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Patterns of variation in glycogen, free glucose and lactate in organs of supercooled hatchling painted turtles (*Chrysemys picta*)

Mary J. Packard* and Gary C. Packard

Department of Biology, Colorado State University, Fort Collins, CO 80523-1878, USA

*Author for correspondence (e-mail: kpackard@lamar.colostate.edu)

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Summary

Hatchling painted turtles (*Chrysemys picta*) typically spend their first winter of life in a shallow, subterranean hibernaculum (the natal nest), where they may be exposed for extended periods to ice and cold. The key to their survival seems to be to avoid freezing and to sustain a state of supercooling. As temperature declines below 0°C, however, the heart of an unfrozen turtle beats progressively slower, the diminished perfusion of peripheral tissues with blood induces a functional hypoxia, and anaerobic glycolysis assumes ever greater importance as a source of ATP. We hypothesized that diminished circulatory function in supercooled turtles also reduces the delivery of metabolic substrates to peripheral tissues from central stores in the liver, so that the tissues depend increasingly on endogenous stores to fuel their metabolism. We discovered in the current investigation that part of the glycogen reserve in hearts and brains of hatchlings is mobilized during the first 10 days of exposure to –6°C but that glucose from hepatic glycogen supports metabolism of the organs thereafter. Hatchlings that were held at –6°C for 10 days and then at +3°C for another 10 days were able to reconstitute some of the reserve of glycogen in heart and liver but not the glycogen reserve in brain. Patterns of accumulation of lactate in individual organs were very similar to those reported for whole animals in a companion study, and point to a high degree of reliance on anaerobic metabolism at –6°C and to a lesser degree of reliance on anaerobiosis at higher subzero temperatures. Lactate had returned to baseline levels in organs of animals that were held for 10 days at –6°C and for another 10 days at +3°C, but free glucose remained elevated. Indeed, carbohydrate metabolism probably does not return to the pre-exposure state in any of the major organs until well after the exposure to subzero temperatures has ended, circulatory sufficiency has been restored, and aerobic respiration has fully supplanted anaerobic respiration as a source of ATP.

Key words: turtle, hibernation, supercooling, metabolism, glycogen, glucose, lactate, heart, brain, liver.

Introduction

The North American painted turtle [(Family Emydidae: *Chrysemys picta* (Schneider 1783)] has a natural history unlike that of most other chelonians occurring in regions characterized by long, cold winters. Whereas hatchlings of other aquatic turtles typically emerge from their subterranean nest in late summer and move to a nearby marsh, lake or stream to spend their first winter, hatchling painted turtles usually spend their first winter inside the shallow chamber where they completed incubation (Wilbur, 1975; Ultsch, 1989; Ernst et al., 1994). As a result of this behavior, hatchling painted turtles are commonly exposed for extended periods in mid-winter to subzero temperatures and frozen soil (DePari, 1996; Packard, 1997; Packard et al., 1997; Weisrock and Janzen, 1999). The animals apparently survive exposure to ice and cold by becoming supercooled, which is a state in which bodily fluids remain unfrozen at temperatures below the equilibrium freezing point (Packard and Packard, 2003, 2004a). Although hatchlings may briefly withstand freezing at high subzero temperatures (Storey et al., 1988; Costanzo et al., 1995; Packard et al., 1999; Packard and Packard, 2004b), such tolerance does not provide a general explanation for survival by animals experiencing conditions like those commonly encountered in the field (Packard and Packard, 2003, 2004a).

The heart rate of supercooled hatchlings slows considerably as temperature declines, and the heart ceases to beat altogether at approximately –9°C (Birchard and Packard, 1997). As heart rate slows, perfusion of peripheral tissues presumably declines, and the requirement of tissues for oxygen outstrips delivery, thereby inducing a functional hypoxia or anoxia (Belkin, 1968) that forces hatchlings to rely on anaerobic metabolism for some portion of their energy requirement (Hartley et al., 2000; Costanzo et al., 2001; Packard and Packard, in press). The proportion of metabolism that is supported anaerobically and the quantity of lactate that accumulates in hatchlings depend on both the temperature to which the animals are supercooled and the time spent at that temperature (Hartley et al., 2000; Costanzo et al., 2001; Packard and Packard, in press).

The preceding observations led us to hypothesize that

reductions in cardiac activity at subzero temperatures also reduce the delivery of metabolic substrates to peripheral tissues from stores in the liver, thereby causing the tissues to rely more on endogenous reserves to support their metabolism. If this hypothesis is correct, exhaustion of these endogenous stores could be a factor in limiting the time that overwintering hatchlings can survive in a supercooled state, and the rate at which the stores are later replenished could influence the timing of emergence by hatchlings in the spring.

We therefore undertook the current study to examine patterns of depletion/repletion of glycogen, as well as patterns of variation in free glucose and lactate, in heart, brain and liver from neonatal painted turtles exposed to the same semi-natural thermal regimes that were used in an earlier study of lactate and free glucose in whole animals (Packard and Packard, in press). Our primary goals were to elucidate the extent to which glycogen reserves in key organs might be depleted during hibernation and to assess the potential for hatchlings to restore organ glycogen reserves once temperatures rise and all metabolism can be supported aerobically.

