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Hatchling turtles survive freezing during winter hibernation

(*Chrysemys picta marginata*/freeze tolerance/cryopreservation/cold hardiness)

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ABSTRACT **Hatchlings of the painted turtle (*Chrysemys picta marginata*) are unique as the only reptile and highest vertebrate life form known to tolerate the natural freezing of extracellular body fluids during winter hibernation. Turtles survived frequent exposures to temperatures as low as −6°C to −8°C in their shallow terrestrial nests over the 1987–1988 winter. Hatchlings collected in April 1988 had a mean supercooling point of −3.28 ± 0.24°C and survived 24 hr of freezing at −4°C with 53.4% ± 1.98% of total body water as ice. Recovery appeared complete after 20 hr of thawing at 3°C. However, freezing at −10.9°C, resulting in 67% ice, was lethal. A survey of possible cryoprotectants revealed a 2- to 3-fold increase in glucose content of liver and blood and a 3-fold increase in blood glycerol in response to freezing. Although quantitatively low, these responses by spring turtles strongly indicate that these may be the winter-active cryoprotectants. The total amino acid pool of blood also increased 2.25-fold in freezing-exposed turtles, and taurine accounted for 52% of the increase. Most organs accumulated high concentrations of lactate during freezing, a response to the ischemic state imposed by extracellular freezing. Changes in glycogen phosphorylase activity and levels of glucose 6-phosphate and fructose 2,6-bisphosphate were also consistent with a dependence on anaerobic glycolysis during freezing. Studies of the molecular mechanisms of natural freeze tolerance in these turtles may identify protective strategies that can be used in mammalian organ cryopreservation technology.**

Few reptile species occur in northern latitudes. Those that do must have well-developed strategies for winter survival, the most frequent solution being hibernation in thermally buffered sites (underwater or in deep underground dens) (1). However, the survival mechanisms of hatchling painted turtles (*Chrysemys picta marginata*) have presented an enigma. Hatchlings, born in early autumn from eggs laid in May–June, overwinter in their nests. This strategy combines predator avoidance with delayed emergence until a time when environmental conditions are favorable for juvenile development (2). In the northern regions of their range, the consequence of this behavior must be long exposures to subzero temperatures, since nesting sites (sandy or clay banks without vegetation cover) and nest depths (average, 10 cm) offer limited insulation value (3, 4). How do hatchlings survive? The choices are two: (*i*) freeze tolerance (a regulated freezing of extracellular water coupled with molecular strategies that render cytoplasmic water unfreezable), and (*ii*) freeze avoidance (extensive undercooling of body fluids) (5). We report here that hatchling *C. picta marginata* are freeze tolerant, able to survive for extended times with >50% of total body water frozen as extracellular ice. This is the only known report of natural freezing survival in a reptile. These turtles, along with four species of terrestrially hibernating frogs (5), are the only known vertebrate animals that have developed natural freeze tolerance as a winter survival strategy.

MATERIALS AND METHODS

Nest Monitoring and Animal Collection. Nests of *C. picta marginata* were located and marked at sites in Algonquin Park, Ontario (45°34′ N, 78°41′ W), over the summer of 1987. Temperatures in two nests were monitored at intervals over the 1987–1988 winter using a copper-constantan thermocouple placed at the bottom of each nest (4). On April 25th, 1988, the nests were excavated from under a covering layer of snow. Hatchlings from three nests [mean body weight, 3.8 ± 0.82 g (SD); range, 2.8–4.7 g; n = 13] were pooled, placed in cold soil, packed in snow, and then transported to the Carleton laboratory. Animals were transferred to plastic boxes containing damp sphagnum moss and placed at 3°C. Experimental tests of cold hardiness were conducted within 3–4 days after excavation of the nests.

Survival at Subzero Temperatures. One box containing four turtles was transferred to −4°C; a second box with two turtles was placed at −10.9°C. Both were exposed to subzero temperature for 24 hr and then returned to 3°C. One turtle from the −10.9°C exposure was taken immediately for the determination of body ice content, as described below, before placement at 3°C. Survival was assessed after 20 hr of thawing.

