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Physiological responses to supercooling and hypoxia in the hatchling painted turtle, *Chrysemys picta*

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Abstract We investigated physiological responses to supercooling in hatchling painted turtles (*Chrysemys picta*) which remain in their natal nests over winter and therefore may become exposed to subzero temperatures. These turtles are freeze tolerant but also must rely on supercooling to survive exposure to the lower temperatures occurring in nests during winter. We compared whole-body concentrations of lactate, glucose, glycerol, and ATP in turtles chilled at 0 °C, –4 °C, or –6 °C for 5 days, or at –6 °C for 19 days. In a companion experiment, we measured metabolite concentrations in turtles exposed to a hypoxic environment for 1 day, 4 days, or 8 days. Supercooling and hypoxia exposure were both associated with an increase in concentrations of lactate and glucose and a decrease in glycerol concentrations (albeit no change in the ATP pool), suggesting that supercooling induces functional hypoxia. We conclude that hypoxia tolerance may be an important pre-adaptation for surviving exposure to subzero temperatures in hatchling *C. picta*.

Keywords *Chrysemys picta* · Supercooling · Hypoxia · Metabolite · Hibernation

Abbreviation T_b body temperature

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Introduction

Hatchling painted turtles (*Chrysemys picta*) commonly hatch in late summer but remain in their shallow subterranean nests over winter and emerge after warm weather returns in spring. In northern areas, particularly those where snow cover is ephemeral or lacking, hatchling *C. picta* may become exposed to temperatures that fall well below the equilibrium freezing point of their tissues. This species apparently tolerates the freezing of body fluids so long as body temperature (T_b) remains above approximately –4 °C and the period of exposure is relatively brief (Storey et al. 1988; Churchill and Storey 1992). However, survival of the turtles at T_bs lower than this depends on their capacity for supercooling, which is exceptional among cold-hardy vertebrates (see review by Lee and Costanzo 1998). Laboratory studies suggest that hatchling *C. picta* may supercool to T_bs as low as –20 °C so long as they are isolated from contamination or contact with ice nuclei, which otherwise would trigger the freezing of their body fluids (Costanzo et al. 2000).

Relatively little is known about the tolerance and physiological responses of hatchling *C. picta* to supercooling. Some work suggests that these turtles can recover from acute chilling to T_bs as low as –10 °C (Costanzo et al. 1999), but they succumb at T_bs lower than this (Packard and Packard 1993, 1999). Hartley et al. (2000) reported that some turtles died during a 25-day exposure to –8 °C. These workers also found that lactate accumulated during supercooling and that turtles having especially high lactate loads exhibited delayed recovery of behavioral function during rewarming.

Although Hartley et al. (2000) provided convincing evidence that supercooling can perturb physiological function in hatchling *C. picta*, the conclusions drawn from their investigation were limited. Measuring concentrations of metabolites in addition to lactate might have provided important insight into the nature of the physiological stress, metabolism, and potential use of

cryoprotectants in supercooled turtles. Therefore, our primary objective in the present investigation was to collect information about the fundamental physiological responses of hatchling *C. picta* to supercooling under ecologically relevant conditions of temperature and exposure duration. Secondly, we wished to test the hypothesis that the physiological changes occurring in turtles exposed to low temperatures are induced by functional hypoxia.

Materials and methods

Animals

Eggs of 32 *C. picta bellii* (Gray) endemic to Garden County, west-central Nebraska (41°N, 102°W) were collected in summer 1999 by inducing females to oviposit by injecting them with oxytocin. Approximately 210 of the eggs were placed in 16 artificial nests constructed at the collection site. These nests were excavated on 9 April 2000 and a group of the recovered hatchlings were placed in canisters containing soil from the nest cavities and shipped under refrigeration to laboratory facilities at Miami University. Turtles were kept in boxes containing damp soil indigenous to the collection site and held at 4 °C in a darkened incubator until used in the experiments in May-June. Our previous experience indicates that hatchling *C. picta* retain cold hardiness under these conditions for many months.