Materials and methods

Collection of eggs and acclimation of turtles

We collected eggs of painted turtles from newly constructed nests on the Valentine National Wildlife Refuge, Cherry County, Nebraska, USA, in June 2003. The eggs were transported by automobile to our laboratory, where they were assigned to boxes of damp vermiculite (water potential=–150 kPa) and incubated at 27°C for 6 weeks. Eggs then were cleaned thoroughly and transferred to boxes containing nesting soil from the study site, so that incubation was completed and hatchlings emerged on natural substrata.

All viable eggs hatched by mid-August. Neonates were then acclimated to 3°C by reducing temperature in the environmental chamber by approximately 0.75 deg. day^{-1} until the final temperature was reached in mid-September. During acclimation, the turtles were held in the boxes in which they hatched, and they remained in those boxes until experimental tests commenced.

General procedures

Neonates were removed from acclimation boxes and cleaned of adhering soil. Turtles used to establish a baseline for organ metabolites were frozen immediately in liquid nitrogen (see below) whereas other hatchlings were prepared for further study. We attached a thermocouple to the carapace of each of the latter using quick-setting epoxy resin, after which the turtles were placed singly onto platforms of dry Styrofoam® in pint-volume canning jars (0.47 liter). By placing the hatchlings on dry surfaces, we minimized the risk that they would later contact ice and freeze by inoculation.

The jars were sealed and then placed in an environmental chamber set at 2°C. The leads from the thermocouples were attached to a Campbell CR-10 datalogger (Logan, UT, USA) to record body temperature every 10 min. The microprocessor controlling the chamber was set to hold temperature in the chamber at 2°C for 4 h (to ensure thermal equilibration of turtles with the environment) and then to change the temperature down over the next 3 h to –6°C, which was maintained for varying times depending on the treatment (see below). Our choice of thermal regimes was dictated by our desire to expose hatchlings to ecologically relevant temperatures (Packard, 1997; Packard et al., 1997) while simultaneously avoiding temperatures so low that turtles would be at substantial risk of freezing spontaneously by heterogeneous nucleation (Packard and Packard, 2003). Also, a lower limit of –6°C ensured that hatchlings experienced a meaningful and challenging reduction in heart rate (Birchard and Packard, 1997) and a functional hypoxia (Hartley et al., 2000) but avoided the circulatory collapse that would have occurred at lower temperatures.

Temperature profiles recorded for each turtle were later downloaded to a PC and searched for evidence of a freezing exotherm. The only data reported here are for turtles that remained unfrozen for the duration of their exposure.

Exposure to semi-natural thermal regime

The experimental design called for two replicates of 18 turtles in each of five treatments (plus another two replicates for acclimated but untreated animals) for a total of 180 hatchlings. We planned initially to use two environmental chambers to apply the several treatments in each replicate but were forced to incorporate a third chamber into the design when one of the original chambers failed early in the study. The treatments were as follows: treatment 1, 10 days at –6°C; treatment 2, 20 days at –6°C; treatment 3, 10 days at –6°C then 10 days at –3°C; treatment 4, 10 days at –6°C then 10 days at 0°C; treatment 5, 10 days at –6°C then 10 days at +3°C. Temperatures like these have been recorded repeatedly in natural nests (Packard, 1997; Packard et al., 1997).

The first test of cold tolerance began in mid-November and the last began in mid-February. The several treatments in each replicate (and untreated controls) were applied in a random order to avoid introducing a temporal bias into the investigation.

At the end of each exposure, 12 jars were removed from the chamber, one at a time, and the turtle contained in each was detached from its thermocouple, wrapped quickly in aluminum foil together with an identifying label, frozen in liquid nitrogen and stored at –80°C. Six of these turtles were used subsequently to assess glycogen, free glucose and lactate in liver, heart and brain, and six were used in a companion study assessing whole-body lactate and free glucose (see Packard and Packard, in press). The temperature in the environmental chamber was then reset to +2°C, and the six remaining jars with turtles were left overnight to rewarm to that temperature. The turtles were then removed from the jars, placed into cups containing a small quantity of water and monitored for several days. These last six turtles in each replicate served as a reference group that provided an index to the effect, if any, of the various treatments on survival.

Processing organs

Processing of hatchlings did not begin until all the treatments had been applied. Random groups of turtles then were transferred from the –80°C freezer to a –20°C freezer, from which animals were removed singly for processing. Each hatchling was weighed (mean body mass, 4.003 g for 61 turtles), thawed while buried in chipped ice, and dissected on a cold surface. The carcass was covered with a plastic bag containing chipped ice while individual organs were weighed. The entire liver was removed first and placed in a pre-weighed, 1.5 ml microcentrifuge tube. The tube was reweighed so that mass of the liver could be determined by subtraction. A known volume of cold 10% trichloroacetic acid (TCA) was then added to the tube to precipitate proteins and thereby prevent enzymatic activity that might modify the constituents of interest. The tissue was homogenized using a mini-pestle attached to an Arrow 1750 stirrer (Hillside, NJ, USA), residual tissue was washed into each tube with a known volume of cold TCA, and the tube was stored on ice. The entire heart and brain were processed similarly. The only opportunity for warming of organs was during weighing, i.e. the ~60 s that elapsed between the time the organ was removed from the carcass and the addition of cold TCA to the microcentrifuge tube. Earlier work showed that samples can be maintained at room temperature for up to 5 min without detectable change in tissue constituents (Daw et al., 1967). Total volumes of TCA used were 1 ml for livers and 200 µl for heart and brain. The quantity of water (in ml) in each organ was estimated from information on percent hydration of each organ (M.J.P. and G.C.P., unpublished) so that we could estimate better the total quantity of solvent present (Keppler and Decker, 1974). Masses of organs are reported in the captions to the figures.

