Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology

Freezing Impairment of Male Reproductive Behaviors of the Freeze-Tolerant Wood Frog, Rana sylvatica
Author(s): Jon P. Costanzo, Jason T. Irwin and Richard E. Lee Jr.
Source: *Physiological Zoology*, Vol. 70, No. 2 (Mar. - Apr., 1997), pp. 158-166
Published by: University of Chicago Press . Sponsored by the Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology
Stable URL: http://www.jstor.org/stable/30164298
Accessed: 08-03-2016 13:56 UTC

Freezing Impairment of Male Reproductive Behaviors of the Freeze-Tolerant Wood Frog, *Rana sylvatica*

Jon P. Costanzo
Jason T. Irwin
Richard E. Lee, Jr.
Department of Zoology, Miami University, Oxford, Ohio 45056

Accepted by C.P.M. 8/16/96

ABSTRACT

The wood frog (*Rana sylvatica*), a temperate-zone anuran that overwinters within the frost zone, is adapted to tolerate the freezing and thawing of its tissues. Because the effects of freezing on complex neurobehavioral function are unknown and because *R. sylvatica* encounters subfreezing temperatures during its late-winter breeding season, we investigated the reproductive behaviors and physiology of male frogs after freezing (minimum body temperature, −2°C) and postthaw recovery (4°C). In tests simulating conditions at the breeding pool, these frogs, which otherwise behaved normally, exhibited reduced mate-searching effort and fewer assaults on mates and did not amplex females until 16–24 h after thawing. Although amplectic ability was ultimately restored in most frogs, they competed poorly for mates against never frozen controls. Further study suggested that the level of behavioral impairment depends on the severity of the freezing exposure. During freezing, tissues accumulated large quantities of the cryoprotectant glucose and desiccated extensively, responses that promote freezing survival. Freezing also caused marked hydroosmotic and metabolic perturbations, which may have impaired neurobehavioral function, perhaps by interfering with the processing of audio, visual, and tactile stimuli. Individuals that encounter subfreezing temperatures shortly before arriving at the breeding pools may incur reduced reproductive success.

Introduction

Several species of Holarctic amphibians and reptiles survive winter's cold by tolerating the freezing of their body tissues. The biophysical and physiological adaptations promoting vertebrate freeze tolerance have been most thoroughly investigated in the wood frog (*Rana sylvatica*), a temperate species that overwinters in relatively exposed terrestrial sites (recent reviews: Costanzo and Lee [1994]; Layne and Lee [1995]). Freeze tolerance in *R. sylvatica* is promoted by the rapid mobilization of glucose from hepatic glycogen reserves (Storey and Storey 1984). Accumulating to high concentrations in most tissues, glucose enhances the survival of cells, tissues, and organs (Costanzo et al. 1993). Another response promoting freeze tolerance is the withdrawal of up to 60% of the tissue water and the sequestration of this water in coelomic and lymphatic compartments (Lee et al. 1992). Collectively, these responses mitigate the osmotic and physical stresses of ice forming and melting within extracellular compartments that would otherwise cause irreparable damage to cells and tissues (Mazur 1984).

The nature of its cryoprotective system requires that *R. sylvatica* tolerate profound disturbances in glycemic and hydroosmotic status. Freezing also depletes high-energy phosphates and causes accumulation of harmful anaerobic metabolites in ischemic tissues (Storey and Storey 1984; Storey 1987). Despite these events, recovery from freezing is remarkably rapid, with all basic physiological and behavioral functions usually returning within several hours of thawing. The dynamics of postthaw recovery is characterized by sequential restoration of fundamental to progressively more complex vital functions (Costanzo and Lee 1994; Layne and Lee 1995). For example, cardiac contractions return even before ice in the body has completely melted, and pulmonary respiration and perfusion of peripheral tissues are restored soon thereafter (Layne and First 1991). Contractility in hindlimb muscles returns 1–2 h after thawing commences (Layne 1992), whereas capacity of the innervating sciatic nerve to propagate action potentials is restored within approximately 5 h (Kling et al. 1994). Fundamental nervous functions, such as hindlimb retraction and righting reflexes, return several hours later, and frogs ultimately exhibit normal body postures and coordinated motor functions within 14–24 h of the onset of thawing (Costanzo and Lee 1994; Layne and Lee 1995).

The effects of freezing on neurologically complex behaviors are essentially unknown. The suite of stereotypic reproductive behaviors of male *R. sylvatica* is a useful experimental model for such study, since these functions require complex integration of various sensory stimuli, neuronal, and motor systems operating under appropriate endocrine contexts (Wilczynski et al. 1993). The effects of freezing on mating behaviors may also have ecological relevance, since *R. sylvatica,* which spawns earlier in the year than sympatric anurans, often encounters

Physiological Zoology 70(2):158–166. 1997. 0031-935X/97/7002-9644$02.00

subfreezing temperatures during its breeding season (Cummins 1920; Martof and Humphries 1959; Kessel 1965; Herreid and Kinney 1967; Howard 1980; Seale 1982; Waldman 1982).

Material and Methods

Experimental Animals

Wood frogs were collected on February 27, 1995, and February 21, 1996, from a traditional breeding pool in Adams County, south-central Ohio. Weather data and field observations, including the absence of egg masses on the day preceding each collection, indicated that frogs had only recently emerged from hibernation and arrived at the pool. Within 4–6 h of capture, frogs were chilled and transported to laboratory facilities where they were housed (unfed) on damp moss within darkened plastic boxes (4°C). Unless otherwise stated, each frog was used in only one experiment.

