Metabolic responses to freezing by organs of hatchling painted turtles *Chrysemys picta marginata* and *C. p. bellii*

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Organ-specific metabolic responses to freezing were monitored over the course of 4 h exposure to freezing at −4°C for autumn-collected hatchlings of midland (*Chrysemys picta marginata*) and western (*Chysemys picta bellii*) painted turtles. Both subspecies accumulated glucose and lactate in seven organs during freezing for possible use as cryoprotectants. Overall levels of both were higher in *C. p. bellii* than in *C. p. marginata*, with glucose ranging up to 16 μmol/g wet weight and lactate up to 25 μmol/g in *C. p. bellii* organs. Glucose accumulation by *C. p. bellii* organs appeared to be initiated during prefreeze cooling, whereas glucose production in *C. p. marginata* was triggered by the initiation of freezing; this difference may explain the higher overall accumulations of both glucose and lactate in *C. p. bellii* organs. Glucose content in most organs declined over the course of the 4-h freezing exposure, suggesting that the main function of the sugar may be as the substrate for lactate production. Analysis of changes in the levels of glycolytic intermediates in liver revealed an accumulation of glucose-6-phosphate, indicating activation of liver glycogenolysis during freezing. An increase in liver fructose-6-phosphate and a decrease in fructose-1,6-bisphosphate concentrations over the course of freezing were consistent with an inhibition of liver phosphofructokinase that would support glucose export to other organs.

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Les réactions métaboliques de certains organes au gel ont été enregistrées au cours d'une exposition de 4 h à une température de −4°C, chez des tortues peintes (*C. picta marginata* et *Chrysemys picta bellii*) fraîchement écloses recueillies à l'automne. Les deux sous-espèces accumulent du glucose et du lactate dans sept de leurs organes au cours du gel, ce qui leur assure probablement une cryoprotection. Les concentrations globales des deux produits sont plus élevées chez *C. p. bellii* que chez *C. p. marginata* et les concentrations de glucose atteignent jusqu'à 16 μmol/g masse fraîche, les concentrations de lactate jusqu'à 25 μmol/g chez *C. p. bellii*. L'accumulation de glucose chez *C. p. bellii* semble déclenchée au cours de la période de refroidissement avant le gel, alors que l'accumulation du glucose ne commence à se produire qu'au début du gel chez *C. p. marginata*; cette différence est probablement responsable de l'accumulation plus considérable de glucose et de lactate dans les organes de *C. p. bellii*. Le contenu en glucose de la plupart des organes diminue graduellement au cours de la période de 4 h d'exposition au gel, ce qui indique probablement que le sucre sert surtout de substrat pour la production de lactate. L'analyse des fluctuations des produits intermédiaires de la glycolyse dans le foie a mis en lumière l'accumulation de glucose-6-phosphate, ce qui reflète l'activation de la glycogénolyse dans le foie au cours du gel. L'augmentation du glucose-6-phosphate dans le foie et la diminution des concentrations de fructose-1,6-biphosphate durant le gel s'accompagnent d'une inhibition de la phosphofructokinase hépatique qui pourrait favoriser le transport du glucose vers d'autres organes.

[Traduit par la rédaction]

Introduction

For many animals the ability to endure the freezing of extracellular body fluids is an important component of winter cold hardiness. Freeze tolerance occurs widely among invertebrates, and in recent years a number of terrestrially hibernating amphibians and reptiles with this capacity has also been identified (Storey and Storey 1988; Storey 1990). Physiologically relevant natural freezing survival has been reported for several species, including five frogs, one salamander, two turtles, and one snake (Storey 1990; Schmid 1982; Berman et al. 1984; Storey and Storey 1984, 1985, 1986a; Storey et al. 1988; Costanzo and Claussen 1990; Costanzo et al. 1988, 1990; Churchill and Storey 1992*a*, 1992*b*).

Hatchlings of the painted turtle *Chrysemys picta* are one of the types of animal that survive extended bouts of freezing during winter hibernation (Storey et al. 1988). Young turtles break out of their eggs in late summer but do not emerge from their nests. Instead, they remain protected from predators within the nest cavity until the warm temperatures of spring create conditions conducive to rapid juvenile growth (Gibbons and Nelson 1978). In the northern areas of their range, this habit, combined with the placement of the shallow nests (approximately 10 cm deep) on exposed stream banks or lakeshores, means that hatchlings may experience subzero temperatures within the nest for a substantial part of the winter. Indeed, sustained subzero temperatures within the nest cavity, reaching as low as −6 to −8°C, have been recorded by several authors over the midwinter months, with live hatchlings removed from these nests in the spring (Breitenbach et al. 1984; Storey et al. 1988; Packard et al. 1989).