Determinations of Supercooling Point (SCP) and Ice Content. Cooling/freezing profiles were monitored as described for frogs (6), with a thermistor placed in contact with the plastron and with air temperature in the incubator set at −10.9°C. After nucleation (indicated by the appearance of the freezing exotherm), animals were held for a further 75–85 min in this incubator until body temperature had again cooled to the SCP. Then turtles were transferred to a second incubator at −4°C, placed under a layer of frozen moss, and held 24 hr. Each turtle was then rapidly thawed in an insulated vacuum flask containing 20 ml of water at 23°C and the decrease in water temperature was recorded (7); complete thawing required 5–7 min. Animals were removed, left at 23°C for a further 40–50 min, and then dissected. The percentage of body water as ice was calculated in two ways: method 1 used specific heats of wet and dry masses (8), whereas method 2 used calorimetry of equivalent masses of −4°C water and ice (7). For method 1, dry weights were determined for two turtles frozen at −10.9°C for 24 hr; specific heat content of the body dry mass was calculated at 0.064 cal·g^{-1}·°C^{-1} (1 cal = 4.18 J) (8).

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Abbreviations: SCP, supercooling point; FF/FT, fast frozen/fast thaw; SF/ST, slow frozen/slow thaw.

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Organ Sampling and Preparation of Extracts. Control turtles were sampled after 3 days at constant 3°C. The fast frozen/fast thaw (FF/FT) group of turtles were those used for SCP and percentage of ice determinations. The slow frozen/slow thaw (SF/ST) group of turtles were those given 24 hr freezing at −4°C followed by 20 hr thaw at 3°C. Turtles were killed by severing the spinal cord at the neck and brain pithing. The plastron was removed and a sample of blood was taken from the aorta. This was immediately mixed with perchloric acid and processed as described (9). Organs were rapidly removed, frozen in liquid nitrogen, and then transferred to −80°C for storage. Perchloric acid extracts of organs were made as described (9) and used for the quantification of acid-stable metabolites. For the analysis of enzyme activities and fructose 2,6-bisphosphate, samples of frozen liver and skeletal muscle were rapidly homogenized (1:4, wt/vol) in buffer containing EDTA, EGTA, and NaF (10). An aliquot of the homogenate was immediately transferred to a second tube containing hot NaOH and was processed for the extraction of fructose 2,6-bisphosphate (11). An aliquot of the remaining homogenate was taken for measurement of phosphorylase activity; the rest was centrifuged and the resulting supernatant was used for assay of pyruvate kinase and lactate dehydrogenase (10).

Contents of glycogen, sugars, polyols, lactate, and ATP were determined fluorometrically via coupled enzyme assays (9). Fructose 2,6-bisphosphate was determined by the method of van Schaftingen (11), amino acids were quantified by a Beckman HPLC, and protein was determined by the Coomassie blue dye-binding method using the BioRad prepared reagent. Assay conditions for enzymes were as described (9, 10).

RESULTS

Recordings of temperature in two *C. picta marginata* nests over the 1987–1988 winter showed frequent subzero temperatures. Lows of −6°C and −8°C were recorded on January 15, 1988, with −5°C and −7°C on February 26 and readings ranging between −2°C and −6°C at several other times in January and February. Hatchlings had survived these exposures, for on April 25th live turtles were removed from these still snow-covered nests; some dead embryos in eggs were also found but there were no dead hatchlings.

To identify the strategy of cold hardiness taken by *C. picta marginata*, hatchlings were initially exposed to selected subzero temperatures, both within (−4°C) and below (−10.9°C) the range of nest temperatures recorded outdoors. Turtles at both temperatures froze within 12 hr. Heads and appendages were drawn up closely around the body and were stiff to the touch. Breathing was stopped. After 24 hr of freezing exposure, animals were returned to 3°C and thawed for 20 hr. All turtles exposed to −4°C revived. Both externally (breathing, limb movements) and internally (heart beat, blood flow, and organ appearance), animals appeared normal and indistinguishable from control turtles held at constant 3°C. Turtles frozen at −10.9°C, however, did not survive.