When organs from each turtle in a group had been removed and homogenized in TCA, each tube was vortexed, and a 100 µl subsample of the homogenate was transferred by pipette into a 2.0 ml microcentrifuge tube. Tubes containing the original homogenate were centrifuged, and all tubes were stored overnight at 4°C because it was not possible to dissect and extract organs and hydrolyze homogenates in a single day.

The following day, all 2.0 ml tubes were removed from the refrigerator, and gylcogen in the subsamples was hydrolyzed to glucose using the procedure of Keppler and Decker (1974). We added 50 µl of 1.0 mol l^{-1} $KHCO_3$ and 1 ml of amyloglucosidase (at a concentration of 1 mg ml^{-1} in 0.2 mol l^{-1} acetate buffer, pH 4.8; nominal activity of 48 U mg^{-1} of protein; Sigma, St Louis, MO, USA) to each tube (Keppler and Decker, 1974). Tubes were vortexed gently, capped and then incubated at 40°C in a shaking water bath for 2 h, after which 500 µl of cold TCA was added to each tube to stop the reaction. The tubes were centrifuged, and the soluble fractions of the original homogenate and the hydrolysate were pipetted into separate 1.5 ml tubes. All tubes were stored at –80°C until organs from all carcasses had been processed.

The concentrations of lactate and free glucose in the original homogenate and the concentration of glucose in the hydrolysate were determined with a YSI 2300 Stat Plus (Yellow Springs, OH, USA) that was calibrated against standard solutions (Packard and Packard, 2004b, in press). Measurements taken with the YSI 2300 Stat Plus are similar to those obtained with traditional spectrophotometry (D. C. Jackson, personal communication). The total quantity of each metabolite was estimated from the product of concentration and volume.

Calculations for glycogen were corrected for free glucose, and the quantity of glycogen in each organ was estimated as outlined by Keppler and Decker (1974). Data for lactate and free glucose are presented in µmoles, whereas data for glycogen are presented as µmoles of glucosyl units (Keppler and Decker, 1974).

Statistical procedures

Data for lactate, free glucose and glycogen in individual organs were examined first by analysis of covariance (ANCOVA) in mixed-model designs, using experimental manipulation (treatment) as a fixed effect and both chamber (which is used here as a surrogate for replicate) and treatment × chamber as random effects. Masses of organs were used as covariates in these analyses to remove effects of variable organ size and to normalize the data. The ANCOVA procedure thereby reduced the data to the equivalent of mass-specific units, or concentrations, for individual organs, but it did so without introducing the statistical biases and artifacts that are commonly associated with the formation of mass-specific units to scale physiological data (Allison et al., 1995; Poehlman and Toth, 1995; Raubenheimer, 1995; Packard and Boardman, 1999). Computations were performed using the Mixed Procedure in SAS release 8.2 (SAS Institute, Cary, NC, USA).

These preliminary analyses revealed that chamber and the treatment × chamber interaction had no detectable influence on the outcome. Thus, we deleted the random factors from the statistical model so that we might include, in the final analyses, data for the samples of turtles taken directly from the acclimation chamber (i.e. for turtles that were never exposed to subzero temperatures). Protected, *post-hoc* comparisons among treatment groups in these one-way analyses were based on pair-wise *t*-tests that used pooled errors from the ANCOVAs. Although our interpretations of data from this study were clearly guided by results of these statistical tests, we were also guided by our examination of the graphical displays, which necessarily focused on the magnitude of differences among means as well as patterns in the data. Thus, we relied on multiple criteria to assign biological significance to our findings.

Results

We detected exotherms in temperature profiles for five of the neonates that were sampled for measurement of organ metabolites (i.e. one in each of the five treatments) and one exotherm in a temperature profile for an animal used to assess survival. These six animals clearly froze during the course of their exposures (presumably by heterogeneous nucleation), so

the data for these hatchlings were not considered further. The only data submitted to graphical and statistical analysis were for organs obtained from turtles that remained unfrozen throughout their exposure to subzero temperatures.