Our initial analysis of freezing survival by hatchlings of the midland subspecies, *Chrysemys picta maginata*, from central Ontario showed that spring-collected turtles newly removed from their nests readily survived 24 h of freezing at −4°C with 53% of their total body water as ice (Storey et al. 1988). A survey of putative cryoprotectants revealed an increase of 2- to 3-fold in organ glucose levels as a result of freezing, although the net accumulation was low, e.g., 16 μmol/g in blood. Very low amounts of glycerol were also produced. Despite the low levels, we speculated that glucose might be the main cryoprotectant used by *C. p. marginata* hatchlings, as it is by most of the freeze-tolerant frog species (Storey and Storey 1984, 1986*a*). The low levels of glucose produced by these hatchlings might have been influenced by the spring season, for in frogs the capacity for cryoprotectant synthesis declines rapidly after spring emergence

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(Storey and Storey 1987). In addition, we also noted a strong increase in organ lactate levels as well as an increase in the pool of free amino acids in blood during freezing (Storey et al. 1988). Although these compounds are not typically considered to be cryoprotectants, they could be the specific choices of freeze-tolerant turtles.

The present study further explores the metabolic adaptations for freeze tolerance by hatchling painted turtles. We analyze autumn-collected hatchlings to determine the responses to freezing by animals that are prepared for entry into the natural period of winter cold exposure. Hatchlings from two subspecies are compared, the midland painted turtle, *C. p. marginata*, from central Ontario and the western painted turtle *Chrysemys picta bellii*, from western Manitoba, both chosen from populations that are near to the northern range limits of the species (Cook 1984). The two subspecies are quite similar in their physical tolerance of freezing, including their supercooling capacity, survival rate for different durations and temperatures of freezing, and the percentage of body ice tolerated (Churchill and Storey 1992*a*). As the present study shows, however, they differ quite substantially in their cryoprotectant response to freezing, as well as in their metabolic response to freezing-induced ischemia.

Materials and methods

Animals and chemicals

Eggs of midland painted turtles, *C. p. marginata*, were obtained from Algonquin Provincial Park, Ontario (45°34′N, 78°41′W), in mid-July 1989 and were reburied in Ottawa in artificial nests. Hatchling turtles were excavated in early November and transferred to a laboratory incubator set at 5°C. Eggs of the western subspecies, *C. p. bellii*, were gathered from Turtle Mountain Provincial Park, Manitoba (49°3′N, 100°15′W), in July and transported to Winnipeg, and were similarly incubated in an artificial nest. Hatchlings were shipped to Ottawa in late September and were held until use in a 5°C incubator. All turtles were held at a constant 5°C for at least 2 weeks before use. Mean weights of the animals used were 4.8 ± 0.11 (SD) g for the midland and 4.3 ± 0.09 g for the western subspecies; hatchlings were of indeterminate sex. All biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim, Montréal, Que.

Preparation of experimental animals

To monitor cooling and freezing of the turtles, animals were fitted with a thermistor taped to the plastron to measure body surface temperature. Thermistors were connected to a YSI telethermometer with output to a linear recorder. Turtles were placed in an incubator set at −4°C and were allowed to cool over 30–40 min until the instantaneous rise in body temperature due to ice nucleation was recorded; the duration of freezing exposure was then timed from this event. In a few instances, turtles cooled to a body temperature of −4°C without freezing. In these cases, the temperature of the incubator was further lowered to −5°C and the animals continued cooling. Within 1 min of nucleation, the temperature of the incubator was raised to −4°C for the duration of the freezing episode.

After an interval of 0.5, 1, 2, or 4 h of freezing at −4°C, animals were removed from the incubator and killed by decapitation, and their organs were quickly excised and frozen in liquid nitrogen. Tissues were then transferred to −80°C for storage until processing. For controls, thermistors were attached to the turtles and the animals were cooled in the −4°C incubator in exactly the same manner as for the experimental animals. However, cooling was continued only until body temperature was lowered to 0°C, at which time turtles were rapidly removed and organs sampled as above.

Preparation of organ extracts

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. Aliquots of the neutralized extracts were immediately removed and assayed for pyruvate and phosphoenolpyruvate; the remaining extracts were frozen for subsequent use. Metabolite levels were measured as described by Lowry and Passonneau (1972), using either fluorometric or spectrophotometric assays. All data are given as the mean ± SE for three animals at each sampling point. For statistical analysis Student's *t*-test was used.

