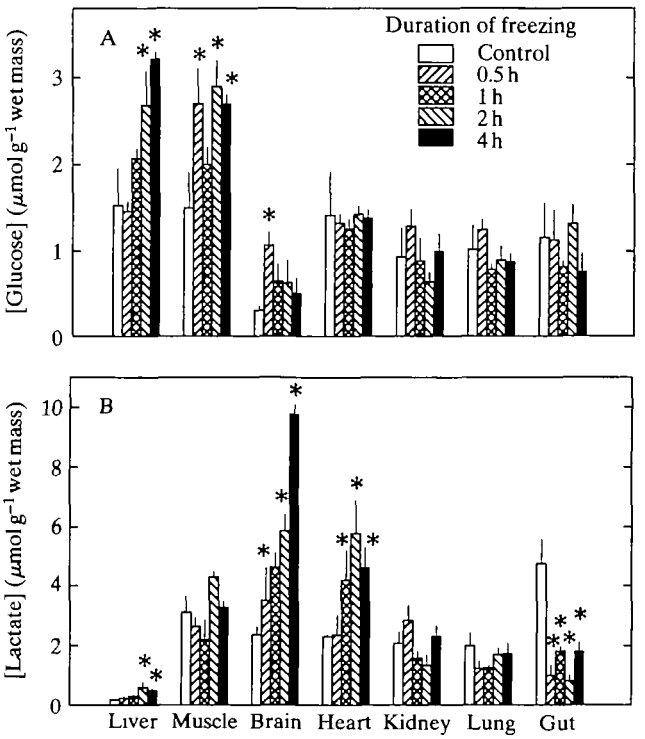

Fig. 3. Effect of the duration of freezing exposure at −4 °C on the levels of glucose (A) and lactate (B) in organs of hatchling *T. s. elegans*. The control turtles were chilled to 0 °C. Gut combines stomach plus intestines. Data are mean±S.E.M., N=3. * Significantly different from the corresponding control value using the Student's t-test, $P<0.05$.

freezing but then dropped steadily towards the ambient temperature as body ice content neared a maximum.

The effect of freezing exposure on metabolite levels in seven organs was assessed in hatchlings exposed to −4 °C for up to 4 h. Levels of glucose in control turtles (chilled only to 0 °C) were low in all organs, less than or equal to 1.5 μmol g^{-1} wet mass (Fig. 3A). During freezing, liver showed a progressive rise in glucose content, culminating in a twofold increase to 3.2 μmol g^{-1} after 4 h. Glucose content in muscle also doubled and glucose rose transiently to 1 μmol g^{-1} in brain after 1 h of freezing. Freezing affected lactate levels in some organs (Fig. 3B). Lactate level increased by two- to threefold in heart and brain over the 4 h of freezing exposure, and rose slightly in liver but no change in lactate content occurred in muscle, kidney or lung. In gut (stomach+intestines combined), lactate content decreased during freezing. Levels of other possible cryoprotectants, glycerol, sorbitol, fructose and mannose, were assessed in liver samples. Glycerol content was 0.36±0.10 μmol g^{-1} in liver of control turtles and 0.67±0.13 μmol g^{-1} (not significantly different) in frozen animals (both N=4).

Sorbitol, fructose and mannose levels were each less than 0.55 μmol g^{-1} and did not change with freezing. Since freezing leads to an ischaemic state in organs, levels of two alternative products of anaerobic metabolism, alanine and succinate, were also assessed in the seven organs. However, there were no significant changes in the level of either compound during freezing exposure. Mean organ alanine content ranged from 0.20±0.02 μmol g^{-1} wet mass in skeletal muscle to 2.80±0.23 μmol g^{-1} in gut, whereas succinate levels ranged from 0.45±0.03 μmol g^{-1} in muscle to 3.17±0.14 μmol g^{-1} in heart (control+freezing-exposed combined, N=15).