Experimental Treatment

Frogs were frozen and thawed following a protocol that promotes survival and presumably mimics natural freezing and thawing episodes (i.e., slow freezing followed by gradual warming; Costanzo et al. 1991). Briefly, each frog was purged of bladder fluid via cloacal cannulation, weighed to 0.1 g, placed in a 50-mL polypropylene tube, and outfitted with an insulated thermocouple probe positioned against the abdomen. Frogs were cooled by packing the tubes into an insulated glass jar submerged in a refrigerated bath (~ -3°C). During cooling, body surface temperature (T_b) was recorded at 30-s intervals on a multichannel data logger (Omega Electronics OM-500).

After each frog had supercooled slightly (T_b: ~ -1°C), freezing was initiated by inoculating the skin with small ice crystals and confirmed by observing the exothermy associated with a change in the physical state of body water. Freezing continued for approximately 24 h, as each frog gradually cooled to the ultimate target T_b, typically -2°C. Control frogs were sham-treated, instrumented as described above, and chilled in an ice bath (T_b: $\sim$0°C). Frozen and sham-treated frogs were gently removed from their tubes, transferred to humidified plastic cages, and kept in darkness at 4°C. Frogs were permitted to thaw and/or recover under these conditions for 12 or 20 h, after which their general state of neuromuscular coordination was ascertained by determining whether each subject could, within 2 s, retract its manually extended hindlimb (hindlimb reflex) and right itself when placed on its dorsum (righting reflex). Specimens meeting these criteria were then used in behavioral or physiological experiments. Additionally, untreated controls, taken directly from the cold room with minimal handling, were used to establish baseline levels of various physiological parameters.

Behavioral Experiments

We maximized the sensitivity of our behavioral analyses by using only male frogs (mean ± SE body mass: 12.6 ± 0.2 g; $n = 48$) exhibiting obvious signs of reproductive readiness. Candidates for use in behavioral experiments were screened prior to freezing or sham treatment for their propensity to emit breeding calls (mate-searching, vocalization, and mate-assault tests) or engage in amplexus (amplexus time course and competition tests). Generally, 50%–75% of the frogs met these screening criteria. All experiments were blocked using pairs, with pair members matched for body mass and randomly assigned to either the frozen or sham-treatment group. Gravid female *Rana sylvatica* used in several experiments weighed approximately 25 g.

In 1995, behavioral experiments were conducted inside a fiberglass basin (floor area = 50 cm × 60 cm; wall height = 35 cm) containing dechlorinated tap water to a depth of 5 cm. The testing arena was housed in a test chamber that provided overhead lighting (1.86 lm m^{-2}) and maintained air and water temperature at 15.0° and 14.5°C, respectively. An overhead microphone and camera (Pentax PV-C850A) were used to document frog movements and vocalizations for subsequent analysis; the recorder (Panasonic AG-6010) and video monitor were stationed in a blind outside the chamber.

Mate-searching, vocalization, and mate-assault behaviors were studied in male frogs frozen to an ultimate target T_b of -2°C or sham-treated for 24 h. Twenty hours after treatment, previously frozen and sham-treated frogs were transferred from the recovery chamber (4°C) to the 15°C testing chamber and primed by exposing them for 20 min to a recording of chorusing conspecifics (indigenous to the state of New York). Primed frogs were individually released into the center of the testing arena, with the chorus continuing, and videotaped for 20 min. During playback of the tapes we determined the linear distance traveled and total area of the testing arena covered by each subject and whether each subject vocalized. The distance traveled by each frog was computed using image-analysis software; the area covered (expressed as a percentage of the total available) was determined by partitioning the arena's floor into a 6 × 8 cell grid and summing the cells each frog entered. At the trial's conclusion each frog was returned to its cage in the 15°C chamber, further primed for 2 h, and finally used in mate-assault experiments. In the latter trials we recorded the responses of experimental subjects to a gravid female that was confined inside a narrow (diameter = 7 cm) transparent glass jar positioned in the center of the arena. This scheme permitted uniform visibility of the female while also eliminating tactile cues. Male subjects were released into a randomly chosen corner of the arena and videotaped for 5 min with the chorus tape playing. Four frogs in each treatment group were tested for mate-searching, vocalization, and mate-assault behaviors in 1995, whereas five additional matched pairs were studied in spring 1996. Although the latter trials used a smaller testing

arena (floor area = 30 cm × 45 cm; wall height = 30 cm), the experimental protocol was essentially unchanged. The hypotheses that freezing reduces the distance traveled and area covered by frogs were tested using one-tailed student's *t*-tests. The hypotheses that freezing diminishes proclivity to vocalize and to initiate amplexus in response to appropriate visual stimuli were tested using one-tailed Fisher's exact tests.

The effect of freezing on amplectic ability was studied in 1995 using 12 pairs of male frogs. Twelve hours after treatment, previously frozen and sham-treated frogs were transferred from the recovery chamber to the testing chamber and promptly used in amplexus trials. Subjects were individually released on a level platform (10 cm × 10 cm) centrally positioned 1 cm above the water surface in the testing arena and permitted up to 15 min to embrace any of three receptive females. Subjects who failed this initial (12 h posttreatment) trial were returned to their cages and retested, as necessary, at successive posttreatment intervals of 16, 20, 24, and 48 h. Testing of all 12 matched pairs was completed within 4 d. Amplexus time was compared between previously frozen and sham-treated groups using the Mann-Whitney *U*-test.