Physiological responses to supercooling

We measured concentrations of key metabolites in the bodies of turtles exposed to target minimum T_bs of 0 °C, −4 °C, or −6 °C ($n = 5$, each group) for 5 days. To investigate responses to long-term chilling, additional turtles ($n = 5$) were kept supercooled at −6 °C for 19 days. Control turtles ($n = 5$) were taken for study directly from their holding boxes in the 4°C incubator. Turtles were prepared for study by gently brushing adherent soil from their surfaces and then holding them at 4 °C in sheltered boxes for approximately 24 h. This procedure permitted evaporation of surface moisture which otherwise might freeze and inoculate tissues of the turtles exposed to subzero temperatures (see Packard and Packard 1993). The clean, dry turtles were then weighed, placed individually in 25 × 200 mm glass tubes, and insulated by loosely filling the space above them with plastic foam. The tubes were then suspended in a crushed-ice bath (to achieve a T_b of 0 °C) or a refrigerated ethanol bath (to achieve a T_b of −4 °C or −6 °C) that was initially set at 0 °C. Turtles in the refrigerated baths were cooled 2 °C each day until they attained the target minimum T_b. We monitored temperature inside each tube by placing the tip of a copper-constantan thermocouple near the turtle and recorded temperature at 30-s intervals on a datalogger (Omega model RD3752; Stamford, Conn., USA). At the conclusion of the exposure, turtles were promptly removed from their tubes and killed by submerging them in liquid N_2.

Metabolite analyses

In preliminary tests, we were unable to collect blood expeditiously from supercooled turtles, even if they were rewarmed before sampling. Therefore, we prepared whole-body homogenates by removing turtles from the liquid N_2, quickly crushing them with hammer blows, and homogenizing them in 12 ml of ice-cold $HClO_4$ (7% v/v) in a Waring mini blender. The homogenates were transferred to 50-ml polypropylene centrifuge tubes and the blender was rinsed thrice with 1-ml volumes of the acid solution, which were added to the homogenate. The tubes were incubated on ice for 30 min and then centrifuged (4,000 *g*, 10 min) to precipitate the proteins. The supernatants were reserved on ice while the pellets were washed in 5 ml $HClO_4$ and again centrifuged. The initial and final supernatants were combined, filtered with suction through Whatman no. 1 paper, and the resulting filtrate was brought to volume (20 ml) with additional acid solution.

We used enzymatic assays to measure concentrations of lactate (Sigma, St. Louis, Mo., USA; no. 735), glucose (Sigma, no. 510), glycerol (Sigma, no. 337), and ATP (Sigma, no. 366). We measured the ATP concentration, in duplicate, of freshly prepared homogenates, whereas concentrations of the other metabolites were measured in acid extracts that were briefly stored at −80 °C, thawed, and neutralized with KOH.

Recovery and survival of supercooled turtles

We investigated physiological recovery from supercooling by sampling additional turtles, after exposure to −6 °C for 5 days, at intervals of 6 h ($n = 5$) or 24 h ($n = 5$) following their return to the 4 °C incubator. These turtles were killed, homogenized, and analyzed as described above. To determine the effect of supercooling on survival, turtles held for 5 days at target T_bs of 0 °C, −4 °C, or −6 °C ($n = 5$–8 per treatment group) were returned to their holding boxes in the 4 °C incubator and monitored over the course of 1 week.

Physiological responses to hypoxia exposure

Turtles were prepared for study by gently brushing adherent soil from their surfaces and then holding them in sheltered boxes for approximately 24 h. The clean, dried turtles were then weighed and placed individually in 25 × 200 mm glass tubes, which were then flushed with N_2 gas for 10 s and quickly closed with a rubber stopper. The turtles ($n = 5$, each group) were kept at 4 °C, in darkness, until sampled 1 day, 4 days or 8 days later. They were quickly removed from their tubes, immersed in liquid N_2, homogenized, and assayed for metabolite concentrations as described above.