Table 1 shows that total amino acid levels rose significantly in *T. s. elegans* liver, muscle and blood after 4 h of freezing at −4°C. In liver, the major peaks were identified as glutamine, valine, taurine and alanine, as also occurs for *C. picta* hatchlings (Storey *et al.* 1988; Churchill and Storey, 1992*a*). The increase in liver amino acid content with freezing was primarily due to a 4.1 μmol g^{-1} rise in taurine. In muscle, aspartate, serine and glycine contents increased during freezing. Levels of eight amino acids increased in *T. s. elegans* blood during freezing, but about 50 % of the total rise in the amino acid pool was due to increased taurine plus alanine concentrations.

Levels of some glycolytic intermediates in liver changed over the course of freezing at −4°C (Fig. 4). Glucose 6-phosphate (G6P) content rose threefold over the first 2 h, suggesting an activation of liver glycogenolysis, but subsequently declined. Fructose 1,6-bisphosphate ($F1,6P_2$) and pyruvate (PYR) contents remained constant over the first 2 h, but then increased sharply at 4 h of freezing exposure. These increases in the products of the two regulatory enzymes of glycolysis, phosphofructokinase and pyruvate kinase, suggested an activation of flux through both loci. Levels of fructose 6-phosphate (F6P), dihydroxyacetone-phosphate (DHAP), glyceraldehyde 3-phosphate (GAP) and PEP were unchanged over the freezing exposure.

In skeletal muscle, levels of F6P, DHAP and PYR had increased after 2 h of freezing, but declined thereafter (Fig. 5). G6P, GAP and PEP levels showed no change over the course of freezing. The level of $F1,6P_2$ declined at 30 min, but subsequently returned to the control value.

Discussion

Trachemys s. elegans hatchlings tolerated short-term freezing exposures at either −2.5 or −4°C as long as the percentage of body water as ice remained relatively low. All animals recovered fully after 2 h of freezing and with ice contents of less than 30 % of total body water. Survival was also 100 % for turtles exposed to −4°C for 4 h and with a mean ice content of 49.6±2.4 %. However, slightly longer exposure times, 5 h at −4°C or 6 h at −2.5°C, and/or higher ice contents resulted in a sharp drop in survival rate. Thus, both time spent frozen and the amount of ice were significant factors in survival; all animals survived 4 h at

Table 1. *Effect of 4 h of freezing exposure at –4 °C on amino acid concentrations (nmol g^{-1} wet mass) in liver, skeletal muscle and blood of* Trachemys scripta elegans *hatchlings*

	Liver		Leg muscle		Blood	
	Control	Frozen	Control	Frozen	Control	Frozen
Aspartate	366±90	414±33	397±45	530±23*	84±21	332±121
Glutamate	564±86	696±73	1257±141	1370±73	210±46	666±117*
Serine	292±49	399±65	321±67	781±33*	102±25	327±34*
Glutamine	3460±934	2018±115	1044±296	1324±94	366±91	776±76*
Glycine	193±23	463±55*	946±28	1925±381*	256±69	552±35*
Threonine	–	–	148±2	151±2	68±27	257±50*
Arginine	19±7	10 (*N*=1)	2958±320	3275±201	62±27	37 (*N*=1)
Taurine†	7394±529	11 455±806*	7447±757	9299±455	2 201±580	6777±668*
Alanine†	950±90	730±40	180±10	210±20	–	–
GABA	111±47	184±8	224±27	205±16	138±36	257±46
Tyrosine	113±24	137±12	76±25	82±7	51±13	75±28
Valine	1339±301	1940±158	1387±274	1710±186	553±96	1307±165*
Phenylalanine	37±9	98±10*	12±3	12±1	31±13	71±25
Leucine	317±57	648±71*	64±12	53±2	212±48	390±119
Lysine	320±61	501±96	266±70	225±38	285±90	599±8*
Total amino acids	14620±1469	19786±1473*	16927±1654	21307±656*	4619±1183	12 521±1438*

Data are means±S.E.M., *N*=3, unless indicated.

GABA is gamma-aminobutyric acid.

Mean levels of asparagine and isoleucine were 7–14 and 79–90 nmol g^{-1} in all cases and did not change with freezing exposure; histidine and methionine were not detected in any samples.