Competition tests were conducted after both members of a matched pair had achieved amplexus. However, because the posttreatment timing of amplexus varied considerably between matched-pair members, the earlier-finishing subject was retried in the arena immediately prior to the competition test to ensure that it retained amplectic ability. Contests were begun by releasing both frogs on a central platform inside the arena, which contained a single gravid female. The trial continued until one male achieved amplexus, at which time the female was removed from his grasp and returned to the arena. Four additional trials were conducted in rapid succession, with the majority of trial wins determining the overall contest winner. The proportion of contests won by previously frozen frogs was compared against the expected random frequency (0.50) using the *Z*-test.

A follow-up experiment determined the effect of a relatively severe freezing regimen on postthaw recovery of amplectic ability. Groups of three frogs were frozen to −4°C for 48 h, or sham-treated for an identical period, and used in amplexus trials following an appropriate recovery period.

Physiological Assays

Studies of the physiological effects of freezing and sham treatment on male frogs (mean ± SE body mass: 13.2 ± 0.6 g; n = 18) were conducted in 1995. To mimic treatments used in behavioral experiments, frogs were frozen to an ultimate target T_b of −2°C, or sham treated, for 24 h and (when appropriate) permitted to recover in the 4°C chamber. Frozen frogs were sampled in the still-frozen state (0 h), or 12 or 24 h after treatment; control frogs were sampled immediately after sham treatment (0 h) or 12 h later. Each sample group contained three frogs. Baseline measurements were made on untreated frogs (n = 3) taken directly from their holding cages in the 4°C chamber.

Frogs were voided of bladder urine (except for still-frozen frogs, whose bladders were empty), weighed to 0.1 g, double-pithed, and dissected. Blood was collected from the aortic trunk (or harvested from the ventricle of still-frozen frogs) and centrifuged at 2,000 *g* in heparinized microcapillary tubes. After measuring hematocrit, plasma was separated from the cellular fraction. Carcasses were dissected on ice, and portions of the liver, brain, and skeletal muscle from one forelimb (flexor carpi) and one hindlimb (gracilis) were rapidly excised, blotted to remove excess surface moisture, and weighed to 0.1 mg. Deproteinized tissue extracts were prepared by homogenization of samples in 7% (wt/vol) perchloric acid, centrifugation to remove the precipitate, and neutralization with KOH. Urine, plasma, and neutralized organ extracts were stored frozen at −80°C for less than 3 wk prior to analysis.

Plasma and urine osmolalities were measured on 10-μL samples with a vapor pressure osmometer (Wescor 5500). Free hemoglobin in plasma, an indicator of catastrophic erythrocyte injury, was measured with a cyanmethemoglobin procedure (Sigma no. 525). Glucose and lactate concentrations in plasma, urine, and organ extracts were determined with glucose oxidase (Sigma no. 510) and lactate oxidase (Sigma no. 735) procedures. Differences in physiological parameters among untreated, sham-treated, and previously frozen frogs were analyzed by comparing group means using one-factor ANOVA; multiple contrasts were based on Fisher's Protected Least Significant Difference.

Results

Effect of Freezing on Reproductive Behaviors

In experiments involving mate-searching, vocalization, and mate-assault behaviors, nine frogs, having attained a mean (± SE) ultimate T_b of −2.1° ± 0.01°C during freezing episodes lasting 23.7 ± 0.7 h, exhibited leg retraction and righting reflexes, and otherwise appeared healthy 12 h after treatment. Although it was conceivable that the performance of frogs in our tests might differ from year to year, no differences occurred in distance traveled (two-factor ANOVA, F = 0.2, P = 0.690) or area covered (two-factor ANOVA, F = 0.0, P = 0.867) between 1995 and 1996; hence the data sets were combined. Freezing significantly reduced mean distance traveled and area covered within the testing arena, parameters that likely reflect mate-seeking effort (Table 1). Whereas two-thirds of sham-treated frogs emitted breeding calls during the 20-min trials, only two of the nine previously frozen frogs did so; however, the difference between these proportions was not quite significant (P = 0.077). The use of visual stimuli, in the absence of tactile cues, to elicit amplexus behavior proved to be a discriminating test of the intensity of reproductive readiness. Only sham-treated frogs were motivated to amplexus by appro-

Table 1: Behavioral responses of frozen and control wood frogs (*Rana sylvatica*) in tests simulating conditions at the breeding pool

Parameter	Previously Frozen (n = 9)	Sham-Treated (n = 9)	P[a]
Mean ± SE distance traveled (cm)	442 ± 140	943 ± 164	.017
Mean ± SE area covered (% of total)	53 ± 11	78 ± 6	.044
Percent vocalizing	22.2	66.7	.077
Percent attempting amplexus	.0	44.4	.041

[a] Probability that differences between the two groups differed.

priate visual stimuli (Table 1). These subjects briskly assailed the jar from distances of up to 20 cm, making vigorous attempts to clutch the female inside.