Other hatchlings were monitored individually during freezing and thawing (Table 1). The mean SCP was −3.28°C with a range from −2.8°C to −4.1°C. The average rate of cooling between 0°C and the SCP was −0.365°C ± 0.02°C·min^{-1}. All individuals nucleated immediately upon reaching the SCP; none persisted in an undercooled state. The exotherm due to heat release from ice formation lasted for an average of 80 min, after which animals were transferred to −4°C. Ice content after 24 hr at −4°C averaged 52–53% of total body water; both methods of calculating ice content gave the same result. After thawing, turtles were left at 23°C for 40–50 min and then dissected. At this time, all were well progressed in recovery from freezing with strong heart beats and good blood flow; one had recovered a twitch response to leg pinching with forceps. One turtle frozen at −10.9°C for 24 hr had an ice content of 59–66% of body water; this animal did not survive freezing.

Table 1. Parameters of cooling and freezing in hatchling painted turtles

Supercooling point	−3.28 ± 0.24°C (n = 4)	
Freezing point	−2.10 ± 0.21°C (n = 4)	
% body water	79.7 ± 0.82 (n = 2)	
	Method 1	Method 2
% ice		
At −4°C	53.4 ± 1.98 (n = 4)	52.1 ± 0.96 (n = 4)
At −10.9°C	66.9 (n = 1)	59.1 (n = 1)

Values are means ± SEM.

Blood and organs of the hatchling turtles were surveyed for the commonly occurring low molecular weight cryoprotectants. Freezing stimulated an increase in glucose content, 2-fold in blood and 3-fold in liver with smaller increases in other organs (Table 2). Levels reached 15 μmol/g (wet weight) in blood of the FF/FT group. Glycerol also increased by 3-fold to 0.75 ± 0.11 μmol/g in blood of the FF/FT animals; blood levels in the SF/ST group were 0.44 ± 0.07 μmol/g (both experimental groups were significantly different from control values of 0.25 ± 0.03 μmol/g, $P < 0.05$). Sorbitol, fructose, and mannose were not detected. The increase in glucose (plus lactate) levels in liver was inversely correlated with a 30% drop in liver glycogen content, but activation of liver glycogen phosphorylase was not apparent (Table 2).

Freezing also resulted in a large increase in lactate concentration in most organs (Table 2), a clear indication that anaerobic glycolysis supports organ energy metabolism during the ischemia imposed by extracellular freezing. Lactate

Table 2. Effects of freezing on glucose, glycogen, and lactate concentrations in turtle organs

	Control	FF/FT	SF/ST
		Glucose	
Blood	7.6 ± 0.91	15.9 ± 1.5*	11.1 ± 1.22*
Liver	3.9 ± 0.58	13.7 ± 0.9*	8.3 ± 0.33*
Heart	4.2 ± 0.59	6.0 ± 0.58*	5.8 ± 0.52*
Brain	4.8 ± 0.53	5.8 ± 0.43*	6.6 ± 0.57*
Kidney	4.0 ± 1.27	5.0 ± 0.73	5.1 ± 0.63
Intestine	5.2 ± 0.60	7.1 ± 0.56*	6.1 ± 0.48
Muscle	6.8 ± 1.09	6.9 ± 0.61	8.1 ± 1.04
		Glycogen	
Blood	—	—	—
Liver	140 ± 13	130 ± 19	87 ± 9*
Heart	170 ± 9	122 ± 20*	138 ± 14*
Brain	44 ± 24	61 ± 10	55 ± 12
Kidney	57 ± 2	46 ± 4*	54 ± 19
Intestine	39 ± 10	42 ± 6	34 ± 2
Muscle	61 ± 5	43 ± 4*	40 ± 3*
		Lactate	
Blood	14.0 ± 2.5	38.2 ± 7.2*	30.9 ± 2.11*
Liver	1.4 ± 0.4	20.4 ± 2.5*	5.9 ± 1.22*
Heart	19.2 ± 2.8	11.9 ± 2.5*	7.5 ± 1.1*
Brain	8.1 ± 0.4	25.1 ± 2.8*	14.7 ± 2.6*
Kidney	4.4 ± 1.1	17.3 ± 1.6*	5.7 ± 0.8
Intestine	2.9 ± 0.2	15.0 ± 1.4*	10.9 ± 1.0*
Muscle	9.3 ± 1.1	11.6 ± 2.0	10.8 ± 2.2