* Significantly different from the corresponding control value using Student's *t*-test, $P<0.05$.

† Taurine and alanine co-eluted in the HPLC system. Values for alanine in liver and muscle were determined separately by enzymatic assay, and taurine content was then determined by subtraction. The alanine enzymatic assay was not carried out on blood, so the value reported for taurine represents the combined taurine plus alanine pool.

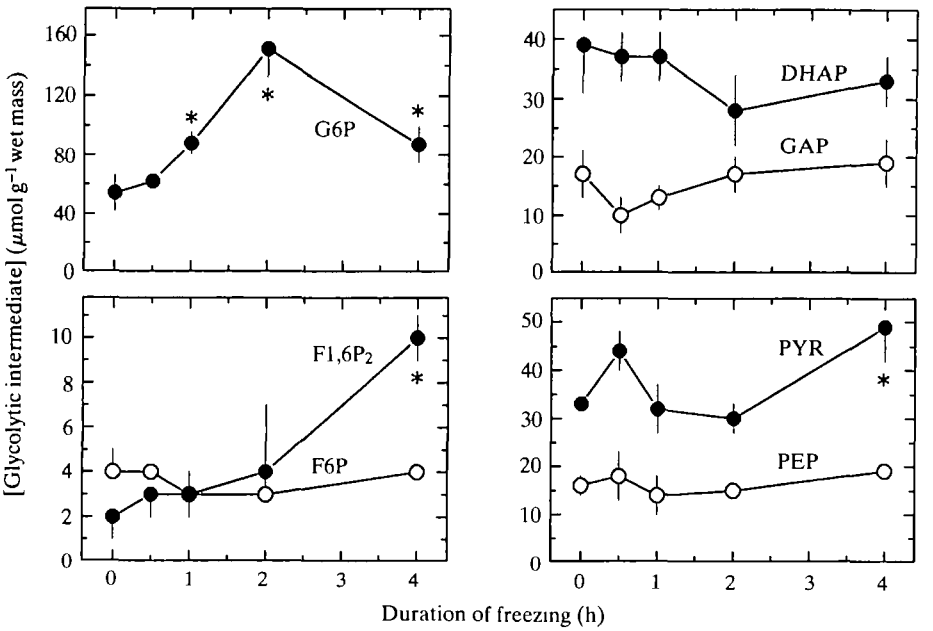

Fig. 4. Effect of the duration of freezing at −4°C on the levels of glycolytic intermediates in liver of hatchling *T. s. elegans*. G6P, glucose 6-phosphate; F6P, fructose 6-P; F1,6P$_2$, fructose 1,6-bisphosphate; DHAP, dihydroxyacetonephosphate; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate. Data are mean±S.E.M., N=3. * Significantly different from the corresponding control value using the Student's t-test, $P<0.05$.

−4°C with 50 % ice but only one-quarter of turtles revived after 12 h freezing at −2.5°C when ice content was also 50 % of body total water. It should be noted that the rapid thawing in the calorimeter could be detrimental to the animals and that survival rates might be higher if thawing were done slowly at a lower temperature, as would occur in nature.

Freezing survival by *T. s. elegans* hatchlings from Louisiana was much poorer than the freeze tolerance exhibited by both midland and western subspecies of painted turtles from Canadian populations (Churchill and Storey, 1992*a*). Autumn-collected *C. p. marginata* and *C. p. bellii* hatchlings, when tested using identical methodologies and with rapid thawing in the calorimeter, showed 100 % survival after 3 days of continuous freezing at −2.5°C. Spring-collected *C. p. marginata* showed even greater tolerance, reviving after 11 days frozen at −2.5°C (Churchill and Storey, 1992*a*). Maximal ice contents were also substantially lower for autumn *C. picta* hatchlings, averaging 43.5–46.6 % at −2.5°C (Churchill and Storey, 1992*a*) compared with the mean 55 % for *T. s. elegans* at the same temperature (Fig. 1). Comparable results were seen at −4°C; maximal ice content was 64.5 % for *T. s. elegans* (Fig. 2) and substantially lower, 49.8–52.6 % of body water, for autumn *C. picta* hatchlings (Churchill and Storey, 1992*a*). Both animal