Statistical analyses

Analysis of variance (ANOVA) was used to compare mean body mass among treatment groups. Within experiments, mean metabolite concentrations were compared among treatment groups using ANOVA. Post-hoc multiple comparisons were made using Student-Newman-Keuls, except that Bonferroni was used to compare treatment groups to the control in the recovery experiment. Statistical significance was set at $P \leq 0.05$.

Results

In pilot tests, turtles were unable to remain supercooled at −8 °C. Several turtles froze spontaneously at this T_b probably because, having hatched in the field, they were contaminated with ice nuclei that commonly occur in nesting soils (Costanzo et al. 2000). However, each of the 12 turtles exposed to −4 °C, and all but one of the 28 turtles exposed to −6 °C, remained supercooled for the duration of the chilling episodes. The turtle that spontaneously froze (and died) was examined for metabolite concentrations, but the data for this individual were excluded from the statistical analyses.

Viability of turtles after chilling

All turtles in each treatment group survived exposure to 0 °C ($n = 5$), −4 °C ($n = 7$), or −6 °C ($n = 8$) for 5 days.

Arousal of the turtles exposed to 0 °C or -4 °C was prompt and they quickly burrowed into the soil in their holding boxes and were responsive to tactile stimulation of their head and limbs. In contrast, turtles exposed to -6 °C for 5 days remained on the soil surface and were lethargic early in the recovery period. However, within 72 h they had burrowed into the soil and exhibited responsiveness to tactile stimulation. We did not examine survival of turtles exposed to -6 °C for 19 days; however, given that ATP concentrations in turtles treated in this way were similar to those of controls (see below), we suspect that our hatchling *C. picta* would have tolerated these conditions.

Metabolite concentrations in supercooled turtles

Body mass (mean ± SD = 3.5 ± 0.1 g; $n = 50$) did not vary among treatment groups in any of our experiments ($P > 0.05$); therefore, metabolite concentrations were expressed as micromoles per gram fresh body mass. We compared metabolite concentrations among turtles held at 4 °C (controls), turtles exposed to 0 °C, -4 °C, or -6 °C for 5 days, and turtles exposed to -6 °C for 19 days, finding marked differences in the concentrations of lactate ($F_{4,19} = 32.1$, $P < 0.0001$), glucose ($F_{4,19} = 14.1$, $P < 0.0001$), and glycerol ($F_{4,19} = 4.1$, $P = 0.015$). Lactate levels in turtles exposed to -6 °C for 5 days were three-fold higher than in controls (Fig. 1). Holding turtles at -6 °C for an additional 14 days further increased their lactate concentrations. Glucose levels also increased markedly (up to six-fold) in supercooled turtles; however, unlike the case with lactate, concentrations of glucose decreased slightly during prolonged exposure to -6 °C. Chilling the turtles tended to decrease their glycerol levels, but the change was significant in only two cases (Fig. 1). Mean (±SEM) ATP concentrations ranged from 0.74 ± 0.04 to 0.88 ± 0.05 μmol·g^{-1} but did not differ statistically ($F_{4,19} = 1.0$, $P = 0.41$) among treatment groups.

Concentrations of lactate (15.5 μmol g^{-1}) and glycerol (0.03 μmol g^{-1}) in the turtle that froze during exposure to -6 °C were similar to those of turtles that remained supercooled at -6 °C. However, concentrations of both glucose (1.0 μmol g^{-1}) and ATP (0.23 μmol g^{-1}) in this turtle were markedly lower than concentrations in its unfrozen counterparts.

Results for the turtles returned to the 4 °C incubator after being exposed to -6 °C for 5 days indicated that physiological recovery would require more than 24 h (Table 1). Lactate and glucose concentrations in turtles sampled after 6 h or 24 h of recovery were ca. four-fold to five-fold higher than in control animals. Furthermore, lactate concentrations were higher ($P < 0.05$) in these turtles than in turtles sampled immediately after exposure to -6 °C (6.8 μmol g^{-1}), suggesting that lactate production continued even after the animals were rewarmed. Glycerol levels during recovery from supercooling were depressed relative to control levels, but the differences were not statistically significant ($P > 0.05$). Mean ATP concentrations did not vary among treatment groups (Table 1).