Twelve frogs used in the amplexus trials attained a mean (± SE) ultimate T_b of −2.2° ± 0.1°C during freezing episodes lasting 24.8 ± 0.1 h. Following freezing or sham treatment, and the subsequent 12-h recovery period, all frogs met fundamental criteria for neuromuscular and reflex behaviors and so were tested promptly. Eleven of the 12 sham-treated frogs engaged in amplexus during the initial trial, and the remaining individual succeeded when it was retested 4 h later. In contrast, only one previously frozen frog initiated amplexus 12 h after treatment; most required an additional 4–12 h of recovery, and one subject failed even after 48 h (Fig. 1). Although treatment strongly influenced the testing interval in which amplexus was achieved (U = 9.5, df = 11,12, $P < 0.0001$), the within-trial time to amplexus was comparable (t = 0.152, df = 21, P = 0.881)

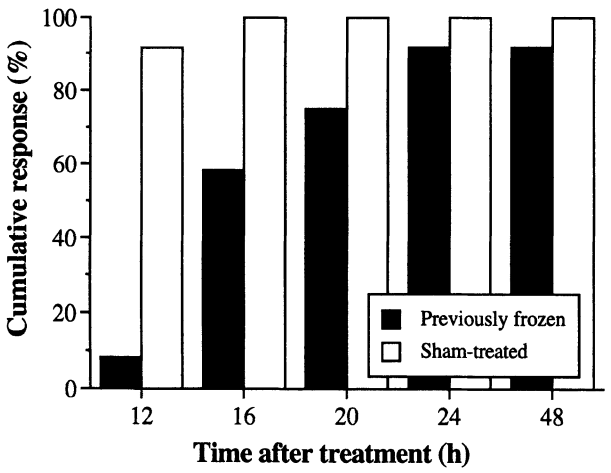

Figure 1. Cumulative sample proportions of previously frozen (n = 12) and sham-treated (n = 12) wood frogs (*Rana sylvatica*) engaging in amplexus with a female conspecific.

between previously frozen frogs (mean ± SE time: 5:22 ± 0:57 min; n = 11) and sham-treated frogs (mean ± SE time: 5:35 ± 1:03 min; n = 12).

Contests in which the previously frozen and sham-treated members of each matched pair vied for amplexus with a single female were conducted using 10 of the 12 original pairs. One pair was disqualified from testing because the previously frozen subject never regained amplectic ability. Another pair was omitted because the sham-treated subject, although it exhibited amplexus 12 h after treatment, ultimately failed the screening trial conducted prior to the competition contest. Results based on the remaining 10 pairs showed that previously frozen frogs were relatively inferior competitors, since their sham-treated counterparts had a disproportionately high frequency of wins (Z = 1.90, P = 0.029; Table 2). Previously frozen frogs fared poorly in most contests, winning at most only one of the five replicate trials. Only two previously frozen frogs won contests, and these wins were only marginally decisive (i.e., they were successful in three of five replicate trials; Table 2).

In our study of the effect of a rigorous freezing regimen on amplectic ability, three frogs attained a mean (± SE) ultimate T_b of −3.9° ± 0.01°C during freezing episodes lasting 48.2 ± 0.0 h. Two of these frogs first met qualifying neuromuscular criteria (leg retraction, righting reflexes) 60 h after treatment; they were tested but ultimately failed in amplexus trials conducted 72, 84, and 96 h after treatment. The remaining subject exhibited persistent neuromuscular dysfunction and was euthanized 60 h after treatment. In contrast, each sham-treated counterpart, tested 12 h after treatment, readily initiated amplexus.

Physiological Responses to Freezing

Nine frogs frozen for use in physiological studies attained a mean (± SE) ultimate T_b of −2.2° ± 0.1°C during freezing episodes lasting 23.8 ± 0.2 h. All subjects permitted to recover met qualifying neuromuscular criteria. Relative to baseline values for untreated frogs, significant changes in mean glucose

Table 2: Results of 10 amplexus contests, each composed of five replicate trials in which a previously frozen and sham-treated wood frog (*Rana sylvatica*) contended for a single female conspecific

Treatment Group	Number of Contests Won (Replicate Trial Wins/Losses) 5/0	4/1	3/2	Total
Previously frozen	0	0	2	2
Sham-treated	4	4	0	8

concentrations were associated with freezing and sham treatment in the liver ($P < 0.001$), brain ($P < 0.001$), forearm muscle ($P < 0.001$), thigh muscle ($P < 0.001$), plasma ($P = 0.004$), and bladder urine ($P = 0.002$). Freezing was a particularly potent stimulus of glucose accumulation (Fig. 2). Mean (± SE) glucose concentrations in still-frozen frogs were highest in the liver (238 ± 47 μmol g^{-1}) and plasma (145 ± 50 μmol mL^{-1}), although the brain showed the largest relative increase (147-fold). Smaller accumulations (13- and 15-fold increases) were found in limb muscles. Glucose levels generally declined during recovery, with plasma and liver concentrations decreasing to basal levels within 12 h of treatment. Except for minor elevations in skeletal muscle, tissue glucose levels of frogs sampled 20 h after the onset of thawing closely approximated those of untreated frogs. Sham treatment induced a significant, albeit relatively minor (e.g., five- to sixfold), accumulation of glucose in skeletal muscles, but these concentrations fully returned to basal levels within 12 h of treatment.