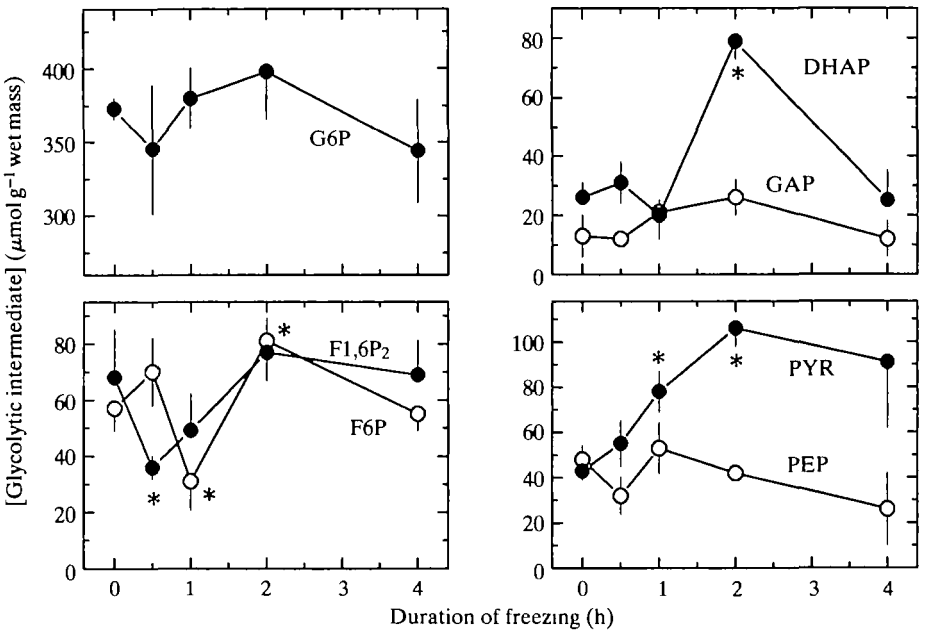

Fig. 5. Effect of the duration of freezing at −4°C on the levels of glycolytic intermediates in skeletal muscle of hatchling *T. s. elegans*. Details are as in Fig. 4.

and cryomedical studies have shown that the ability to minimize both the total amount of ice formed and the rate of ice formation are important to freezing survival. The above comparison of two closely related turtle species, *C. picta*, which routinely employs freeze tolerance for hibernation, and *T. s. elegans*, which does not, appears to confirm this for reptiles.

Although freeze tolerance is clearly less well developed in Louisiana *T. s. elegans* hatchlings than it is in northern *C. picta* hatchlings, freezing survival by *T. s. elegans* compared favourably with that of garter snakes (Costanzo *et al.* 1988; Churchill and Storey, 1992*b*) and far exceeded that of wall lizards (Claussen *et al.* 1990). Garter snakes frozen at −2.5°C recovered fully after freezing exposures of 3 h or less, which produced ice contents of up to about 40 % of total body water; with longer times and greater ice contents, however, survival declined (Churchill and Storey, 1992*b*). Studies of wall lizards found that only one-third of animals recovered after freezing for 2 h or less and the maximal ice content tolerated was only 28 % (Claussen *et al.* 1990).

The limited freeze tolerance displayed by the Louisiana *T. s. elegans* hatchlings of the present study probably has little ecological relevance for the natural overwintering of southern populations of the species since the probability of experiencing subzero temperatures within the nest cavity would be very low in their southern homeland. Furthermore, for the hatchlings to survive, the net freezing exposure would have to be very mild (combining a short freezing time and only a few degrees below 0°C) and such circumstances would not often occur,