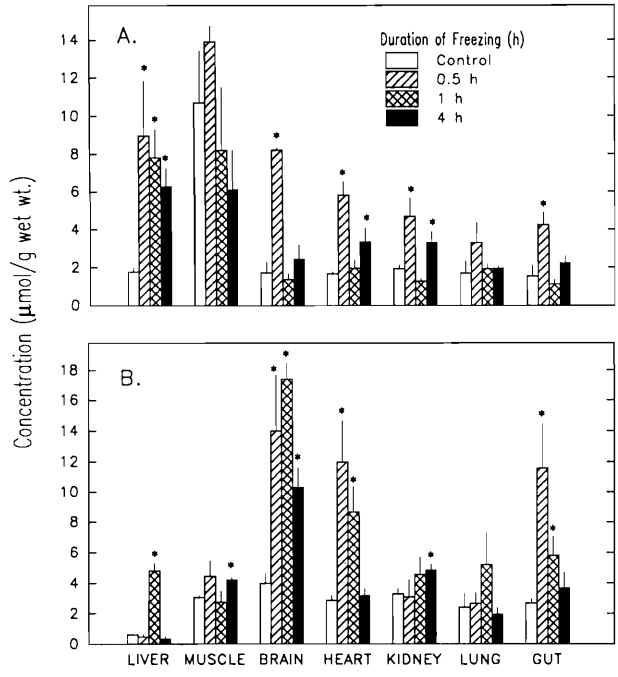

FIG. 1. Effect of duration of freezing exposure at −4°C on the levels of glucose (A) and lactate (B) in organs of hatchling midland painted turtles, *C. p. marginata*. The controls were chilled to a body temperature of 0°C, all others to −4°C. "Gut" represents data for the stomach plus intestines. Data are given as the mean ± SE; $n = 3$. *, significantly different from the corresponding control value ($P < 0.05$).

Results

Previous studies had shown that both subspecies showed 100% recovery after 4 h freezing at −4°C with 50–52.5% of total body water as ice (Churchill and Storey 1992*a*). All animals tested after this exposure and 24 h of thawing at 5°C had completely normal appearance and normal locomotory responses and were still healthy when reexamined after 2 weeks.

Midland painted turtle hatchlings

Figure 1A shows the effect of freezing on glucose levels in seven organs of hatchling *C. p. marginata*. In all organs except skeletal muscle (pectoral and pelvic muscle combined) and lung, freezing resulted in a rapid increase in glucose content; levels rose 2- to 5-fold after 30 min of freezing exposure. Glucose content remained high in liver over the full 4 h of freezing, but in other organs glucose levels had returned to near control values after 1 h of freezing exposure. Heart and kidney showed levels that were again significantly higher than control values after 4 h of freezing. In skeletal muscle glucose levels were high (and variable) in control turtles and did not change significantly over the time course of freezing.

The effect of freezing on organ lactate levels is shown in Fig. 1B. Freezing stimulated a rapid rise in lactate levels in oxygen-sensitive organs, the brain and heart (4.7- and 4.1-fold, respectively), as well as a 4.3-fold rise in the gut (stomach + intestines). Levels in brain and heart remained high after 1 h of

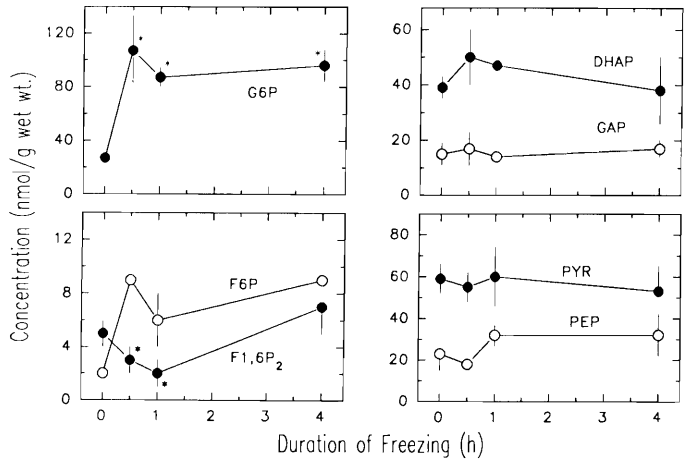

FIG. 2. Effect of duration of freezing at −4°C on the levels of glycolytic intermediates in liver of hatchling midland painted turtles. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6P_2, fructose-1,6-bisphosphate; DHAP, dihydroxyacetonephosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate. Data are given as the mean ± SE; $n = 3$. *, significantly different from the corresponding control value ($P < 0.05$).