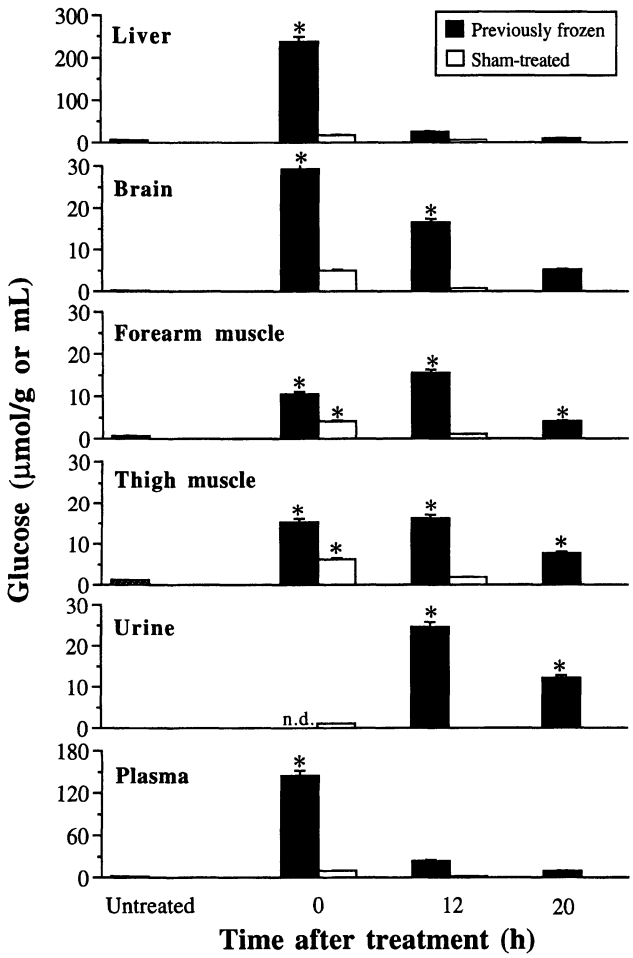

Figure 2. Glucose concentrations in tissues and body fluids of previously frozen and sham-treated wood frogs (*Rana sylvatica*) during recovery at 4°C relative to baseline values for untreated specimens. Each point represents a mean (± SE) based on $n = 3$ frogs/group. Means identified by an asterisk differed significantly ($P < 0.05$) from the untreated group.

Dynamics of organ water content were associated with freezing in the liver ($P < 0.001$), forearm muscle ($P = 0.002$), and thigh muscle ($P < 0.001$). Respective mean water contents of these organs in still-frozen frogs were 35%, 26%, and 33% lower than those in untreated frogs; however, hydration state was restored within 12 h after thawing began (Fig. 3). Sham treatment did not alter organ water content.

Freezing markedly increased the osmolality of plasma ($P < 0.001$) and urine ($P = 0.021$), as well as hematocrit ($P = 0.002$) and plasma hemoglobin ($P = 0.006$; Fig. 4). Mean (± SE) plasma osmolality was 763 ± 36 mOsmol kg^{-1} in still-frozen frogs, threefold higher than the basal level (260 ± 3 mOsmol kg^{-1}), but returned to normal within 12 h after the onset of thawing. Twelve hours after thawing began, the mean (± SE) osmolality of the bladder urine was approximately twofold higher than the basal level (84 ± 7 mOsmol kg^{-1}), which was resumed by 20 h. Mean (± SE) hematocrit was 66% ± 4% in still-frozen frogs but declined to the baseline level (31% ± 2%) within 12 h after treatment. The concentration of hemoglobin in plasma of still-frozen frogs exceeded the background levels of untreated frogs by eightfold and remained elevated even 20 h after thawing began. Sham treatment did not affect plasma or urine osmolality, hematocrit, or plasma hemoglobin levels.

Freezing induced substantial increases (from three- to 17-fold) in lactate concentration in the plasma ($P < 0.001$), liver ($P < 0.001$), and brain ($P < 0.001$), but not in forearm muscle ($P > 0.610$) or thigh muscle ($P > 0.130$), relative to untreated control frogs (Fig. 5). Tissue lactate levels generally declined during recovery and ultimately reached basal levels in the blood within 12 h and in the brain within 20 h after thawing began; in contrast, the liver concentration remained elevated throughout this period. Organ and blood lactate levels were unaffected by sham treatment.

Discussion

The primary purpose of this study was to improve our understanding of vertebrate freeze tolerance by investigating the postthaw recovery of neurobehavioral function. Although most basic physiological and behavioral faculties are restored within 2–14 h of thawing (Costanzo and Lee 1994; Layne and Lee 1995), the present work revealed that complex neurobehavioral functions may be impaired for 24 h or longer. Our secondary objectives were to probe the physiological basis and the ecological implications of the inhibiting effect of freezing on male reproductive behaviors.

Physiological Aspects of Recovery

Recovery from natural freezing episodes involves mitigation of the myriad stresses imposed by freezing and thawing, repair of damaged cells and tissues, and resumption of homeostasis. In the laboratory, complete recovery requires hours to days,

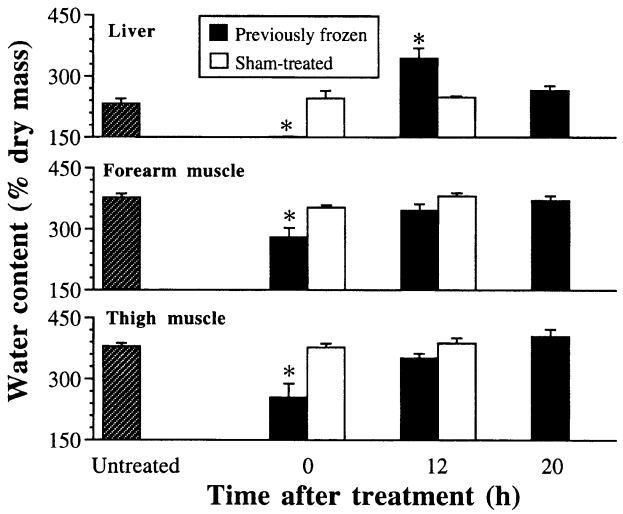

Figure 3. Tissue water contents of previously frozen and sham-treated wood frogs (*Rana sylvatica*) during recovery at 4°C relative to baseline values for untreated specimens. Each point represents a mean (± SE) based on $n = 3$ frogs/group. Means identified by an asterisk differed significantly ($P < 0.05$) from the untreated group.

depending on the severity of the freezing episode (Costanzo and Lee 1994; Layne and Lee 1995). Complex neurobehavioral functions are among the last to be restored since the nervous system of *Rana sylvatica,* which otherwise readily tolerates low temperature (Miller and Dehlinger 1969), is particularly sensitive to freezing (Kling et al. 1994).