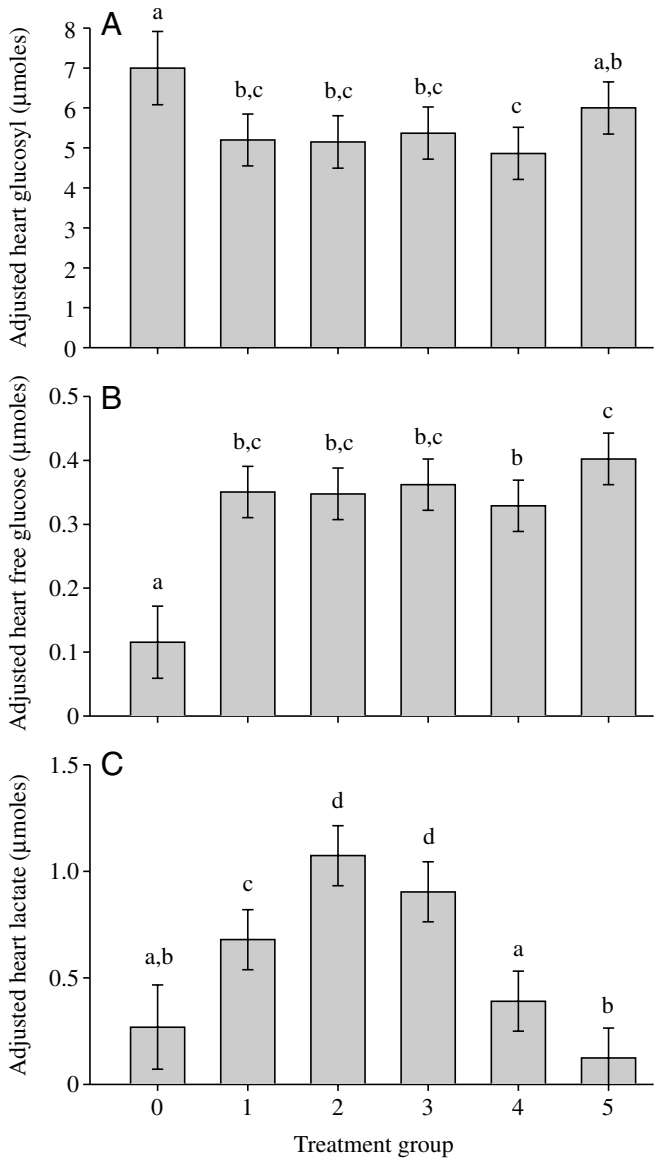

Fig. 1. Means (±2 S.E.M.) for (A) glycogen (expressed as glucosyl units), (B) free glucose and (C) lactate in hearts of hatchling painted turtles. Values were adjusted by ANCOVA to the mean mass of 32 mg for the 61 hearts analyzed in this study. ANCOVA for cardiac glycogen, $F_{5,54}$=3.74, P=0.006; for free glucose, $F_{5,54}$=14.78, P<0.001; for lactate, $F_{5,54}$=25.96, P<0.001. Treatment 0 represents controls that were not exposed to subzero temperatures; treatment 1 represents animals exposed to –6°C for 10 days; treatment 2 represents animals exposed to –6°C for 20 days; treatment 3 represents animals exposed first to –6°C for 10 days and then to –3°C for another 10 days; treatment 4 represents animals exposed first to –6°C for 10 days and then to 0°C for another 10 days; treatment 5 represents animals exposed first to –6°C for 10 days and then to +3°C for another 10 days. Bars that share the same letter cannot be distinguished statistically at P=0.05.

All the unfrozen turtles in the reference groups withstood their respective exposures to subzero temperatures. We assume, therefore, that all the unfrozen hatchlings used to investigate metabolites in organs were also alive when they were immersed in liquid nitrogen.

Hearts of neonates exposed continuously to subzero temperatures (i.e. treatments 1–3) contained less glycogen than those removed from control turtles (Fig. 1A), and the reserve of glycogen in hearts of turtles exposed to treatment 4 (10 days at –6°C, 10 days at 0°C) was also lower than that of controls (Fig. 1A). The quantity of glycogen in hearts of animals in treatment 5, however, cannot be distinguished statistically from that of control turtles, but neither can it be distinguished from values for hatchlings in treatments 1–3 (Fig. 1A). Graphical analysis leads us to believe, therefore, that some slight replenishment of glycogen may have occurred in hearts of turtles in treatment 5 but that the reserve had not been fully restored (Fig. 1A). Note, however, that the variation in cardiac glycogen among treatments is relatively small (Fig. 1A).

Appreciable reserves of glycogen were mobilized in brains of hatchlings that were exposed to –6°C for 10 days (treatment 1), but no further depletion of these reserves occurred in brains of turtles that were exposed for 20 days to subzero temperatures (treatments 2, 3) (Fig. 2A). Turtles in treatments 4 and 5 were exposed for the last 10 days to either 0°C or +3°C, respectively, but glycogen reserves were not replenished (Fig. 2A).

Livers of untreated hatchlings contained substantial glycogen, regardless of whether the findings are expressed as μmoles of glucosyl in a liver of mean mass or as μmoles of glucosyl in a liver regardless of its mass (Fig. 3A,B). The glycogen reserve was lower in hatchlings that were exposed to –6°C for 10 days, and it seemed to be diminished further in turtles in treatments 2, 3 and 4. However, liver glycogen may have increased somewhat (Fig. 3A,B) in hatchlings in treatment 5 (10 days at –6°C then 10 days at +3°C) compared with the preceding three treatments.