Values are μmol/g (wet weight) ± SEM (control, n = 3; experimental, n = 4). Glycogen is expressed in glucose units. Muscle is pectoral skeletal muscle. Experimental groups: FF/FT animals were used for SCP and percent ice determinations; SF/ST animals were exposed to −4°C for 24 hr followed by 3°C for 20 hr.
*Significantly different from control values, $P < 0.05$.

Table 3. Levels of ATP, glucose 6-phosphate, and fructose 2,6-bisphosphate in liver and pectoral skeletal muscle of hatchling painted turtles

	ATP, μmol/g	Glucose 6-phosphate, μmol/g	Fructose 2,6-bisphosphate, nmol/g
Liver			
Control	0.43 ± 0.14	0.04 ± 0.01	<0.50
FF/FT	0.16 ± 0.02*	0.16 ± 0.02*	2.60 ± 0.20*
SF/ST	0.31 ± 0.03	0.12 ± 0.02*	1.10 ± 0.11*
Muscle			
Control	1.10 ± 0.16	0.36 ± 0.08	0.22 ± 0.13
FF/FT	1.21 ± 0.16	0.59 ± 0.10	0.69 ± 0.04*
SF/ST	0.67 ± 0.05*	0.37 ± 0.05	0.11 ± 0.02

Values are means ± SEM (control, $n = 3$; experimental, $n = 4$). Experimental groups are as defined in Table 2.
*Significantly different from control values, $P < 0.05$.

levels were highest in organs of the FF/FT group and were uniformly reduced for the equivalent organs of the SF/ST group, suggesting that lactate is rapidly cleared soon after thawing. Production of glucose and lactate was supported by a decrease in glycogen content occurring in most organs during freezing. The distribution of glycogen reserves was organ specific, with highest levels occurring in liver and heart.

Table 3 shows the levels of other metabolites in liver and skeletal muscle. ATP content in the FF/FT group was reduced to 37% of the value in control liver but had recovered substantially in animals that were slow thawed over 20 hr at 3°C. Muscle showed a different response, with ATP content being unaffected in the FF/FT group, but levels were reduced in the SF/ST animals. Glucose 6-phosphate and fructose 2,6-bisphosphate levels were both increased significantly in liver of freezing-exposed turtles; this is consistent with an increased rate of glycogenolysis supporting both glucose and lactate production during freezing. Levels of both were slightly lower in the SF/ST group than in the FF/FT animals, in line with the more advanced stage of recovery of the slow-thawed animals.

Levels of amino acids in blood and liver of control and freezing-exposed turtles are shown in Table 4. The total amino acid content in blood from turtles of the FF/FT group was increased 2.25-fold over that of controls. Fifty-two percent of the increase was due to increased levels of taurine, with the remainder made up of several other amino acids. Levels of blood amino acids had returned to control values, however, in the SF/ST group, indicating that slow thawing over 20 hr allowed ample time for metabolic recovery. Blood of all groups showed high levels of γ-aminobutyric acid, but this was essentially absent from liver tissue. Freezing had lesser effects on amino acid contents in liver than were seen in blood. Levels of several amino acids were elevated in liver of FF/FT and SF/ST groups, but freezing did not affect the contents of glutamine, taurine, and valine, the three major free amino acids. Of note, however, were significantly increased levels of alanine in both freezing-exposed groups, alanine being an alternative product of fermentative glycolysis.