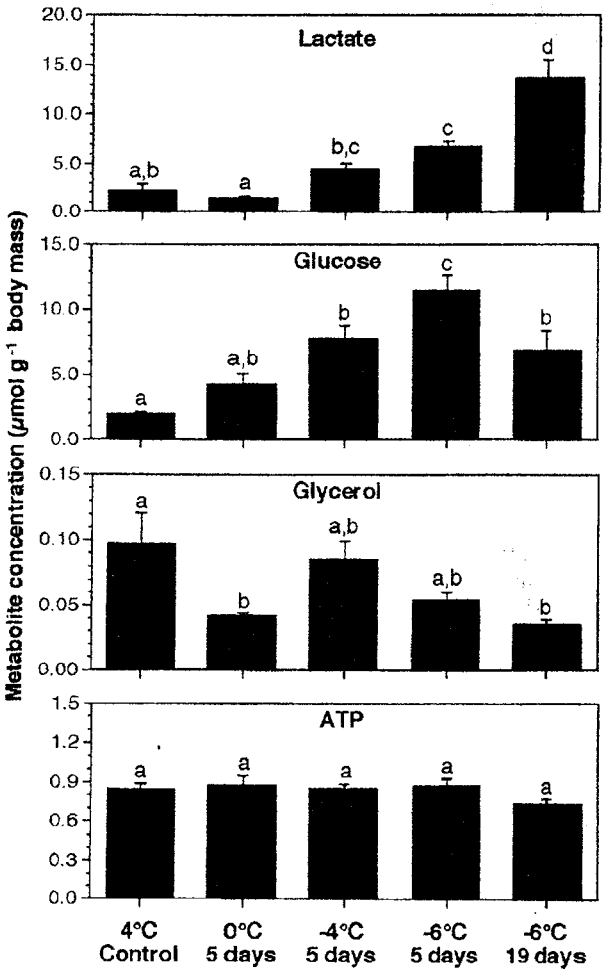

Fig. 1 Whole-body concentrations of metabolites in hatchling turtles held at 0 °C or held supercooled at -4 °C or -6 °C, compared to control animals. Mean values (± 1 SEM) are based on $n = 5$ turtles per group, except $n = 4$ turtles in the -6 °C, 19-day group. Values identified by similar letters were statistically indistinguishable (ANOVA, Student-Newman-Keuls; $P > 0.05$)

Metabolite concentrations in hypoxic turtles

Turtles tolerated environmental hypoxia for up to 8 days. They were generally quiescent, albeit responsive to external stimuli, during the trials.

We found marked differences in the concentrations of lactate ($F_{3,16} = 5.3$, $P = 0.010$), glucose ($F_{3,16} = 3.9$, $P = 0.028$), and glycerol ($F_{3,16} = 11.1$, $P < 0.001$) among control animals (held at 4 °C, under normoxic conditions) and turtles exposed to 4 °C in a hypoxic environment for 1 day, 4 days, or 8 days (Fig. 2). Turtles showed no change in lactate or glucose concentration

Table 1 Metabolite concentrations (μmol·g^{-1} body mass) in hatchling painted turtles (*Chrysemys picta*) after a 6-h or 24-h period of recovery from exposure to −6 °C relative to control animals maintained at 4 °C. Means (± 1 SEM) are based on $n = 5$ turtles per group. P column = probability that means within the same row differed significantly (ANOVA)

Metabolite	Control	6-h recovery	24-h recovery	$F_{2,12}$	P
Lactate	2.2 ± 0.7	10.2 ± 0.8*	12.0 ± 2.3*	12.8	0.001
Glucose	1.9 ± 0.1	7.8 ± 1.4*	8.2 ± 2.0*	6.2	0.014
Glycerol	0.10 ± 0.02	0.06 ± 0.01	0.06 ± 0.02	1.4	0.29
ATP	0.84 ± 0.05	0.96 ± 0.04	0.75 ± 0.15	1.4	0.29