Changes in hydroosmotic status of *R. sylvatica* during freezing and thawing are caused by the transmembrane water fluxes associated with the freezing and melting of water in extracellular spaces, as well as the cryoprotective accumulation of an osmotically active solute (glucose) and extensive tissue desiccation. Freezing also promotes hypovolemia, which in our still-frozen frogs was evidenced by marked increases in plasma osmotic concentration and hematocrit (Fig. 4). Osmotic perturbations are a primary threat to freezing survival (Mazur 1984). The minor cryohemolysis observed during even relatively mild freezing episodes attests that cells are indeed subject to osmotic injury (Fig. 4; Costanzo et al. 1993). Impaired behavioral capacity of frozen frogs may be attributed to osmotic effects on the nervous system (e.g., Hillman 1988). However, the hydration state of neural (Kling et al. 1994) and visceral (Costanzo et al. 1991; Lee et al. 1992) tissues generally returns to prefreeze levels 2–4 h after thawing begins. Similarly, in the present study normal levels of tissue water content, hematocrit, and blood osmolality were rapidly reestablished. Our analyses of the urine demonstrate the importance of the kidney—which apparently is nonfunctional during freezing—in restoring hydro-osmotic balance during postthaw recovery (Fig. 4).

The freezing-induced mobilization of glucose from hepatic glycogen reserves and its accumulation in tissues is crucial to freezing survival: glucose is the definitive chemical cryoprotectant in *R. sylvatica* (Costanzo et al. 1993). After thawing, frogs resolve hyperglycemia through renal excretion (Layne et al. 1996; Fig. 2) and by reconverting glucose into liver glycogen (Storey 1987; Costanzo and Lee 1993). Nevertheless, glucose levels in the brain and limb muscles remained elevated until 12–20 h after thawing began. The coincidental resumption of amplectic ability (Fig. 1) and glycemic homeostasis (Fig. 2) suggests that high glucose levels impair behavioral capacity, perhaps by inhibiting metabolic processes (Storey and Storey 1988). That restoration of osmotic and glycemic homeostasis is delayed in frogs subjected to particularly rigorous freezing episodes (e.g., >8 d to recover from exposure to −5°C for 48 h; Costanzo et al. 1993) may explain why frogs frozen to −4°C for 48 h in this study were refractory to amplexus behavior for at least 60 h.

Impaired behavioral capacity may also occur if freezing hampers neuroendocrine function, which strongly modulates anuran reproductive behaviors (Wilczynski et al. 1993). Freezing may promote qualitative and quantitative changes in hormone efficacy and/or impair cell recognition and signal processing systems (Hemmings and Storey 1994). Behavioral impairment could also result from freezing-induced changes in metabolic state, such as depletion of high-energy phosphate reserves and energy charge (Storey and Storey 1984; Storey 1987). Because frozen ischemic tissues respire using glycolytic energy produc-

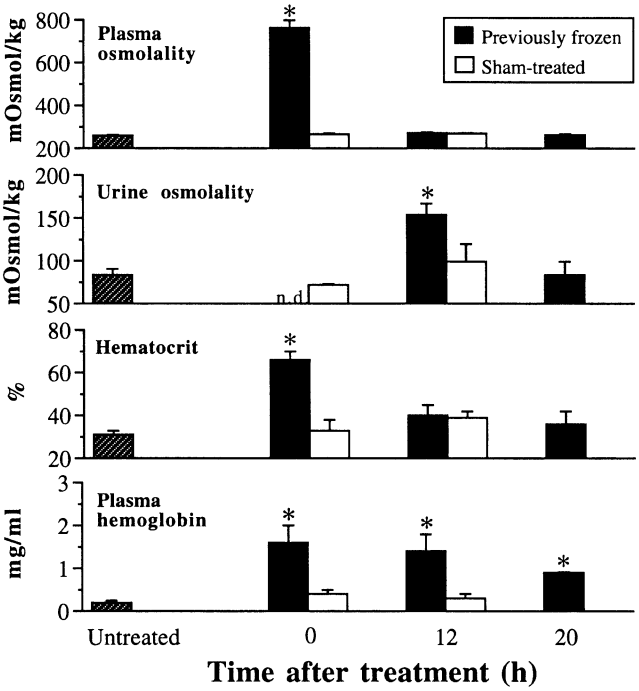

Figure 4. Osmolality of body fluids, hematocrit, and levels of free hemoglobin in plasma of previously frozen and sham-treated wood frogs (*Rana sylvatica*) during recovery at 4°C relative to baseline values for untreated specimens. Each point represents a mean (± SE) based on $n = 3$ frogs/group. Means identified by an asterisk differed significantly ($P < 0.05$) from the untreated group.