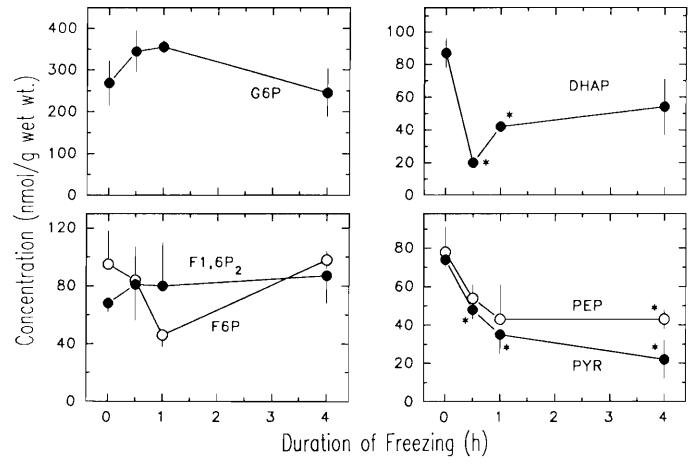

FIG. 3. Effect of duration of freezing at −4°C on the levels of glycolytic intermediates in skeletal muscle of hatchling midland painted turtles. For details see Fig. 2.

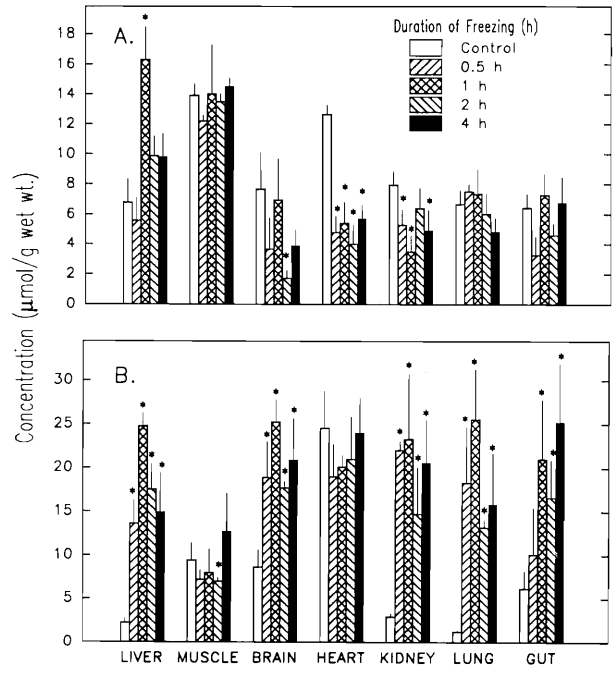

FIG. 4. Effect of length of freezing exposure at −4°C on the levels of glucose (A) and lactate (B) in organs of hatchling western painted turtles, *C. p. bellii*. For details see Fig. 1.

freezing but were reduced after 4 h . Other organs showed little or no net accumulation of lactate over the time course of freezing exposure.

The rapid rise in glucose levels in *C. p. marginata* liver suggested that freezing activated glycogenolysis, as has been shown in freeze-tolerant frog species (Storey and Storey 1984, 1986*b*). To analyze the status of glycolysis in liver, levels of glycolytic intermediates were quantified over the time course of freezing. Changes in the concentrations of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1, 6P_2), dihydroxyacetonephosphate (DHAP), glyceraldehyde-3-phosphate (GAP), phosphoenolpyruvate (PEP), and pyruvate (PYR) are shown in Fig. 2. G6P levels rose sharply at 30 min of freezing exposure and remained elevated over the full 4-h course. F6P levels similarly increased, whereas F1,6P_2 content dropped significantly. The increase in the levels of hexose monophosphates is consistent with an activation of glycogenolysis that supports the rise in glucose concentration. Furthermore, the rise in concentration of the substrate (F6P) and decrease in that of the product (F1,6P_2) of the phosphofructokinase (PFK) reaction indicates an inhibition of glycolytic flux at this locus, the effect of which could be to promote glucose output from the liver. Levels of other intermediates of glycolysis did not change significantly over the time course of freezing exposure.

Changes in glycolytic intermediates in skeletal muscle of *C. p. marginata* are shown in Fig. 3. Overall, freezing had little effect on the levels of these metabolites, in line with the minor effects of freezing on glucose and lactate contents of muscle. PYR levels decreased consistently over the time course of freezing, and PEP content was reduced after 4 h. Levels of other intermediates were not affected by freezing.

Western painted turtle hatchlings

The effect of freezing on glucose and lactate levels in western painted turtle hatchlings is shown in Fig. 4. Changes in glucose levels with freezing were very different from those seen for the midland hatchlings. Glucose levels were generally very high in all organs of these turtles. Glucose concentration showed a transient increase in liver after 1 h of freezing, but in brain, heart, and kidney glucose levels decreased significantly during freezing (Fig. 4A). Lactate levels were low in liver, kidney, and lung of control turtles but increased rapidly during freezing exposure. Control levels of lactate were much higher in brain and gut but continued to increase during freezing. Lactate contents of 15–25 μmol/g were measured in all organs except skeletal muscle (Fig. 4B). Lactate in muscle and heart showed little or no change over the course of freezing exposure.

