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Postfreeze Reduction of Locomotor Endurance in the Freeze-Tolerant Wood Frog, *Rana sylvatica*

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ABSTRACT

Considerable study has focused on the physiological adaptations for freeze tolerance in the wood frog, *Rana sylvatica*, a northern species that overwinters within the frost zone, but little attention has been paid to the associated costs to organismal performance. Here we report that freezing causes transient impairment of locomotor endurance and adverse changes in exercise physiology that persist for at least 96 h. Wood frogs frozen at $-2°C$ for 36 h exhibited normal behaviors and hydro-osmotic status and near-normal metabolite (glycogen, glucose, and lactate) levels within 24 h after thawing began. However, when exercised to exhaustion on a treadmill, these frogs showed a 40% reduction in endurance as compared to sham-treated (unfrozen) controls, a reduction that persisted for at least 96 h. Previously frozen frogs exhibited higher rates of lactate accumulation during exercise than controls, suggesting that prior freezing forces greater reliance on the glycolytic pathways of energy production to support exercise. Given that this species breeds in late winter, when subzero temperatures are common, freezing may result in reduced fitness by hampering their ability to reach the pond, avoid predators, and successfully obtain mates.

Introduction

Several ectothermic vertebrates tolerate the freezing of their body tissues (Schmid 1982; Storey and Storey 1992), an adaptation that allows survival in relatively exposed terrestrial hibernacula. Whereas the biochemical adaptations supporting freeze tolerance are beginning to be understood, the physiological costs associated with this adaptation, some of which may directly affect fitness (Huey 1991), have only recently been explored. To this end, we investigated the effect of freezing on one aspect of organismal performance, locomotor endurance.

The physiological mechanisms promoting freeze tolerance have been best studied in the wood frog, *Rana sylvatica*, which survives the freezing of up to 70% of its body water at temperatures as low as $-6°C$ (see reviews by Costanzo and Lee [1994]; Lee and Costanzo [1998]). Upon the initiation of freezing, large quantities of glucose are produced, primarily from hepatic glycogen stores (Storey 1987). Glucose protects cells and tissues from freezing damage (Canty et al. 1986; Costanzo and Lee 1991; Costanzo et al. 1993), may suppress metabolism in frozen tissues (Storey and Storey 1988), and provides a substrate for metabolism in hypoxic tissues, with lactate accumulating as the chief metabolic by-product (Storey and Storey 1986).

Another primary adaptation to protect the tissues during freezing is the redistribution of water within the body. During the first 24 h of freezing, skeletal muscles lose from 22% (gracilis major) to 36% (gastrocnemius) of their water to the lymphatic spaces, whereas the liver and intestine lose up to 60% (Lee et al. 1992). Organ dehydration may improve freeze tolerance by increasing the concentration of glucose in the unfrozen fluid and by reducing the amount of ice that forms within the tissues (Costanzo et al. 1992). Because ice forms mainly in the lymph spaces (Storey and Storey 1984; Layne and Lee 1987), the blood becomes concentrated, resulting in increased hematocrit and osmolality.

Despite the profound effects of freezing, recovery of basic physiological functions occurs remarkably soon after thawing (Costanzo and Lee 1994). Within 1 h of the onset of thawing, the heart resumes beating (Layne et al. 1989), and pulmonary respiration follows shortly (Layne and First 1991). In vitro studies show that muscle contractility returns within 1–2 h (Layne 1992) and sciatic nerve excitability is restored 4–5 h after thawing begins (Kling et al. 1994). However, not all physiological systems recover so quickly. Free hemoglobin in the plasma, an indicator of erythrocyte damage, is detectable for at least 12 h after frogs are frozen for 24 h at $-2°C$ (Costanzo et al. 1991). Following a 72-h freeze to $-2.5°C$, several days are required for restoration of normal glycogen, glucose, and lactate levels, as well as for repletion of ATP and energy charge in various tissues (Storey and Storey 1986).

Whereas other studies have investigated physiological recov-

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ery following freezing, only one (Costanzo et al. 1997) has addressed the costs of freezing in terms of reduced organismal performance. This study of reproductive behaviors revealed that previously frozen male wood frogs were largely unsuccessful when competing with unfrozen controls to achieve amplexus with a female. Previously frozen frogs also spent less time searching for females and were less likely to emit breeding calls than control conspecifics. Thus, freezing adversely affected the physiological systems responsible for male reproductive behaviors in this species. This raised the question of whether other aspects of physiological performance may be similarly affected.