particularly since, once frozen, the hatchlings cannot thaw until the temperature rises above the melting point (−0.5°C) of their body fluids. However, we cannot rule out a natural use for freezing abilities displayed by this species, since the range of the subspecies extends to northern Illinois (Behler and King, 1979), where colder winters would be encountered. Among *T. s. elegans* populations in colder climates, the freeze tolerance of hatchlings could be much more highly developed. Indeed, Ultsch *et al.* (1985) have demonstrated a north–south cline in physiological responses that are vital to hibernation success among adult painted turtles. Northern *C. p. bellii*, which may spend up to 6 months hibernating under water, showed far less metabolic stress during long-term anoxic submergence at 3°C than did southern *C. p. dorsalis*, which may not hibernate at all. We have previously postulated that anoxia tolerance is an important factor in freezing survival (Storey and Storey, 1988) and turtles of the *Trachemys* and *Chrysemys* genera are among the premier vertebrate facultative anaerobes (Ultsch, 1985; Kelly and Storey, 1988). Latitudinal clines in anoxia tolerance among these genera may be a key element of freezing survival by the hatchlings in their winter nests.

Two of the specific adaptations typically displayed by freeze-tolerant animals are ice-nucleating proteins and low molecular weight cryoprotectants (Storey and Storey, 1988). Plasma of hatchling *C. p. marginata* contains proteinaceous ice-nucleators, whereas that of adult painted turtles does not (Storey *et al.* 1991). The status of ice nucleators in *T. s. elegans* is not yet known. The addition of cryoprotectants to body fluids helps to reduce, *via* colligative effects, the amount of ice that can form at any given temperature. Freeze-tolerant frogs accumulate glucose or glycerol for this purpose in amounts ranging up to several hundred millimoles per litre (Schmid, 1982; Storey and Storey, 1984, 1985, 1986). However, our studies with *C. picta* hatchlings indicated only low amounts of cryoprotectants. Levels of glucose, glycerol, lactate, taurine and glutamate increased during freezing exposure in spring-collected *C. p. marginata* (Storey *et al.* 1988), but analyses of autumn-collected turtles indicated that only glucose and lactate levels showed sufficiently large changes to have an impact on cryoprotection (Churchill and Storey, 1992*a*,*c*). Box turtles also produced only low amounts of these compounds during freezing (K. B. Storey, J. R. Layne, M. M. Cutwa, T. A. Churchill and J. M. Storey, in preparation). Organs of *T. s. elegans* hatchlings accumulated glucose, lactate and some amino acids during freezing. Net accumulations of glucose and lactate were low (a maximum of 3.2 μmol g^{-1} for glucose; 9.8 μmol g^{-1} for lactate) and restricted to selected organs (glucose in liver and muscle; lactate in brain and heart) with a pattern that was similar to the effects of anoxia on these metabolites in organs of adult *T. s. elegans* (Kelly and Storey, 1988). The accumulation of these compounds in *T. s. elegans* organs may have been due, therefore, to the anoxia and ischaemia stresses associated with freezing. By comparison, organs of *C. p. bellii* hatchlings built up greater amounts of these compounds during equivalent 4-h freezing exposures at −4°C (up to 16 μmol g^{-1} glucose and 25 μmol g^{-1} lactate) (Churchill and Storey, 1992*c*). This could suggest that the established responses to anoxia have been accentuated by *C. p. bellii* in

order rapidly to build up pools of glucose and lactate for cryoprotection during freezing.

Changes in glycolytic intermediates in liver of *T. s. elegans* hatchlings revealed increased levels of both F1,6P_2 and PYR after 4 h of freezing. Elevated levels of these products of the regulatory enzymes of glycolysis, phosphofructokinase and pyruvate kinase, indicate enzyme activation and suggest that the 4 h of freezing exposure caused an energetic stress on liver that resulted in glycolytic activation. For long-term survival while frozen, such a metabolic activation would be detrimental. Not unexpectedly, the opposite response occurred in liver of *C. picta* hatchlings, with changes in the levels of glycolytic intermediates that were consistent with metabolic rate depression during freezing (Churchill and Storey, 1992*c*). Thus, another critical element of the development of freeze tolerance by reptiles may be the ability to use metabolic arrest strategies to reduce metabolic rate further during freezing and, thereby, maximize the time that an animal can survive in the frozen state.

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