Effects of freezing on the activities of some enzymes in turtle liver and pectoral skeletal muscle are shown in Table 5. Both the total activity of glycogen phosphorylase ($a + b$) and the percentage of enzyme in the a form were modulated slightly by freezing exposure. Net activity of the a form (total multiplied by percent a) was increased by 66% in the SF/ST groups but not significantly altered for the FF/FT group. Pyruvate kinase activity was also slightly increased in liver of freezing-exposed animals, but the K_m for phospho*enol*pyruvate was little affected. Activities of lactate dehydrogenase and total protein content of liver were not affected by experimental treatment. The effect of freezing on enzyme activities in muscle was different. Activities of all three enzymes increased, measured as units/g (wet weight), the average being a 99% increase. Similarly, protein content, again measured per g (wet weight), increased by an average of 103%. This suggests a dehydration of muscle tissue during freezing. Freezing in muscle stimulated a 2.7-fold increase in the percentage of phosphorylase in the a form, suggesting activation of glycogenolysis. Affinity of pyruvate kinase for phospho*enol*pyruvate was not affected by freezing-exposure.

DISCUSSION

Hatchling *C. picta* overwinter in the nest, even near the northern limits of the range (2–4). Indeed, of 55 nests monitored over 6 years at the Algonquin park site, no

Table 4. Amino acids in blood and liver of control and freezing-exposed turtles

	Blood			Liver		
Amino acid	Control	FF/FT	SF/ST	Control	FF/FT	SF/ST
Aspartate	78 ± 14	113 ± 33	54 ± 9	134 ± 19	151 ± 22	274 ± 36*
Glutamate	122 ± 25	465 ± 77*	169 ± 15	195 ± 44	451 ± 48*	404 ± 18*
Asparagine	4 ± 1	5 ± 1	0	0	0	0
Serine	55 ± 9	106 ± 16*	74 ± 8	17 ± 2	19 ± 3	25 ± 5
Glutamine	162 ± 32	253 ± 24*	157 ± 54	1090 ± 349	1123 ± 208	1330 ± 125
Histidine	47 ± 7	67 ± 25	101 ± 35	202 ± 119	73 ± 25	220 ± 19
Glycine	105 ± 7	173 ± 28*	135 ± 24	—	—	—
Taurine	684 ± 124	2043 ± 269*	663 ± 110	4242 ± 113	4463 ± 517	3570 ± 247
Alanine	71 ± 15	192 ± 9*	99 ± 15	60 ± 1	322 ± 30*	168 ± 25*
GABA	2420 ± 190	2570 ± 420	2440 ± 490	0	155 ± 31*	0
Tyrosine	7 ± 1	18 ± 6	0	14 ± 1	115 ± 43*	18 ± 3
Valine	658 ± 129	1097 ± 104*	676 ± 154	1020 ± 57	1072 ± 65	1110 ± 121
Phenylalanine	13 ± 2	24 ± 1*	13 ± 1	12 ± 5	7 ± 3	15 ± 3
Isoleucine	23 ± 8	21 ± 3	13 ± 2	67 ± 1	13 ± 2*	8 ± 2*
Leucine	35 ± 7	33 ± 4	14 ± 2*	8 ± 3	30 ± 3*	20 ± 3*
Lysine	25 ± 3	80 ± 8*	33 ± 3	27 ± 5	73 ± 9*	49 ± 2*
Total amino acids (−GABA)	2084 ± 340	4697 ± 433*	2103 ± 410	7368 ± 533	7906 ± 451	7165 ± 311

Values are nmol/g (wet weight) ± SEM (control, $n = 3$; freezing-exposed, $n = 4$). GABA, γ-aminobutyric acid. Glycine peaks in liver and arginine/threonine peaks in all samples were obscured by the presence of Tris buffer in the tissue samples.
*Significantly different from corresponding control value by Student's t test, $P < 0.05$.