We tested the hypothesis that previous exposure to freezing would significantly reduce locomotor endurance. Also, given the importance of carbohydrate reserves (especially glycogen) and lactate accumulation in both freezing and vigorous exercise, we followed recovery of these and other physiological responses following freezing to seek correlations with the hypothesized reductions in locomotor endurance.

Material and Methods

Specimens

Male wood frogs, *Rana sylvatica* LeConte, were collected during their migration to a breeding pond in Adams County, Ohio, on February 23, 1996. After transport on ice to the lab, they were stored unfed at 4°C in dark boxes containing wet moss (as in Costanzo et al. 1997). Frogs were stored from 1 to 4 wk before experimentation. Before use, each frog's bladder was emptied via a glass cannula, and the frogs were weighed to ±0.1 g.

Freezing/Thawing Protocol

Frogs were frozen inside 50-mL plastic centrifuge tubes (Costanzo et al. 1992). Thermocouples, which were connected to an OM-500 (Omega Electronics) multichannel datalogger, were placed on their ventral surfaces to monitor temperature during freezing. After the tubes were lowered into a circulating alcohol bath (model 2095, Forma Scientific), the frogs supercooled to between $-0.5°$ and $-1.0°C$. At this point, aerosol coolant was applied to the outside of the tube, which caused freezing of the condensation on the inner wall of the tube. These ice crystals quickly spread to regions of contact with the frog's skin and thus stimulated freezing of the frog's tissues. Once the frogs began to freeze, they were slowly cooled to a target minimum temperature of $-2°C$ and held frozen for a total of 36 h. Control counterparts for each frozen frog, matched by standard body mass, were held in tubes submerged in an ice bath (i.e., chilled but unfrozen) for the same duration. During recovery from freezing or the control treatment, frogs were kept on moist filter paper in ventilated cups, in darkness, at 4°C.

Tests of Endurance

No less than 1 h before being exercised, the frogs were transferred from the recovery room to a test chamber at 15°C. Individual frogs were tested on a cylindrical treadmill (diameter = 26.5 cm; track width = 15.0 cm) that was rotated at 5.8 cm s^{-1} by an electric motor. Initially, frogs jumped to keep up with the rotation of the treadmill. Once fatigued, however, the frogs were flipped on their dorsa by the rotation of the chamber, but they still righted themselves. Endurance was measured as the time elapsed until the frogs reached exhaustion, operationally defined as the point at which they failed to right themselves three times consecutively. Exhausted frogs were immediately removed from the treadmill, killed by double pithing, and used in physiological assays.

Experimental Design

Time to exhaustion was measured in previously frozen and control frogs after 24 h ($n = 9$ per group) or 48 h ($n = 5$ per group) of recovery at 4°C. In addition to the physiological assays performed on the exhausted frogs, other frogs were assayed 24 and 48 h after recovery from freezing, or 24 h after the control treatment, to determine their physiological states before exercise ($n = 3$ per group). We did not analyze control frogs recovered for 48 h because their physiological condition likely would not differ from that of control frogs recovered for 24 h (Costanzo et al. 1997). Time to exhaustion was measured on additional groups of previously frozen and control frogs ($n = 3$ per group) after recovery for 96 h, but the physiological condition of these frogs was not assessed.

For comparison, physiological assays were also performed on fully frozen frogs (i.e., not provided any time to recover; $n = 3$). Collectively, these measurements allowed us to (1) assess the nature of the physiological perturbations induced by freezing, and whether the frogs had recovered from these effects before exercise, and (2) compare changes in physiology due to exercise in previously frozen and control frogs.

Physiological Assays

The blood of thawed, dissected frogs was removed from an incision in the truncus arteriosus, whereas in fully frozen frogs, which had no circulation, the ventricle was removed and centrifuged (1,000 *g*, 1 min) to expel its contents. Blood was collected in heparinized hematocrit tubes and centrifuged (2,000 *g*, 5 min) to determine the hematocrit and to isolate the plasma. We sampled portions of the liver, body wall musculature (internal and external obliques), and thigh musculature (gracilis minor and gracilis major), which were weighed to ±1 mg upon removal. These samples and the remaining carcass (weighed to ±0.1 g) were sealed in vials, promptly frozen in liquid nitrogen,

and stored at −80°C until bioassays were performed 3–15 wk later.