Exposure of hatchlings to –6°C for 10 days (treatment 1) elicited an increase in cardiac free glucose over baseline levels (Fig. 1B). Free glucose in hearts of turtles in other treatments, however, was indistinguishable from the concentration in hearts of animals in treatment 1 (Fig. 1B), despite the fact that animals in two of those treatments were exposed for longer periods to subzero temperatures (treatments 2, 3) and those in two other treatments were exposed for 10 days to temperatures above the equilibrium freezing point for bodily fluids (treatments 4, 5). Again, note that the amount of free glucose in hearts was never particularly high (Fig. 1B).

Free glucose was substantially higher in brains of hatchlings from treatment 1 (10 days at –6°C) than in those of untreated animals (Fig. 2B), and it was progressively higher still in brains of turtles from treatments 2 (20 days at –6°C) and 3 (10 days at –6°C then 10 days at –3°C). The quantity of free glucose in brains of hatchlings from treatment 4 (10 days at –6°C then 10 days at 0°C) was also elevated compared with controls and treatments 1 and 2 (Fig. 2B). Brains of turtles

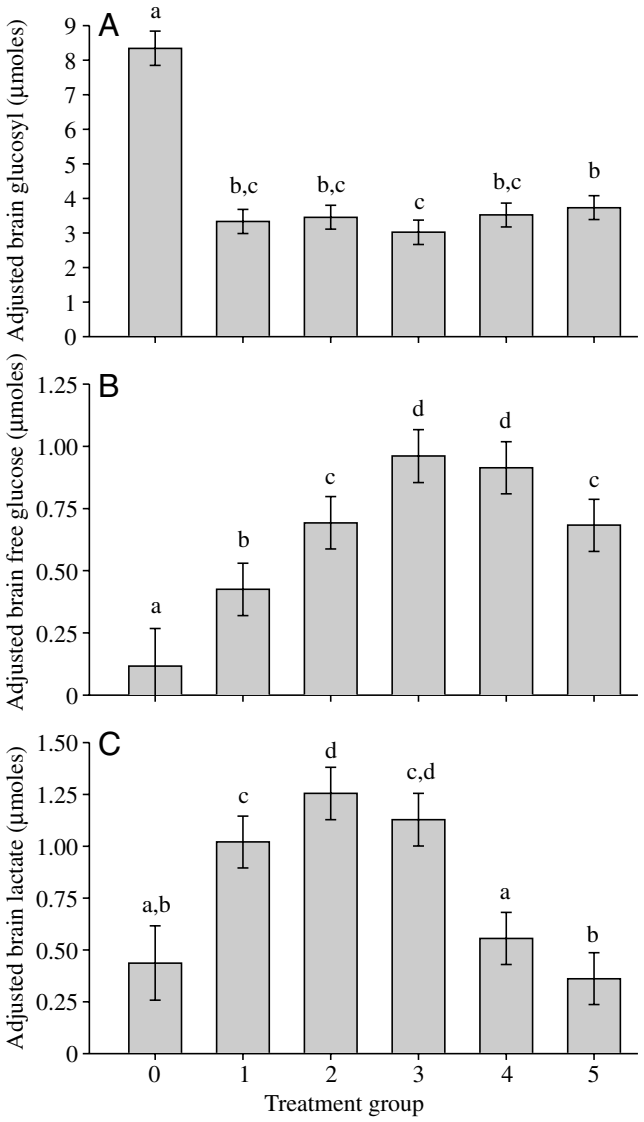

Fig. 2. Means (±2 S.E.M.) for (A) glycogen (expressed as glucosyl units), (B) free glucose and (C) lactate in brains of hatchling painted turtles. Values were adjusted by ANCOVA to the mean mass of 37 mg for the 61 brains examined in this study. ANCOVA for brain glycogen, $F_{5,54}$=71.23, P<0.001; for free glucose, $F_{5,54}$=25.53, P<0.001; for lactate, $F_{5,54}$=31.97, P<0.001. Treatment 0 represents controls that were not exposed to subzero temperatures; treatment 1 represents animals exposed to –6°C for 10 days; treatment 2 represents animals exposed to –6°C for 20 days; treatment 3 represents animals exposed first to –6°C for 10 days and then to –3°C for another 10 days; treatment 4 represents animals exposed first to –6°C for 10 days and then to 0°C for another 10 days; treatment 5 represents animals exposed first to –6°C for 10 days and then to +3°C for another 10 days. Bars that share the same letter cannot be distinguished statistically at P=0.05.

exposed to treatment 5 contained less free glucose than did brains of turtles from treatments 3 and 4 but nonetheless remained considerably elevated above baseline values (Fig. 2B), despite the fact that turtles spent the last 10 days of their exposure at +3°C.

Exposure of neonates to treatments 1, 2 and 3 (all of which involved continuous exposure to subzero temperatures) led to levels of free glucose in liver that were considerably higher than those observed in control animals, and free glucose was highest among neonates exposed to the harshest conditions (treatment 2; 20 days at –6°C) (Fig. 3C). Lower levels of free glucose were observed in livers from hatchlings exposed to treatment 5 (10 days at –6°C then 10 days at +3°C), but free glucose had not returned to control levels (Fig. 3C).