Changes in glycolytic intermediates in the liver of *C. p. bellii* over the course of freezing exposure are shown in Fig. 5. G6P levels did not change over this time, a result that correlates with the lack of glucose accumulation by the organ. Changes in F6P (an increase) and F1,6P_2 (a decrease) levels again suggested an inhibitory block on glycolysis at the PFK reaction. Levels of DHAP, GAP, and PEP did not change over the freezing time

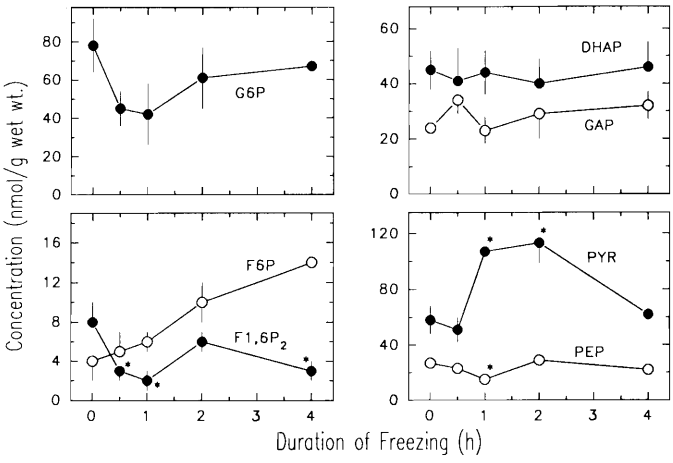

FIG. 5. Effect of duration of freezing at −4°C on the levels of glycolytic intermediates in liver of hatchling western painted turtles. For details see Fig. 2.

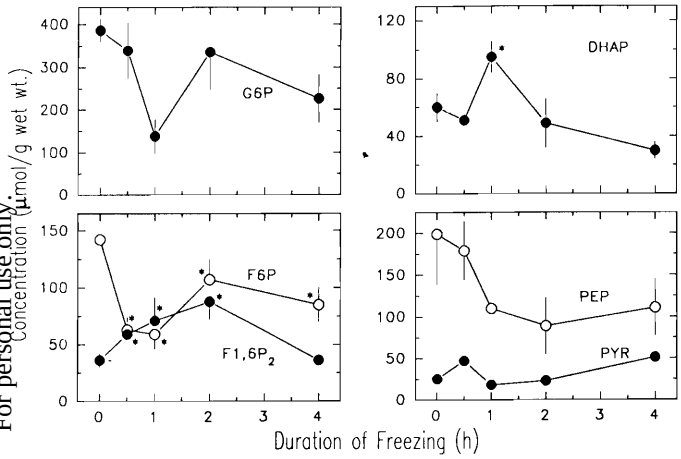

FIG. 6. Effect of duration of freezing at −4°C on the levels of glycolytic intermediates in skeletal muscle of hatchling western painted turtles. For details see Fig. 2.

course, but PYR levels rose about 2-fold at 1 and 2 h of freezing, a result that correlates with the high lactate accumulation by the organ at these times.

Changes in levels of glycolytic intermediates in skeletal muscle of *C. p. bellii* are shown in Fig. 6. Contrary to the situation in liver, changes in F6P (a decrease) and $F1,6P_2$ contents (an increase) were consistent with an activation of flux through the PFK locus, indicating an increased glycolytic rate during freezing exposure. G6P concentration oscillated, whereas levels of other glycolytic intermediates were unchanged over the course of freezing.

Alanine and succinate

Many invertebrate and some vertebrate animals accumulate alternative end products (e.g., alanine and succinate) in addition to lactate under anoxic conditions. To determine whether these compounds might also be produced during freezing-induced ischemia, their levels were measured in the seven organs of the turtles. However, we found no significant changes in the level of either alanine or succinate over the time course of freezing exposure. Control values and all lengths of freezing exposure were combined, therefore, and Table 1 shows the mean levels of alanine and succinate in each organ of the two subspecies.

TABLE 1. Levels (μmol/g wet weight) of L-alanine and succinate in organs of midland and western painted turtle hatchlings

	Chrysemys picta marginata		*Chrysemys picta bellii*	
	Alanine	Succinate	Alanine	Succinate
Liver	1.21±0.08	1.55±0.12	1.09±0.07	1.62±0.10
Muscle	0.10±0.01	0.36±0.08	0.35±0.02	0.53±0.04
Brain	2.22±0.21	3.16±0.19	2.01±0.10	2.44±0.11
Heart	2.42±0.18	3.18±0.42	2.06±0.19	3.54±0.22
Kidney	3.12±0.18	3.55±0.15	3.01±0.19	3.61±0.28
Lung	2.54±0.18	2.30±0.16	2.74±0.20	2.60±0.16
Gut	0.79±0.09	1.88±0.11	1.55±0.16	2.31±0.13

NOTE: Since no significant changes in the levels of either compound were noted over the time course of freezing, control and experimental values were combined. Data are given as the mean ± SE; n = 12 for *C. p. marginata* and n = 15 for *C. p. bellii*. "Gut" represents data for stomach and intestines combined.