*Means that differed significantly from the control (Bonferroni)

within the 1st day; however, after 4 days, concentrations of these metabolites were approximately 2.5-fold higher than in control animals (Fig. 2). Unexpectedly, lactate and glucose levels fell, rather than increased further, with additional hypoxia exposure, and concentrations in the 8-day turtles did not differ from those of control animals. Concentrations of glycerol were reduced by 80–90% in hypoxic turtles, even in those sampled after the first day of hypoxia exposure (Fig. 2). In contrast, mean ATP concentrations did not vary ($F_{3,16} = 1.8$, $P = 0.18$) among the treatment groups (Fig. 2), indicating that turtles maintained ATP homeostasis during hypoxia exposure.

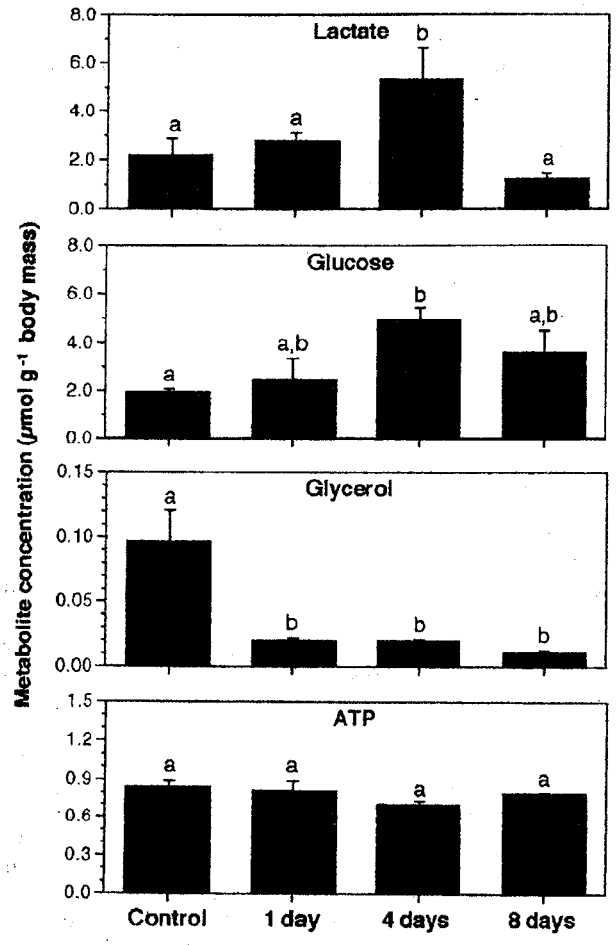

Fig. 2 Whole-body concentrations of metabolites in hatchling turtles held at 4 °C under hypoxic conditions for 1 day, 4 days, or 8 days, or held at 4 °C under normoxic conditions (control). Means (± 1 SEM) are based on $n = 5$ turtles per group. Values identified by similar letters were statistically indistinguishable (ANOVA, Student-Newman-Keuls; $P > 0.05$)

Discussion

Hatchling *C. picta* survive episodic exposures to subzero T_bs by tolerating the freezing of their tissues or by remaining supercooled. Some workers have investigated biochemical and physiological adaptations of these turtles to freezing (Storey et al. 1988; Churchill and Storey 1992; Hemmings and Storey 2000). However, with the exception of one behavioral study (Costanzo et al. 1999) and two physiological studies (Birchard and Packard 1997; Hartley et al. 2000), little attention has been paid to the responses of the turtles to supercooling. Our primary objective in this project was to examine changes in metabolite concentrations in turtles supercooled under ecologically relevant conditions of temperature and exposure duration.