Table 5. Effect of freezing on enzyme activities and on the contents of protein and fructose 2,6-bisphosphate in turtle liver and skeletal muscle

Protein	Liver			Pectoral skeletal muscle		
	Control	FF/FT	SF/ST	Control	FF/FT	SF/ST
Glycogen phosphorylase						
Total, units/g	0.94 ± 0.05	1.04 ± 0.11	1.42 ± 0.08*	2.02 ± 0.26	2.82 ± 0.12*	3.59 ± 0.36*
% phosphorylase *a*	12.3 ± 0.84	9.7 ± 0.58*	13.6 ± 0.75	0.75 ± 0.20	2.03 ± 0.18*	1.92 ± 0.59*
Pyruvate kinase						
Total, units/g	5.6 ± 0.09	6.9 ± 0.24*	6.9 ± 0.45*	72.1 ± 5.7	108 ± 2.7*	119 ± 9.9*
K_m PEP, mM	0.37 ± 0.02	0.35 ± 0.04	0.31 ± 0.003*	0.07 ± 0.004	0.07 ± 0.002	0.09 ± 0.007
Lactate dehydrogenase, units/g	62.8 ± 2.9	63.1 ± 5.1	62.8 ± 1.6	65.2 ± 17.6	170 ± 7.3*	192 ± 15.9*
Total protein, mg/g	126 ± 10.9	129 ± 7.3	136 ± 3.7	72 ± 10.7	153 ± 8.1*	139 ± 8.9*

All values are means ± SEM, expressed per g (wet weight) (control, $n = 3$; experimental, $n = 4$). Experimental groups are as described in Table 2. For pyruvate kinase, Hill coefficients for the K_m phospho*enol*pyruvate (PEP) were 2.7–3.0 for the liver enzyme and 1.3–1.8 for the muscle enzyme.

*Significantly different from control values, $P < 0.05$.

instances of hatchling emergence in autumn have been noted, yet all nests examined between August and October contained active hatchings (R.J.B., unpublished observations). Several authors have reported subzero temperatures within the nests of *C. picta* and speculated on how the hatchlings could survive such exposures (12–14). Breitenbach *et al.* (12) suggested that the normal supercooling capacities of reptiles [SCPs range from −2°C to −8°C (15)] would be sufficient to limit winter kill by freezing for a Michigan population of *C. picta*, where nest temperatures rarely dropped below −2°C. However, when long-term exposure to colder temperatures occurs, a specific strategy of cold hardiness is required. Freeze avoidance by deep supercooling is a potential solution but is probably impractical when nests are in wet soil or sand, since the chances of inoculative freezing by environmental ice would be high.

The present study demonstrates that *C. picta marginata* use the alternative strategy, that of freeze tolerance. These turtles share natural freezing survival with only one other group of vertebrates, four species of terrestrially hibernating frogs (*Rana sylvatica, Hyla versicolor, Hyla crucifer*, and *Pseudacris triseriata*). Various cold-hardy invertebrates also tolerate freezing, including many species of terrestrial insects and several types of intertidal invertebrates (mussels, barnacles) (5, 16). These turtles represent the highest vertebrate animal known to survive natural freezing with the long-term presence of ice in all extracellular fluid spaces. No bird or mammal has such a natural freeze tolerance, and studies of experimental freezing in mammals have shown only a very limited tolerance in one hamster species, work that has never been repeated (17). Hatchlings tolerated 24 hr of freezing at −4°C and survived both fast and slow regimens of freezing and thawing. Those that were fast-thawed had regained a strong heart beat within 1 hr after thawing, whereas those that were slow-thawed over 20 hr at 3°C were indistinguishable from controls in vital signs (breathing, heart beat), motor reflexes (head and eye movements, walking, response to pinching), and internal appearance of organs. Freeze-tolerant wood frogs of equivalent size show the same time scale and sequence (heart beat, then breathing, then skeletal muscle responsiveness) of recovery from freezing (16).

With a mean SCP of −3.28°C and measured nest temperatures that frequently dropped to −6°C to −8°C, it is obvious that *C. picta marginata* hatchlings must routinely experience one or more bouts of freezing during a normal winter. Since melting would occur only at −2.1°C (or higher) (Table 1), these freezing episodes may range from days to weeks. High SCPs are, in fact, adaptive for freeze-tolerant animals and ensure that undercooling below the freezing point is minimized, thereby lessening the osmotic shock to cells when freezing is initiated (5).