Portions of solid tissues were homogenized in cold 7% $HClO_4$ and centrifuged (13,000 g, 5 min); the supernatant was then neutralized with KOH. Glucose and lactate were measured in the protein-free supernatants, and in plasma, using glucose oxidase (no. 510, Sigma) and lactate oxidase (no. 735, Sigma) procedures, respectively. Liver and the skeletal muscles were assayed for glycogen using a calorimetric micromethod (Kemp and Kits Van Heijningen 1954); concentrations are expressed in glucosyl units. Metabolite concentrations in solid tissues and plasma are expressed in micromoles per gram of fresh weight or millimoles, respectively. Plasma hemoglobin, an indicator of erythrocyte destruction due to freezing and thawing stress (Costanzo et al. 1991), was quantified using the cyanmethemoglobin procedure (no. 525, Sigma).

Statistical Analyses

Differences in physiological parameters among control, fully frozen, and previously frozen groups were analyzed using one-factor ANOVA and Dunnett's multiple comparison against controls. Exercise-induced physiological changes were compared with ANOVA followed by Student-Newman-Keuls multiple comparisons. ANCOVA followed by Student-Newman-Keuls multiple comparisons was used to evaluate differences in time to exhaustion between the groups; log-transformed standard body mass was included in this analysis as a covariate to correct for differences in body size. Statistical analyses were performed using SAS with significance set at $P < 0.05$. Values reported in the text are means ± SEM, except times to exhaustion, which are least-square means ± SE.

Results

Effects of Freezing on Locomotor Endurance

All frogs survived the freezing treatment (mean duration = 36.2 ± 0.1 h; mean minimum temperature = −2.2° ± 0.1°C) and recovered normal posture and behaviors (righting response, spontaneous activity) well before any tests of endurance were performed. Despite their normal appearance, the frogs that were tested 24 h after thawing reached exhaustion in 6.1 ± 0.7 min, whereas it took 11.0 ± 0.7 min in frogs that had not been frozen (Fig. 1). Similarly, previously frozen frogs tested 48 h after thawing began endured forced exercise for 7.8 ± 1.0 min, whereas their control counterparts reached exhaustion much later (12.4 ± 1.3 min). After 96 h of recovery, both the control and previously frozen frogs exhibited improved endurance. However, the time to exhaustion was again lower (11.0 ± 1.9 min) for the previously frozen frogs than in the control group (15.6 ± 2.1 min). Because body size may strongly influence endurance (Bennett et al. 1989; but see Miller et al. 1993), we included body mass (log-transformed) as a
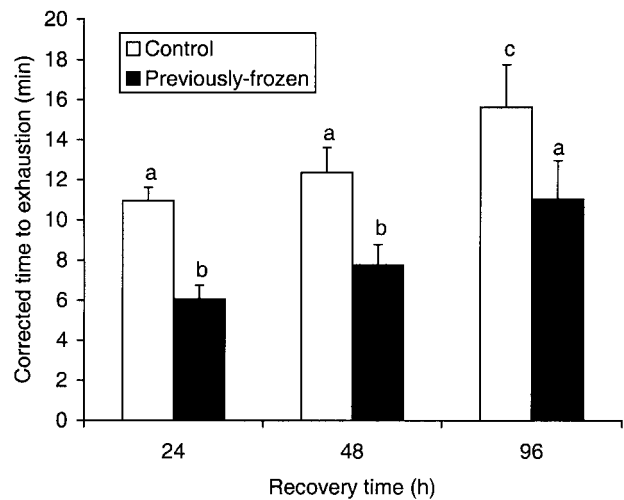

Figure 1. Mean (± SEM) time to exhaustion of male wood frogs (*Rana sylvatica*) tested on a treadmill after 24 h ($n = 9$), 48 h ($n = 5$), or 96 h ($n = 3$) of recovery at 4°C following control (0°C) or freezing (−2°C) treatment. Values are least-square means corrected for the covariate of log-transformed standard body mass. Means not sharing a letter differ significantly (Student-Newman-Keuls multiple comparison).

covariate in the comparisons ($F = 8.7$, $P < 0.01$) between treatment groups and present least-square means above and in Figure 1.