Low levels of lactate were detected in hearts, brains and livers from hatchlings that were not subjected to tests of cold tolerance (treatment 0; Figs 1C, 2C, 3D). However, lactate increased appreciably in organs of animals that were held at –6°C for 10 days (treatment 1), and it was even higher in turtles that were held at –6°C for 20 days (treatment 2). Lactate in organs from hatchlings in treatment 3 (10 days at –6°C then 10 days at –3°C) was generally like that for organs from turtles in treatment 2, despite the fact that animals in the former treatment were not exposed to as stressful a regime as those in the latter treatment (Figs 1C, 2C, 3D). Lactate declined in organs from turtles in treatment 4 (10 days at –6°C then 10 days at 0°C), and it seems to have returned to baseline levels in hatchlings from treatment 5 (10 days at –6°C then 10 days at +3°C).

Discussion

Organ glycogen and free glucose

As temperature falls below the equilibrium freezing point, the heart of an unfrozen hatchling beats progressively more slowly until it stops beating altogether at approximately –9°C (Birchard and Packard, 1997). The resultant graded reduction in perfusion of peripheral tissues with blood induces a similarly graded functional hypoxia that causes the tissues to turn to anaerobic glycolysis for a portion of the ATP needed to maintain cellular function (Hartley et al., 2000; Costanzo et al., 2001). However, circulatory insufficiency has consequences beyond the one on delivery of oxygen to peripheral tissues: delivery of metabolic substrate to those tissues is also impacted. Moreover, individual organs differ in their metabolic response to the ischemia that accompanies supercooling, thereby pointing to organ-specific variation in glycolytic processes (Kelly and Storey, 1988).

Glycogen was mobilized in hearts and brains of hatchlings that were held at –6°C for 10 days, but longer exposure to subzero temperature was not accompanied by any further reduction in size of the glycogen reserve in these organs (Figs 1A, 2A). By contrast, the glycogen reserve in liver appears to have been depleted more among turtles that were held at subzero temperature for 20 days than among those that were sampled after only 10 days at –6°C (Fig. 3A,B). Thus, hatchlings mobilized glycogen in all organs during initial stages of exposure to subzero temperature, but glycogenolysis was then reduced to a minimal level or shut down in heart and brain but not in liver, which thereafter supplied substrate to the other organs. This interpretation is consistent with the

continuing increase in brain glucose even after glycogenolysis in that organ was terminated (Fig. 2A,B), coupled with the continued mobilization of hepatic glycogen (Fig. 3A,B). Moreover, circulation would have continued, albeit at very low levels, under the experimental conditions used here (Birchard and Packard, 1997), so a mechanism would have existed for distribution of glucose from liver to other parts of the body.

A much larger fraction of the glycogen reserve in brain was mobilized during the first 10 days of exposure of turtles to subzero temperature than was the case in hearts (Figs 1, 2). Moreover, free glucose continued to increase in brains of supercooled turtles during the second 10 days of exposure to –6°C (treatment 2) whereas glucose in hearts reached a maximum after just 10 days and did not increase any more with longer exposure (Figs 1, 2). These disparate patterns could reflect a higher requirement of the central nervous system for glucose because of the larger proportion of glycogen mobilized by this organ during initial stages of supercooling and the ongoing augmentation of glucose. Alternatively, the contrasting patterns could reflect a higher requirement of the heart for glucose, because concentrations peaked early and remained high thereafter. In either case, however, the source of glucose after the initial contribution from endogenous glycogenolysis apparently was the liver (Fig. 3). Regardless of the underlying basis for the disparate patterns of variation in free glucose in heart, brain and liver, it is apparent that return of carbohydrate metabolism to the pre-exposure state requires substantial time at a temperature sufficiently high for animals to function aerobically.

If hatchling painted turtles held at –6°C sustained the same level of metabolism during the second 10 days of exposure that prevailed during the first 10 days, turtles in treatment 2 should have mobilized twice as much hepatic glycogen as turtles in treatment 1. This expectation was not realized (Fig. 3), which