In the laboratory, mortality in the absence of somatic freezing occurs in hatchling *C. picta* acutely exposed to T_bs below −10 °C (Packard and Packard 1993, 1999) or held for long periods at slightly higher T_bs (e.g., 25 days at −8 °C; Hartley et al. 2000). However, because turtles infrequently encounter such extreme conditions within their nests, we do not know whether they commonly succumb to supercooling in nature. Analysis of seasonal thermal dynamics in soil at our study site in the Sandhills of west-central Nebraska indicates that, although transient exposure to low T_bs (e.g., <−6 °C) occasionally occur, most subzero chilling episodes are relatively moderate (Costanzo et al. 1995). Other field studies (DePari 1988; Packard 1997; Nagle et al. 2000) also suggest that episodes of supercooling (or somatic freezing) would commonly involve brief exposures to relatively high temperatures (e.g., ≥−4 °C). Nevertheless, the physiological responses of supercooled turtles are of interest inasmuch as they may provide clues to the development of cold hardiness in *C. picta*.

The cause of mortality in hatchling *C. picta* during prolonged supercooling episodes is not known. Hartley et al. (2000) attributed death of their turtles, held at −8 °C for 25 days, to accumulation of lactate in response to a "stagnant hypoxia," which likely reflects reduced perfusion in peripheral tissues and an ultimate failure of the circulatory system. Although the lactacidosis itself is unlikely to be damaging (Levine 1993), our data support their contention that ischemic hypoxia develops in supercooled turtles, possibly as a consequence of low cardiac output and elevated blood viscosity. Notably, heart rate declines by approximately 80% in hatchling *C. picta* cooled from 4 °C to −6 °C (Birchard and

Packard 1997) and the attendant fall in shear rate undoubtedly compounds the effect of cold on blood viscosity (Saunders and Patel 1998). However, we do not know whether mortality associated with prolonged supercooling stems from perturbation of acid-base status or a failure to meet ATP demands. In our study, concentrations of ATP were maintained in turtles that very likely survived 5-day bouts of supercooling to moderate temperatures. They were also maintained in the turtles held for 19 days at –6 °C; however, we did not determine survival rates of turtles exposed to these conditions. Whether ATP homeostasis can be preserved during longer exposures and at lower T_bs remains to be determined.

A common, but perhaps erroneous, belief is that supercooling is a relatively benign state from which animals rapidly recover (Costanzo and Lee 1995). Some evidence suggests that the degree of physiological perturbation incurred during supercooling (and also with somatic freezing; e.g., Layne et al. 1998), increases with decreasing T_b and with increasing duration of exposure. Recovery of normal behaviors took longer in turtles exposed to –6 °C than it did in turtles supercooled to –4 °C, and elevated metabolite levels persisted in these turtles at least 24 h after they rewarmed to 4 °C. Delayed recovery from supercooling may stem from the effects of hypoxia on nervous tissue function (Wegener et al. 1986) and/or the deleterious consequences of reperfusing ischemic tissues (Levine 1993). Hartley et al. (2000), who noted a delayed recovery of behavioral function in turtles held supercooled at –8 °C for ≥15 days, questioned whether turtles might emerge, in spring, from nests in a debilitated state. Further work is needed to assess the costs associated with supercooling.

Hatchling *C. picta* accumulated lactate and glucose during supercooling, as they do during somatic freezing (Storey et al. 1988; Churchill and Storey 1992; Hemmings and Storey 2000), probably in response to functional hypoxia. Possibly, our turtles might have mobilized more of these compounds had they been tested in winter, rather than in spring. On the other hand, our results for lactate seem consistent with those of Hartley et al. (2000), who presumably studied winter turtles: the concentration of lactate in our turtles exposed to –6 °C for 19 days (14 $\mu mol \cdot g^{-1}$) was intermediate in comparison to the concentrations they reported for turtles exposed to –4 °C or –8 °C for 25 days.