Physiological Effects of Freezing

Before comparing the physiological responses to exercise between previously frozen and control frogs, we first determined the effects of freezing and thawing on rested frogs. As expected, hepatic glycogen levels were markedly reduced (20-fold) as this substrate was converted into the cryoprotectant glucose (Table 1). Glycogen levels in the skeletal muscle were unaffected by freezing (Table 1; as in Storey and Storey 1984). Concentrations of glucose in fully frozen frogs were highest in the liver (63.7 $\mu mol\ g^{-1}$) and plasma (49.8 mM) but much lower in the thigh muscle (9.7 $\mu mol\ g^{-1}$) and carcass (12.8 $\mu mol\ g^{-1}$). By 24 h of recovery, glucose in the liver and plasma were still somewhat high (23.3 and 18.7 $\mu mol\ g^{-1}$, respectively) but not significantly different from control values. Glucose levels in the thigh muscle, body wall, and carcass remained significantly elevated (10.0, 9.8, and 8.1 $\mu mol\ g^{-1}$, respectively) after 24 h but recovered within 48 h (Table 1; Costanzo et al. 1997).

The degree of lactate accumulation in tissues, which is caused by ischemia during freezing, was consistent with that observed under similar circumstances (Costanzo et al. 1997). In fully frozen frogs, lactate levels were elevated in the liver (28-fold), plasma (fourfold), and the body wall musculature (threefold) but not in the thigh muscle or the carcass (Table 1). Lactate

Table 1: Effect of freezing and postfreeze recovery on metabolite levels in rested wood frogs, *Rana sylvatica*

	Control	Fully Frozen	Postfreeze Recovery Time 24 h	48 h	ANOVA F	P
Glycogen (μmol glucose g^{-1} fresh weight):						
Thigh muscle	2.6 ± 1.1	.2 ± .1	2.4 ± .8	.8 ± .4	3.2	.08
Body wall	9.7 ± 2.3	4.2 ± .6	5.2 ± 1.7	.9 ± .9	2.4	.14
Liver	202.1 ± 85.8	9.1 ± .7[a]	29.9 ± 15.1	33.6 ± 17.3	27.2	<.01
Glucose (μmol g^{-1} fresh weight or mM):						
Thigh muscle	1.2 ± .2	9.7 ± 2.3[a]	10.0 ± .7[a]	5.2 ± .2	17.2	<.01
Body wall	.7 ± .1	20.9 ± 2.5[a]	9.8 ± .9[a]	5.9 ± 1.1	44.7	<.01
Liver	9.9 ± 2.4	63.7 ± 14.1[a]	23.3 ± 2.1	27.4 ± 4.7	5.3	.02
Plasma	2.4 ± .3	49.8 ± 10.8[a]	18.7 ± 4.3	4.8 ± 1.4	16.4	<.01
Carcass	.7 ± .1	12.8 ± 2.0[a]	8.1 ± 1.3[a]	2.6 ± .1	25.1	<.01
Lactate (μmol g^{-1} fresh weight or mM):						
Thigh muscle	11.7 ± .4	12.9 ± 3.0	6.9 ± 1.5	15.6 ± .4	8.2	<.01
Body wall	11.7 ± 1.5	30.9 ± 7.9[a]	9.3 ± 1.9	19.0 ± 2.0	5.4	.02
Liver	2.8 ± .3	78.1 ± 15.9[a]	3.9 ± .7	3.9 ± .6	24.8	<.01
Plasma	7.2 ± 1.9	27.5 ± 4.6[a]	6.9 ± .9	7.5 ± 1.6	17.6	<.01
Carcass	5.7 ± .4	6.5 ± .5	4.0 ± .8	6.0 ± .7	1.8	.23

Note. All values are means ± SEM; $n = 3$ in all cases.

[a] Mean differs from control (Dunnett's; $P < 0.05$).

concentrations generally returned to control levels within 24 h of recovery; thus, both previously frozen and control frogs began exercise with typically low levels of lactate.