Discussion

Canadian populations of both the midland and western subspecies of painted turtles tolerate the freezing of extracellular body fluids during the winter hibernation of hatchlings in shallow terrestrial nests. Although there are latitudinal and climatic differences between the two regions of the country that could result in the exposure of the *C. p. bellii* population to more severe winter conditions, the inherent capacity for freeze tolerance displayed by autumn-collected hatchlings of the two populations was very similar. Physical parameters associated with freezing were virtually the same for the two subspecies, including body weight (4.3–4.8 g), body water content (76–77% water), osmolality of body fluids of control animals (275 mosmol/L), and percent body water as ice after 3 d at −2.5°C (43.5–46.5%) or 4 h at −4°C (49.8–52.6% ice) (Churchill and Storey 1992*a*). Not surprisingly, then, freezing survival capacity was also similar; autumn-collected hatchlings of both *C. p. marginata* and *C. p. bellii* showed 100% recovery after 3 d of continuous freezing at −2.5°C, whereas 100% recovery extended to 3–5 h at −4°C (Churchill and Storey 1992*a*). Freezing at −4°C was chosen for the present study because we predicted that metabolic changes would be triggered and develop rapidly at this temperature.

Many freeze-tolerant animals produce low molecular weight cryoprotectants, usually sugars or polyhydric alcohols, that are packed into cells and used to help minimize the reduction of cell volume that occurs as the consequence of ice formation in extracellular spaces (Storey and Storey 1988). Among freeze-tolerant amphibians, glucose or glycerol, at high concentrations (often 100–300 μmol/g wet weight), are the typical cryoprotectants (Schmid 1982; Storey and Storey 1984, 1985, 1986*a*). Our initial analysis of painted turtle hatchlings focused on spring-collected *C. p.marginata* and documented a significant increase in glucose levels as a result of freezing (for 24 h at −4°C) in blood and several organs (Storey et al. 1988). Blood amino acid levels also doubled during freezing (largely because of an increase in taurine), and a small increase in the blood glycerol level occurred. The overall accumulation of putative cryoprotectants was small, however, since levels of the major component, glucose, reached a maximum of only 16 μmol/g wet weight in blood. However, earlier studies with freeze-tolerant frogs had shown that the capacity for cryoprotectant synthesis in response to freezing was much reduced in the spring compared with the autumn because liver glycogen reserves were rapidly depleted to fuel activity in the early weeks, before feeding

commences (Storey and Storey 1987). The same situation might also apply to spring-emerged turtles. To test this we examined the effects of freezing on autumn-collected hatchlings. Glycerol was not found in *C. p. bellii*, and only about 5.5 μmol/g built up in liver of *C. p. marginata* (Churchill and Storey 1992*a*). Changes in free amino acid levels during freezing were also minor in turtle organs, but the total pool size was high (18–25 μmol/g, composed mainly of taurine) and so might contribute to cryoprotection.

The present study assesses the role played by glucose and lactate in cryoprotection of turtle organs and the regulation of liver glycolysis that supports the production of these compounds. Time-dependent changes in glucose concentration in the organs of both species occurred over the course of 4 h of freezing at −4°C. For midland painted turtle hatchlings, glucose concentrations were rapidly elevated when freezing began, increasing 2.4- to 5-fold in liver, brain, heart, kidney, and gut within the first 30 min of freezing exposure, with maximal amounts of 8–9 μmol/g in liver and brain and 14 μmol/g in skeletal muscle (Fig. 1). Glucose levels did not remain elevated, however, but decreased over time, though remaining well above control levels in several organs after 4 h, in line with our earlier analysis of 24-h-frozen *C. p. marginata* (Storey et al. 1988). Glucose levels in *C. p. bellii* hatchlings responded somewhat differently during freezing. Glucose levels in all organs of controls were substantially higher for *C. p. bellii* (Fig. 4) than for *C. p. marginata*. Over the time course of freezing, the glucose level continued to increase in liver of *C. p. bellii* (suggesting continued liver glycogenolysis), but in other organs the levels either remained elevated and not significantly different from controls (skeletal muscle, lung, gut) or decreased from the control values (heart, brain, kidney).