The hyperglycemic response in our supercooled turtles likely resulted from hypoxia-induced, β-adrenergic stimulation of glycogenolysis in liver (Keiver and Hochachka 1991). This process may account for the elevated glucose levels found in hatchling *C. picta* extricated from their natal nests during winter (DePari 1988; Churchill and Storey 1992), although hepatic glycogenolysis is also triggered by somatic freezing (Storey et al. 1988; Hemmings and Storey 2000). The mobilization of glucose provides an important substrate in anaerobic metabolism, and possibly a cryoprotectant, in both supercooled and frozen turtles.

Glycerol concentrations tended to fall in supercooled turtles, perhaps because triglyceride catabolism, a primary source of glycerol, is diminished in hypoxic tissues as the primary energy substrate typically shifts from lipids to carbohydrates (e.g., Donohoe and Boutilier 1998). The decrease in glycerol concentration may also indicate that this compound is used in glycolysis and/or gluconeogenesis during chilling. On the other hand, some evidence suggests that glycerol levels in hatchling *C. picta* may increase during somatic freezing (Storey et al. 1988; Churchill and Storey 1992).

Our finding that hatchling *C. picta* can tolerate at least 8 days of hypoxia is perhaps not surprising given the exceptional anoxia tolerance exhibited by adults of this species. Survival without oxygen is promoted by a sizeable hepatic glycogen reserve, abilities to mitigate and cope with metabolic acidosis and redirect blood flow to anoxia-sensitive organs, and a profound decrease in standard metabolism (Keiver and Hochachka 1991; Hochachka 1997). The increase in glucose and lactate levels reflects the so-called Pasteur effect, the body's effort to meet extant demands for ATP via anaerobic metabolism (Lutz and Storey 1997). Anoxic turtles ultimately establish a new metabolic equilibrium where metabolic rates may be only 10% of those seen in normoxic animals (Hochachka 1997), and this transition may explain the reduced production of lactate and glucose in our hatchling *C. picta* after 4 days of hypoxia exposure (Fig. 2). A similar response is seen in hibernating frogs (*Rana temporaria*) undergoing metabolic adjustment to hypothermic hypoxia (Donohoe and Boutilier 1998). Nevertheless, we were surprised to find that the lactate that had accumulated by the fourth day of hypoxia exposure apparently had been lost during the subsequent 4 days of exposure (Fig. 2). The lactate may have been metabolized; however, so far as is known, this is an aerobic process. Although it does not seem likely, we cannot exclude the possibility that a small amount of oxygen remained in the holding vessels.

The remarkable anoxia tolerance in *C. picta*, as previously demonstrated in adults, has been credited as being a major factor in the development of freeze tolerance in this species (Storey et al. 1988; Churchill and Storey 1992). Our results suggest that the chilling of unfrozen turtles imposes a functional hypoxia, and, therefore, that anoxia tolerance may also be an important pre-adaptation to surviving in the supercooled state.

The physiology of our turtles was perturbed more by supercooling than by hypoxia exposure, as judged by the degree of mobilization of lactate and glucose. Furthermore, whereas our turtles apparently eliminated accumulated lactate (and glucose) and slowed development of lactacidosis after 4 days of hypoxia exposure, they continued to accumulate lactate for as long as supercooling was sustained (Fig. 1; see also Hartley et al. 2000). Supercooled turtles, which may lack effective circulation and therefore cannot buffer the lactate load (e.g., Jackson 2000) or distribute glucose to sensitive tissues,

apparently cope less well with oxygen lack than do turtles exposed to environmental hypoxia at higher temperatures. Lactate may reach high levels during supercooling (Hartley et al. 2000) and may even continue to increase after turtles are rewarmed (Fig. 1; Table 1). Glucose levels may ultimately decrease (Fig. 1), perhaps because this compound is used in glycolysis. We do not know how long the lactacidosis and hyperglycemia might persist; however, it is possible that these compounds accumulate with successive chilling episodes throughout the winter. Whether the effects of an unabated lactacidosis ultimately prove harmful, either by causing winter mortality directly, or by diminishing fitness of turtles during or after spring emergence (Hartley et al. 2000), remains to be determined.

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