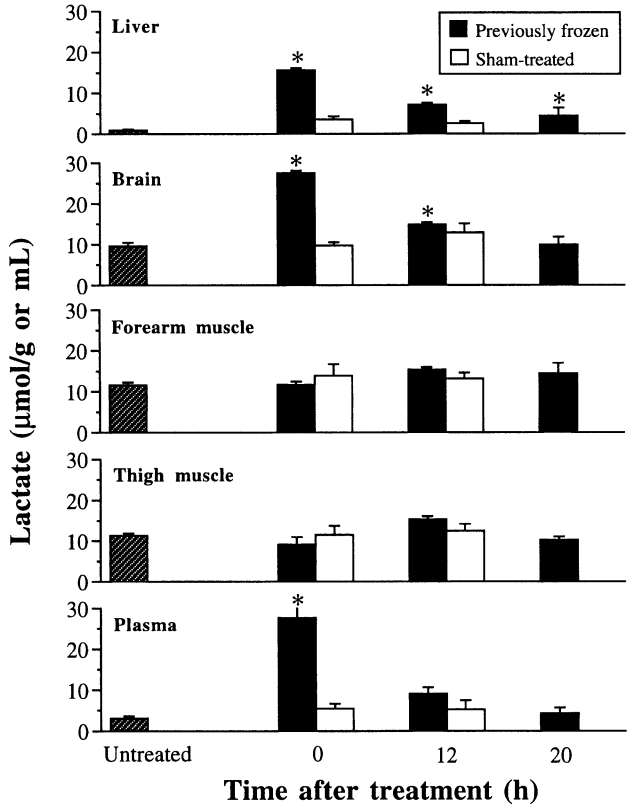

Figure 5. Lactate concentrations in tissues of previously frozen and sham-treated wood frogs (*Rana sylvatica*) during recovery at 4°C relative to baseline values for untreated specimens. Each point represents a mean (± SE) based on $n = 3$ frogs/group. Means identified by an asterisk differed significantly ($P < 0.05$) from the untreated group.

tion, lactate in visceral tissues and the blood increases during freezing (Fig. 5). Our finding that limb muscles did not accumulate lactate is consistent with some earlier results (Storey 1987; but see Storey and Storey 1984) and may stem from early metabolic arrest in these organs, since the freezing front progresses from the periphery toward the core (Rubinsky et al. 1994). Similarly, peripheral tissues accumulate less cryoprotectant and dehydrate less extensively than visceral organs (Lee et al. 1992; Costanzo and Lee 1994; Figs. 2, 3). Organs such as the liver and brain remain metabolically active longer and thus accumulate heavy lactate loads. The general concurrence in restoration of behavioral capacity and lactate clearance from these organs suggests that elevated lactate is limiting. For example, lactate in the brain was elevated 12 h after treatment, when frogs were refractory to amplexus behavior, but concentrations returned to basal levels over the next 8 h (Fig. 5), when amplectic ability was restored (Fig. 1). Ischemic hypoxia not only compromises ATP-dependent ion transport in peripheral (Strupp et al. 1991) and central (Pérez-Pinón et al. 1992) nervous systems but also may hamper nervous function via lactate accumulation and metabolic acidosis.

Implications for Male Reproductive Success

Reproductive success in male *R. sylvatica* is achieved primarily through scramble competition for females (Howard 1980; Berven 1981; Howard and Kluge 1985). Amplexus is initiated by the male responding to female body size, coloration, skin texture, motion, and behavioral responsiveness to clasping; thus, male *R. sylvatica* do not establish breeding territories but, rather, locate potential mates through active, extensive search (Banta 1914; Cummins 1920; Noble and Farris 1929). Given that recently thawed frogs may travel less and cover less area (Table 1), it is plausible that freezing limits reproductive success. In tests intended to simulate conditions of the breeding pool, sham-treated frogs, but not previously frozen frogs, typically maintained the head, dorsal trunk, and thighs at or above the water surface (see also Banta 1914; Berven 1981). Greater buoyancy, possibly due to the lungs being hyperinflated during calling, may provide an elevated vantage from which mates may be located.

Motion is likely a chief stimulus of amplectic behavior in male *R. sylvatica* (Banta 1914; Cummins 1920; Noble and Farris 1929; Howard and Kluge 1985). Previously frozen and sham-treated frogs differed in their ability to be stimulated to amplexus in the presence of a potential mate (Table 1). The failure of visual cues to elicit amplexus in previously frozen frogs could stem from impairment of the ocular sensory and image-recognition system, or it may indicate a generally reduced intensity of reproductive motivation.

Because *R. sylvatica* is an explosive breeder, the synchronous choruses of males may be of primary importance for attracting gravid females to the breeding site, rather than to any particular individual (Wells 1977). Reduced chorus intensity thus may indirectly bear on individual fitness if fewer females are drawn to the pool (e.g., Seale 1982; but see Tejedo 1993). Freezing might inhibit calling behavior by impairing the neural pathways involved in vocalization, such as anterior preoptic and pretrigeminal nuclei (Schneider 1988) and/or pertinent effector systems. However, given the level of significance obtained in our tests, the effect of freezing on vocalization must be considered inconclusive.