The controls used for both subspecies were turtles handled in exactly the same way as the experimental animals but chilled only until a body temperature of 0°C was reached. Glucose levels in liver of these controls, 1.76 ± 0.23 μmol/g for *C. p. marginata* and 6.79 ± 2.57 μmol/g for *C. p. bellii*, can be compared with liver glucose contents of comparable hatchlings sampled directly from the 5°C holding containers: 2.6 ± 0.61 and 1.7 ± 0.4 μmol/g, respectively (Churchill and Storey 1992*a*). Obviously, then, the *C. p. bellii* hatchlings responded to chilling to 0°C (or perhaps to the handling involved in experimentation) with a rapid elevation of glucose level, whereas the *C. p. marginata* hatchlings triggered glucose output only in response to freezing. The former response is reminiscent of the anticipatory synthesis of cryoprotectants that is seen in insects, whereas the latter response is what occurs in freeze-tolerant frogs; cooling and (or) time spent in the supercooled state do not alter glucose levels, but glucose output from frog liver is triggered in less than 5 min following the appearance of the freezing exotherm (Storey and Storey 1988). *Chrysemys picta bellii* hatchlings may have altered the trigger used to stimulate glucose output from liver and made it responsive to cooling rather than to freezing. This may allow the animals to "anticipate" freezing as temperatures within the hibernacula decrease, and prepare ahead of time with an appropriate accumulation of cryoprotectants in all organs. This could enhance the survival chances of the western subspecies, whose range extends substantially farther north than that of the midland subspecies (Cook 1984) despite the generally colder midcontinental climate.

However, is glucose a cryoprotectant for turtle organs? Certainly, the overall glucose levels in the hatchlings are not high; the maximal level measured over the 4 h of freezing exposure was 14 μmol/g in *C. p.marginata* skeletal muscle and 16 μmol/g in *C. p. bellii* liver (Figs. 1, 4) and amounts measured after longer freezing exposure of either autumn or spring hatchlings rose to only 14–18 μmol/g in liver and the same or less in other organs (Storey et al. 1988; Churchill and Storey 1992*a*). Compared with the 50–250 μmol/g rise in glucose level in the organs of freeze-tolerant frogs (Storey and Storey 1986*a*, 1986*b*), this additional glucose would have only a minor colligative effect on cell volume changes in turtle organs during extracellular ice formation. Furthermore, the glucose concentration in all turtles organs rose to its maximum either during the cooling procedure or shortly after freezing began, subsequently declining or remaining constant. In the wood frog, by contrast, glucose accumulation continued for at least 18–48 h after nucleation occurred and remained elevated throughout freezing episodes, to decline only after thawing (Storey and Storey 1986*b*). Thus, although the glucose accumulated by turtle organs would make a contribution to the overall low molecular weight osmolyte pool that is available to contribute to cell volume regulation during freezing, the amounts are too small to have a major effect as a colligative cryoprotectant. Furthermore, the pattern of changes in glucose concentration seen in the hatchlings turtles over the time course of freezing is, in fact, more reminiscent of the expected changes in concentration of a substrate for a newly activated metabolic pathway than the concentration of a stable product that is the output of the pathway (as occurs in frogs).

What could glucose be the substrate for? The obvious answer suggested by the present data is lactate. Glucose can be readily converted to lactate via glycolysis, so glucose distributed via the blood from a central liver glycogen reserve could be readily converted to lactate in all organs. Lactate levels increased rapidly in most organs of both subspecies, with particularly high levels, 15–25 μmol/g, found in the organs of the western subspecies. Although lactate levels showed a tendency to decease over time in organs of *C. p. marginata* in the present study (Fig. 1), our previous analysis of spring-collected *C. p. marginata* showed high levels, 12–38 μmol/g in all organs after 24 h of freezing at −4°C (Storey et al. 1988). One advantage of lactate as a cryoprotectant is the colligative bonus of using a triose rather than a hexose, for 2 moles of lactate can be produced from the same 1 mole of hexose phosphate that produces only a single mole of glucose. A disadvantage of lactate, however, is the acidosis that is typically associated with its accumulation. However, unlike most other vertebrates, painted turtles (and several of their freshwater relatives) have an excellent tolerance for extremely high concentrations of lactate in their body fluids (Jackson and Ultsch 1982; Herbert and Jackson 1985; Ultsch and Wasser 1990). This capacity is part of the anoxia tolerance of the species that allows adult painted turtles to survive up to 3–4 months while hibernating in cold waters (Ultsch 1985). Plasma lactate concentrations as high as 200 μmol/mL are endured by means of adaptive strategies that include a high buffering capacity of body fluids and compensatory ion changes that counteract the accumulation of the lactate anion (Jackson and Ultsch 1982). This capacity for lactate tolerance, developed for anaerobiosis by adult turtles, may be first put to use by hatchlings in northern climates for enduring accumulation of lactate as a cryoprotectant.