Freezing was associated with an increase ($F = 33.3$, $P < 0.01$) in hematocrit, from 31% ± 1% in controls to 63% ± 5% in fully frozen frogs, because vascular water was lost to ice crystals (Lee and Costanzo 1993). Plasma osmolality also increased substantially, from 201 ± 27 mOsm in controls to 370 mOsm, as measured in a single, fully frozen frog. Despite these large increases, both hematocrit and osmolality returned to control levels (32.2% ± 2% and 216 ± 4 mOsm, respectively) within 24 h of recovery. Freezing caused hemolysis, as indicated by the marked increase ($F = 16.2$, $P < 0.01$) in plasma hemoglobin, from 0.6 ± 0.1 to 21.8 ± 5.2 mg mL^{-1}. This 36-fold increase well exceeded the increment that would be expected solely because of the hemoconcentration resulting from the freezing of body water (two- to threefold). By 24 h of recovery, plasma hemoglobin concentration was reduced considerably (3.1 ± 0.6 mg mL^{-1}) yet remained slightly (though not significantly) higher than the control level.

Consequences of Freezing on Exercise Physiology

Changes in carbohydrate metabolism with exercise (Table 2) included a 2.4-fold increase in hepatic glucose levels of control frogs (as in Hutchison and Turney 1975), although an associated decrease in liver glycogen concentration was not detected (as in Fournier and Guderley 1993). In contrast, previously frozen frogs did not show an increase in liver glucose concen-

tration, perhaps because they retained some of the glucose mobilized during freezing (Table 2). In concordance with Fournier and Guderley (1993), the glucose level in skeletal muscle was unaffected by exercise. Although we did not detect a decrease in muscle glycogen concentration with exercise (Table 2), glycogen concentration was negatively correlated with time to exhaustion in control frogs ($F = 5.1$, $P < 0.05$, $r^2 = 0.32$) but not in previously frozen frogs.

Vigorous exercise in ranid frogs is primarily supported by anaerobic respiration (Gatten et al. 1992), and, as expected, lactate levels in our frogs generally increased during exercise. Liver lactate concentration more than doubled in control frogs and in previously frozen frogs that had recovered for 24 h, but no change occurred in previously frozen frogs that had recovered for 48 h (Table 2). Carcass lactate tended to increase with exercise and was significantly higher in both control and previously frozen frogs given 24 h to recover (Fig. 2). Expected increases in thigh muscle lactate with exercise (Fournier and Guderley 1993) were lacking, although plasma lactate levels increased by similar margins in all treatment groups. However, because the previously frozen frogs reached exhaustion sooner, it appears that they accumulated lactate at a much higher rate than controls (Fig. 2). There was a positive correlation ($F = 5.6$, $P = 0.05$, $r^2 = 0.45$) between the time to exhaustion and the concentration of carcass lactate in the previously frozen frogs but not in the controls.

There were no significant changes from rested values in plasma osmolality ($F = 0.1$, $P = 0.79$), hematocrit ($F = 3.1$, $P = 0.09$), or plasma hemoglobin ($F = 0.1$, $P = 0.90$) due to

Table 2: Effect of exercise on metabolite levels in control and previously frozen wood frogs sampled after either 24 or 48 h of recovery

| | Control | | Postfreeze Recovery Time | | | | ANOVA | |
| | | | 24 h | | 48 h | | | |
	Rested	Exhausted	Rested	Exhausted	Rested	Exhausted	F	P
Glycogen (μmol glucose g^{-1} fresh weight):								
Thigh muscle	2.6 ± 1.1	2.3 ± .7	2.4 ± .8	1.8 ± .4	.8 ± .4	2.3 ± .5	.6	.74
Liver	202.1 ± 85.8	172.6 ± 48.4	29.9 ± 15.1	51.8 ± 17.8	33.6 ± 17.3	54.5 ± 11.3	3.0	.03
Glucose (μmol g^{-1} fresh weight):								
Thigh muscle	1.2 ± .2	1.6 ± .3	10.0 ± .7	12.1 ± 1.0	5.2 ± .2	7.9 ± 2.0	19.4	<.01
Liver	9.9 ± 2.4	22.8 ± 1.1[a]	23.3 ± 2.1	25.7 ± 2.7	27.4 ± 4.7	30.0 ± 2.4	5.2	<.01
Lactate (μmol g^{-1} fresh weight):								
Thigh muscle	11.7 ± .4	11.9 ± 1.5	6.9 ± 1.5	11.9 ± 1.8	15.6 ± .4	11.5 ± 1.6	1.3	.30
Liver	2.8 ± .3	7.5 ± 1.0[a]	3.9 ± .7	8.3 ± .8[a]	3.9 ± .6	7.3 ± .7	5.1	<.01

Note. All values are mean ± SEM; $n = 3$ in all cases.