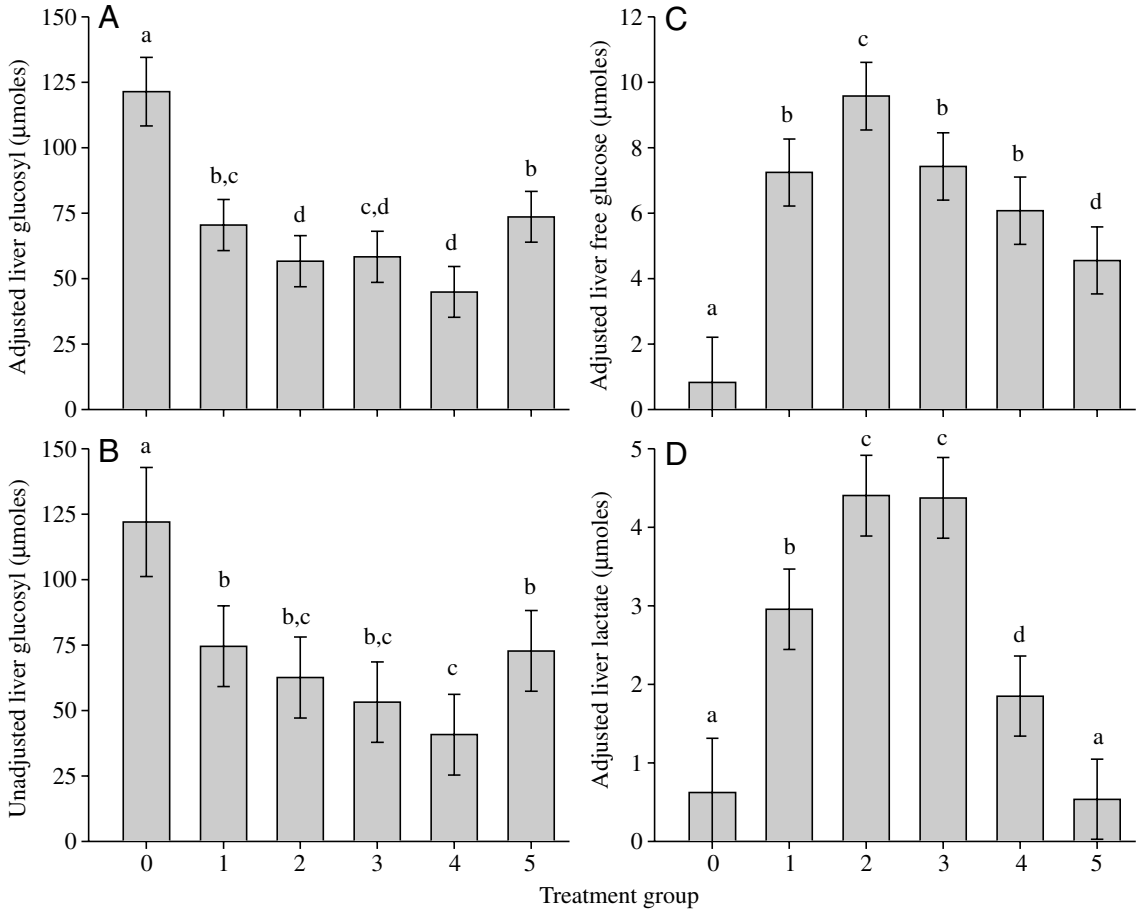

Fig. 3. Means (±2 S.E.M.) for (A,B) glycogen (expressed as glucosyl units), (C) free glucose and (D) lactate in livers of hatchling painted turtles. In A, C and D, values were adjusted by ANCOVA to the mean mass of 246 mg for the 61 livers examined in this study. Unadjusted means for hepatic glycogen are also presented (B) because these values reflect on the size of the carbohydrate reserve that was available to individual hatchlings. ANCOVA for liver glycogen, $F_{5,54}$=20.01, P<0.001; for free glucose, $F_{5,54}$=24.64, P<0.001; for lactate, $F_{5,54}$=41.59, P<0.001. ANOVA for unadjusted glycogen, $F_{5,55}$=8.89, P<0.001. Treatment 0 represents controls that were not exposed to subzero temperatures; treatment 1 represents animals exposed to –6°C for 10 days; treatment 2 represents animals exposed to –6°C for 20 days; treatment 3 represents animals exposed first to –6°C for 10 days and then to –3°C for another 10 days; treatment 4 represents animals exposed first to –6°C for 10 days and then to 0°C for another 10 days; treatment 5 represents animals exposed first to –6°C for 10 days and then to +3°C for another 10 days. Bars that share the same letter cannot be distinguished statistically at P=0.05.

raises the possibility that some downregulation of metabolism occurred in supercooled animals. Downregulation of metabolism has been observed in adult turtles held in anoxic water at 3°C (Jackson, 2000, 2002; Jackson et al., 2001), so such a process might reasonably be expected to occur in hatchlings as well. However, suppression of metabolism in supercooled neonates might not be as pronounced as it is in anoxic adults. Adult turtles in simulated hibernation are generally exposed to total anoxia and thus rely exclusively on anaerobic metabolism for ATP (Jackson, 2000, 2002; Jackson et al., 2001). By contrast, energy requirements of supercooled neonates are met by a mixture of both anaerobic and aerobic metabolism as long as temperature is above –9°C and circulation remains patent.

Turtles that were held at +3°C for the second 10 days of their treatment apparently began to replenish glycogen in their liver, but the reserve remained well below that in liver of untreated controls (Fig. 3A,B). Recovery may also have begun in heart but analysis of glycogen in brains reveals no indication of recovery (Figs 1A, 2A). Hatchlings maintained at 0°C or higher operate aerobically and do not accumulate lactate (Hartley et al., 2000). Thus, restoration of glycogen reserves presumably was an option for turtles that spent their last 10 days at 0°C or +3°C. Nonetheless, our results indicate that liver is the only organ to exhibit compelling evidence of recovery. Hatchlings evidently require longer periods at higher temperatures to achieve substantial replenishment of cardiac or brain glycogen.