The freezing of extracellular body fluids imposes an ischemic state upon organs that quickly leads to a state of anoxia (Storey 1990; Storey and Storey 1988). It could be argued, then, that the observed accumulation of lactate by turtle organs during freezing might have an alternative origin, as the product of anaerobic

energy metabolism within ischemic organs. However, the absolute amounts of lactate generated seem to be too high for the short time (4 h) and low body temperature (between −1 and −4°C over the first 4 h of freezing (Churchill and Storey 1992*a*)) involved. Thus, net lactate accumulation was as much as 13 μmol/g for *C. p. marginata* brain and 24 μmol/g for *C. p. bellii* lung (Figs. 1, 4) within the first hour of freezing exposure. However, in organs of adult *C. picta* submerged in anoxic water at 7°C the net lactate accumulation was only 1.6 μmol/g or less after 5 h of anaerobiosis and 2–4.6 μmol/g after 20 h (Duncan and Storey 1992 and unpublished data); at 10°C, less than 10 μmol/g lactate accumulated in blood over the first 24 h (Ultsch and Wasser 1990). Given these rates for lactate synthesis in anoxia at temperatures above 0°C, we would expect lactate accumulation to be even slower at subzero temperatures if lactate output was linked solely to anoxic ATP production. Thus, a cryoprotectant function for the rapid accumulation of large organ lactate pools seems to be supported.

Overall, however, the levels of glucose, lactate, amino acids, and other putative cryoprotectants accumulated by hatchling painted turtles are low, with total net accumulations of only about 50 μmol/g, far less than the several hundred micromoles per gram found in frogs and freeze-tolerant insects (Storey and Storey 1988). Furthermore, measurements of blood osmolality (275–290 mosmol/L; K. B. Storey and D. G. McDonald, unpublished results) are normal for vertebrates, and there is no unexplained increased in osmolality in freezing-exposed turtles that would indicate the addition of large quantities of an unidentified cryoprotectant. Although the cryoprotectant status of field populations of *C. picta* hatchlings in midwinter has yet to be determined, the available data for both autumn- and spring-collected animals suggest that high levels of colligative cryoprotectants are not needed by this species. The reasons for this are not yet known.

Liver plays a central role in cryoprotectant metabolism in freeze-tolerant frogs (Storey and Storey 1986*b*; Storey 1987), and as in the frogs, turtle liver has the large glycogen pool that suggests that this organ is the central carbohydrate reservoir for cryoprotectant synthesis (Storey et al., 1988; Churchill and Storey 1992*a*). Analysis of changes in the levels of glycolytic intermediates over time in liver of both subspecies indicated the status of cryoprotectant production. In *C. p. marginata* the sharp increase in liver G6P concentrations within the first 30 min indicated activation of glycogen phosphorylase in response to a freezing trigger; the same response by G6P occurred in liver of the freeze-tolerant wood frog (Storey 1987*b*), and in both turtles and frogs is the result of a freezing-induced increase in the percentage of glycogen phosphorylase in the active form, *a* (Storey 1987; Churchill and Storey 1992*a*). Freezing also activated phosphorylase in *C. p. bellii* liver (Churchill and Storey 1992*a*), but G6P levels were not different from the control (chilled to 0°C) values over the time course of freezing exposure (Fig. 5). However, this is not unexpected for *C. p. bellii* liver in light of the increase in glucose concentration stimulated by cooling as opposed to freezing (as discussed earlier). Changes in the levels of F6P (an increase) and $F1,6P_2$ (a decrease), the substrate and product of PFK, in liver of both subspecies were consistent with an inhibitory control on the glycolytic rate at this enzyme locus. This also occurs during cryoprotectant synthesis in frogs (Storey 1987), and inhibition at the PFK locus is a common means of promoting glucose export from vertebrate liver. The data did not support the involvement of other regulatory enzymes (e.g., pyruvate kinase) in the regulation of liver glycolysis during freezing, although elevated levels of PYR in *C. p. bellii* liver are consistent with the high lactate levels accumulated in the organ and the equilibrium nature of the lactate dehydrogenase reaction.

Skeletal muscle of both midland and western hatchlings was consistently different from other organs in showing sustained high glucose levels and substantially lower lactate levels than other organs. It is not known whether this indicates a different cryoprotectant strategy of muscle or slower glucose to lactate conversion by this peripheral organ which typically freezes much more rapidly than internal organs. Changes in F6P and $F1,6P_2$ levels in *C. p. bellii* muscle indicated activation of PFK, suggesting that the glycolytic rate could be increased as a result of freezing-induced ischemia.

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