[a] Metabolite concentrations in exhausted frogs differ from the mean of rested frogs in the same group (Student-Newman-Keuls multiple comparison).

exercise in any of the treatment groups. Variations in mean plasma osmolality and mean hematocrit were ≤5.3%. Plasma hemoglobin showed more variability but never changed by more than 0.5 mg mL^{-1}.

Discussion

Our central hypothesis, that freezing reduces locomotor endurance, was supported by the results of the treadmill experiments. Previously frozen frogs exhibited a 38% reduction in endurance as compared to their control counterparts. This impairment was evident even after 96 h of recovery (Fig. 1), suggesting that the adverse effect of freezing may last several days. Wood frogs exhibited diminished locomotor endurance despite the fact that they had fully recovered normal behaviors and were superficially indistinguishable from control frogs. There was also a slight increase in the endurance of the control frogs over the 96 h period. Thus, confinement within a small tube and/or chilling to 0°C had a transient, adverse effect on locomotor endurance. However, those that experienced freezing had reduced endurance, even after 96 h of recovery.

What caused the reduced endurance? Glycogen stores within the muscle are the major source of glucose during exercise (Fournier and Guderley 1993). Our frogs had very low thigh muscle glycogen concentrations, typically <3 μmol g^{-1} compared to 40–50 μmol g^{-1} of summer- and autumn-collected frogs (Storey and Storey 1986; Fournier and Guderley 1993), but were on par with those reported previously for spring frogs (Storey and Storey 1987). Muscle glycogen levels were low because our wood frogs were collected immediately after overwintering (and possibly natural freezing) and during breeding, all of which deplete muscle glycogen (Smith 1950; Mizell 1965; Pasanen and Koskela 1974; Storey and Storey 1986; Wells and

Bevier 1997). Also, this species does not commence spring feeding until after its reproductive period (Wells and Bevier 1997), and, therefore, our frogs did not have the opportunity to restore glycogen levels before being used in our experiments. Because the muscle glycogen concentrations were so low and variable, it is possible that we lacked the resolution to detect the consumption of glycogen with freezing and exercise when comparing means (Tables 1, 2). However, the control frogs do show a significant decline in thigh muscle glycogen over time. That is, those frogs that spent more time on the treadmill consumed more muscle glycogen. This result suggests that muscle glycogen concentration did not limit locomotor endurance. Why the previously frozen frogs did not show such a relationship is unclear, but it is possible that freezing added additional variability to glycogen concentration, thus preventing the detection of a significant relationship. If concentrations of glycogen are low, then glycogen availability may be the limiting factor in exercise duration (Shulman and Rothman 2001). Whether glycogen availability limited the endurance of the previously frozen frogs is unclear from our data.

In recent years, the role of lactate in exercising muscle and fatigue has been reassessed (Brooks 2001; Gladden 2001; Nielsen et al. 2001). It is now clear that lactate plays a significant role in both aerobic and anaerobic muscle activity (Kemper et al. 2001). In our experiments, lactate concentrations of previously frozen frogs quickly returned to control levels upon thawing (Table 1); therefore, these frogs had little residual lactate when their endurance was tested. However, previously frozen frogs exhibited higher rates of lactate accumulation (Fig. 2). Higher rates of lactate accumulation could be caused by lower rates of lactate degradation and/or a greater reliance on glycolytic pathways of energy production. In amphibians, skel-
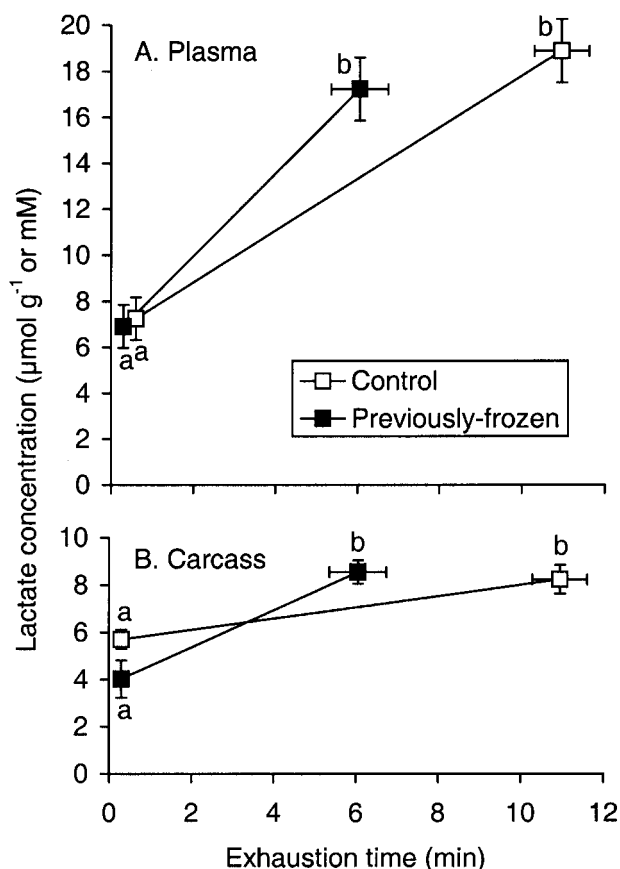
Figure 2. Exercise-induced increase in plasma (*A*) and carcass (*B*) lactate levels in control and previously frozen male wood frogs (*Rana sylvatica*) that had recovered for 24 h at 4°C. Values shown are mean ± SEM (*n* = 3). Means not sharing a letter are significantly different (Student-Newman-Keuls multiple comparison).