Organ lactate

The patterns of variation in lactate in heart, brain and liver are remarkably similar to the pattern of variation in whole-body lactate observed for intact hatchlings (Packard and Packard, in press). Bodies of turtles held at –6°C for 20 days contained more lactate than did bodies of turtles held at that temperature for only 10 days (Packard and Packard, in press), and the same holds true for organs (Figs 1C, 2C, 3D). Moreover, the lactate load in bodies of turtles held at –6°C for 10 days was largely (or completely) removed over the course of another 10 days at either 0°C or +3°C (Packard and Packard, in press), and similar observations were made for organ lactate (Figs 1C, 2C, 3D). A few differences exist between results of our studies of organs and intact animals, but these differences are relatively minor. Indeed, the differences probably reflect little more than different dynamics for handling of lactate in different organs and the cumulative effects of these organ-specific dynamics on intact turtles. We emphasize the major increases in lactate in animals and individual organs during exposure to subzero temperatures and the apparent elimination of the lactacid debt within days of returning to temperatures at or slightly above 0°C (Packard and Packard, in press; Figs 1C, 2C, 3D).

The mechanism whereby neonatal turtles eliminate the lactacid debt that accumulates during supercooling cannot be addressed directly by the data gathered in the present study. Lactate could be excreted (which seems unlikely given that neonates were maintained in a dry environment), oxidized or converted to glucose. We cannot speak to the possibility that lactate is oxidized when neonates are exposed to temperatures above the supercooling point and all metabolism can be supported aerobically. However, we view conversion of lactate to glucose as the most likely scenario for dealing with accumulation of this potentially toxic end product of anaerobic metabolism. We note that organ lactate declined dramatically among turtles in treatments 4 and 5, both of which included exposure of animals during the second 10 days to temperatures that enabled them to be fully aerobic. The continued elevation of free glucose above baseline in turtles exposed to treatments 4 and 5 may indicate that some of the lactate generated during exposure to –6°C was converted to glucose during exposure to higher temperatures. Of course, we cannot rule out the possibility that some lactate was oxidized. Nonetheless, we believe that conversion of lactate to glucose is likely to be the more important mechanism for dealing with the lactacid debt that accumulates during supercooling.

Implications for animals overwintering in the field

Our results confirm that supercooled hatchlings, like adult painted turtles in cold, anoxic environments (Jackson, 2000; Jackson et al., 2001), mobilize glycogen reserves to support metabolic activity. However, adult turtles are thought to rely on glycogen reserves in individual organs for provision of glucose until those reserves are exhausted, at which time they are supplanted by glucose derived from liver glycogen (Reeves, 1963; Bing et al., 1972; Brachfeld et al., 1972; Clark and Miller, 1973; Penney, 1974). Hatchlings in our study, however, did not fully deplete either heart or brain of glycogen during their tests of cold tolerance, so the turtles apparently were in no danger of exhausting their endogenous reserves in these organs. The apparent conservation of glycogen reserves in heart and brain of supercooled hatchlings may have been facilitated by their ability to maintain an aerobic component to metabolism while the heart continues to beat and some metabolic substrate can be supplied from reserves in the liver.

However, animals that were exposed first to –6°C for 10 days and then to 0°C or +3°C for another 10 days were unable to fully replenish glycogen stores in brain and probably not in heart either (Figs 1, 2). How, then, might turtles respond to a second exposure to subzero temperature and supercooling? This question is ecologically relevant because temperatures in natural nests commonly go through daily cycles in which the zenith is near (or even slightly above) 0°C and the nadir is several degrees lower (Packard, 1997; Packard et al., 1997). If the initial response by a hatchling to falling (subzero) temperature is for heart and brain to mobilize endogenous glycogen, regardless of the size of the reserve, then cycling of temperature from below 0°C to slightly above 0°C might lead over time to exhaustion of endogenous glycogen in those organs without seriously depleting glycogen in liver. On the other hand, the continued high levels of free glucose in heart and brain, even in animals exposed for several days to temperatures near 0°C, may enable turtles to endure another

bout of supercooling without the need to draw on endogenous reserves of glycogen at all. These are important issues that merit attention.

We have suggested elsewhere that the accumulation of lactate may also pose a threat to turtles overwintering in the field, where prolonged exposure to subzero temperature conceivably leads hatchlings to accumulate potentially lethal quantities of lactate (Hartley et al., 2000). Results of the present study are consistent with that hypothesis. However, the capacity for hatchlings rapidly to recover from a lactic acidosis is also impressive. For example, turtles in treatment 4 (10 days at –6°C then 10 days at 0°C) were able in the second half of their treatment to remove a substantial quantity of the lactate that accumulated during the first half (Figs 1C, 2C, 3D), and the entire lactacid debt was paid off during the second 10 days by neonates in treatment 5 (10 days at –6°C then 10 days at +3°C). This may mean that a brief exposure during the day to temperatures near 0°C enables turtles in the field to remove lactate that was formed during periods of cold only a few hours earlier. Thus, animals in the field may not accumulate substantial amounts of lactate except on occasions when temperature remains below –3°C for several days without interruption, and only in these instances is lactate accumulation likely to pose a threat to survival.

Eggs of painted turtles were collected at the Valentine National Wildlife Refuge under the authority granted to us by Special Use Permit #VLT–03–04 from the U.S. Fish and Wildlife Service and Scientific Collecting Permit #03–397 from the Nebraska Game and Parks Commission. Our experimental protocol was considered and approved by the Animal Care and Use Committee at Colorado State University (#01–052–01). Our research was supported by a grant from the National Science Foundation (IBN–0112283). We thank M. Lindvall, D. Kime and M. Nenneman for logistical support during our stay at the Valentine NWR.

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