Turtles tolerated 52–53% of total body water as ice but were killed by a slightly higher value (59–66%). Such behavior is typical of freeze-tolerant animals, a value of ≈65% of total water as extracellular ice generally defining the limit of survival (5). The same is true of cell/tissue cryopreservation in the laboratory (18), the reason being that at higher ice contents the dehydration of the cell causes shrinkage beyond the critical minimum cell volume and results in irreversible structural damage (5, 18). Adaptive strategies of freeze-tolerant animals therefore seek to lower the temperature at which such a critical minimum cell volume is reached to a level that is below the normal microenvironmental thermal experience. For gallfly larvae, for example, this temperature is about −25°C (8). For hatchling turtles collected in the spring, this limit was reached by −10.9°C.

One of the strategies for minimizing cell dehydration during freezing is the accumulation of low molecular weight cryoprotectants that limit cell shrinkage by colligative means. Polyhydric alcohols are the common cryoprotectants among insects; glucose or glycerol occurs in freeze-tolerant frogs (5, 16). Our scan of possible cryoprotectants in *C. picta marginata* suggested that glucose or glycerol could prove to be the winter-active cryoprotectant of this species. In addition, the freezing-induced increase in taurine (and total amino acids) suggests a role for amino acids in cryopreservation in this species. For species that must adapt to changing environmental salinities, taurine is a primary osmolyte that is used to regulate cell volume (19). Levels of glucose, glycerol, and taurine were all elevated after freezing exposure of spring-collected turtles, although the amounts accumulated by these hatchlings would not have significant colligative effects in cell volume control during freezing. The absence of high levels of cryoprotectants in spring turtles is perhaps not unexpected in light of the comparable behavior of spring-collected frogs (5, 20). Spring frogs produce only low amounts of cryoprotectants, whereas equivalent freezing conditions in the autumn or winter months induce the synthesis of 200–500 mM glucose or glycerol (5, 9, 16). The difference between the autumn/winter versus spring responses appears to be the commitment of liver glycogen reserves (the substrate for cryoprotectant synthesis) to other uses as winter hibernation ends (20). It is interesting to note, however, that both turtles and frogs retain freeze tolerance in the early spring despite low levels of cryoprotectants; this indicates that colligative function alone cannot be the reason for the presence of cryoprotectants in freeze-tolerant vertebrates (5). Work with autumn-collected hatchlings will be needed to further assess the role of carbohydrates and amino acids as cryoprotectants in freeze-tolerant turtles.

The cessation of breathing and the freezing of blood and extracellular fluids places the organs of the frozen turtle in an anoxic and ischemic state. Molecular adaptations supporting

anaerobiosis are, therefore, a critical part of natural freeze tolerance (5, 9). Indeed, the crucial damage done by ischemia is a primary reason that freezing at ultralow temperatures (e.g., −196°C) is viewed as the goal of medical organ cryopreservation. Freeze-tolerant animals, however, maintain viability while frozen for days or weeks at high subzero temperatures (5). For the hatchling turtles, anaerobic glycolysis provides the major route of ATP generation while frozen. All organs maintained a substantial glycogen content and most rapidly accumulated lactate during freezing. Liver also showed a minor accumulation of alanine, an alternative glycolytic end product. Phosphorylase *a* activity increased in both liver and muscle. Elevated levels of glucose 6-phosphate and fructose 2,6-bisphosphate in liver and muscle of freezing-exposed turtles were also consistent with increased flux through glycolysis. Adults of *C. picta* are excellent facultative anaerobes and survive for months submerged at 3°C during winter hibernation (21). The molecular mechanisms (22) that support anoxia tolerance and hibernation by adults are undoubtedly first put into use for freezing survival by hatchlings.

The phenomenon of natural freeze tolerance, previously thought to be restricted to selected invertebrates, has now within the past 6 years been documented for four species of terrestrially hibernating frogs (5–7, 9, 16, 20) as well as, with this report, hatchling turtles. Occurring in both reptiles and amphibians, the phenomenon must now be accepted as a real physiological capability of ectothermic vertebrates and their organs. As such, studies of the organ-specific mechanisms of natural freeze tolerance in turtles and frogs can illustrate the principles of organ freezing in vertebrates and could hold the key to the development of techniques for mammalian organ cryopreservation.

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