etal muscle is largely responsible for lactate degradation (Withers et al. 1988; Gleeson 1996). Whether freezing stress compromises this function is unknown.

Increased reliance on glycolytic energy production during exercise could have been caused by freezing-induced changes in energy balance. Muscle stores of creatine phosphate, ATP, and myoglobin-bound oxygen support the early stages of exercise before glycolytic ATP production becomes active (Gatten et al. 1992). Freezing decreases creatine phosphate and ATP levels (Storey and Storey 1984) and conceivably may deplete myoglobin oxygen stores, thus forcing muscles to prematurely shift to anaerobic metabolism and, hence, accumulate lactate more quickly. However, given the short duration of exercise that is supported by creatine phosphate stores (on the order of seconds; Gatten et al. 1992; Kemper et al. 2001), the reduction of leg muscle creatine phosphate with freezing probably does not account for the great differences in the rate of lactate accumulation that we observed.

Another factor promoting increased reliance on anaerobic metabolism in previously frozen frogs may be freezing damage to the oxygen-delivery system (somewhat analogous to those reported by Hillman [1980]). Elevated plasma hemoglobin levels following freezing indicated that erythrocyte damage occurred (as in Costanzo et al. 1991), but this extracellular hemoglobin was cleared from the blood in less than 24 h. Hematocrit values had also returned to control levels within 24 h of recovery. Thus, either relatively few erythrocytes were damaged by freezing or the damaged erythrocytes were rapidly replaced. Additional study is needed to determine whether freezing diminishes the capacity of the oxygen transport system.

The general implication of these results is that freezing causes an impairment of locomotor endurance lasting for at least 96 h, which ultimately may adversely impact organismal fitness. Wood frogs breed for a 36–48-h period in late winter (mid- to late February in southern Ohio), when ambient temperatures may drop below freezing (Howard 1980; Costanzo et al. 1997). A frog that freezes shortly before or during migration from its hibernaculum to the breeding pond, after thawing, will have reduced locomotor endurance. Will this affect the frog's fitness? Few studies have assessed the importance of locomotor endurance to survival in nature, and these generally consider the effects of other factors, especially sprint speed, on survival through predator avoidance (see, e.g., Watkins 1996). Although one field study has shown that endurance is not strongly related to survival (Jayne and Bennett 1990), we have several reasons to suspect that locomotor endurance is important to wood frogs during the spring reproductive period. Wood frogs may migrate a considerable distance to the breeding pond (>400 m; Bellis 1965). Even slight delays in covering this distance could cause an individual to miss part or all of the brief breeding period (Wells 1977). Once at the breeding pond, male frogs engage in energetically expensive behaviors such as calling, searching, and competing for mates (Taigen and Pough 1985). Reduced performance in these activities following freezing (Costanzo et al. 1997) may be directly due to the reduction in locomotor endurance found in the current study. Freeze tolerance is an important adaptation promoting the survival of animals that overwinter in the frost zone but apparently is not without costs.

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