Amplexus is a manifestation of behaviors intended to properly align the bodies of the male and female in preparation for spawning. As in previous studies (Costanzo et al. 1991; Layne and First 1991; Layne 1992; Kling et al. 1994), our frogs exhibited normal neuromuscular reflexes and posture and otherwise appeared healthy 12 h after thawing commenced. However, amplectic ability was suppressed in previously frozen frogs for an additional 4–12 h or more. Incidental physical contact with a gravid female occasionally failed to culminate in amplexus for previously frozen specimens but never for sham-treated frogs. Furthermore, although most previously frozen frogs ultimately regained amplectic ability (Fig. 1), when pitted against sham-treated frogs they were inferior competitors (Table 2). The deleterious effect of freezing likely is exacerbated because

male *R. sylvatica* must often vie for relatively few mates (Howard 1980; Berven 1981; Howard and Kluge 1985). Given that frogs frozen to −4°C for 48 h were refractory to amplexus for at least 4 d, our data further suggest that behavioral inhibition may be even greater following more severe freezing episodes. Similarly, postthaw restoration of simple neuromuscular functions is delayed in specimens subjected to rigorous freezing episodes involving rapid cooling (Costanzo et al. 1991), exposure to low temperature (Costanzo et al. 1993; Layne 1995), and prolonged freezing (Layne 1992).

Ecological and Evolutionary Implications

The breeding season of *R. sylvatica* ranges from January in the southern United States to July in northern Canada, but in all locales the onset coincides with an early thaw (Moore 1942; Martof and Humphries 1959; Kessel 1965). Early breeding of *R. sylvatica* minimizes the risk of predation, reduces competition with other amphibians for oviposition sites, and improves the likelihood that larvae metamorphose before natal pools dry or freeze. Having been aroused from hibernation (by some as yet unidentified stimulus), males migrate potentially long distances to traditional breeding pools. Not uncommonly, cold weather may return abruptly, exposing frogs to subfreezing temperatures and interrupting both migration and mating activities (Cummins 1920; Martof and Humphries 1959; Kessel 1965; Herreid and Kinney 1967; Howard 1980; Seale 1982; Waldman 1982). Whereas frogs arriving at the pool can avoid freezing by submerging in water (Licht 1991), frogs whose arousal was delayed or whose migration was particularly long remain abroad and thus may encounter subfreezing temperatures. Freezing risk seems especially high during the migration because active frogs would be relatively exposed, the duff and upper soil layer would provide little geothermal warming or buffering against low air temperatures, and the seasonal minimum temperatures often occur during late winter (Fig. 6).

Although freeze tolerance of *R. sylvatica* extends into spring (Storey and Storey 1988; Layne 1995), freezing is not without consequences for organismal fitness. Due to the brevity of the breeding season (Wells 1977), frogs exposed to even mild freezing episodes (e.g., −2°C for 24 h) may be sufficiently impaired that they forfeit mating opportunities. Although amplectic ability might eventually be recovered, freeze-exposed frogs would compete poorly against frogs that avoided freezing. Furthermore, late breeding typically results in eggs being deposited at the periphery of the communal aggregate, where they may suffer reduced survivorship to hatching (Waldman 1982). This scenario presents an intriguing example of how an environmental stressor may compromise organismal fitness by impairing neurobehavioral function and raises novel questions about the costs and benefits of early breeding in temperate amphibians. Further study should determine the influence of freezing risk on the spatial proximity of overwintering sites to the breeding pool, the stimulus for arousal and synchrony of frog migration, and the effects of freezing on female reproductive success.

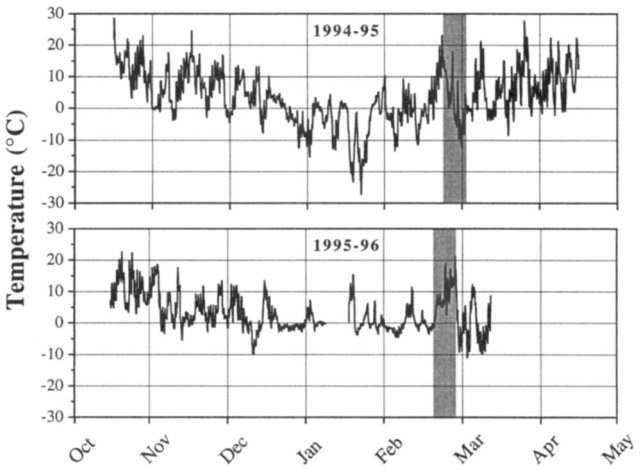

Figure 6. Air temperatures during the winters of 1993–1994 and 1995–1996 near a traditional breeding pool of *Rana sylvatica*, the source of animals used in the present study, in south-central Ohio. Data were collected at 2-h intervals by a miniature data logger (Onset, HoboTemp) and a shaded thermistor positioned about 6 cm above the forest floor. The unit was deployed in an upland deciduous forest, about 30 m from the pool, and gathered data representing temperatures experienced by surface-active frogs. The breeding season is represented by a shaded region on each graph. Some data were lost when snow covered the thermistor in mid-January 1996. Of particular note is that subfreezing temperatures were recorded 60 h before frogs began arriving at the breeding pool on February 21, 1996.

Acknowledgments

We thank R. Knauff for assistance with the collection of frogs, and R. Brown, P. A. Callahan, M. E. Feder, M. P. Frisbie, R. L. Schaefer, D. H. Taylor, M. F. Wright, and two anonymous reviewers for critically reading the manuscript. Research was supported by grants from the National Institutes of Health (NIDDKD 1R15DK48067-01A1) and the National Science Foundation (IBN 9507437). A portion of this work will be submitted by J.T.I. to the faculty of the Department of Zoology, Miami University, in partial fulfillment of the requirements for the M.S. degree.

Literature Cited

Banta A.M. 1914. Sex recognition and the mating behavior of the woodfrog, *Rana sylvatica*. Biol. Bull. 26:171–183.

Berven K.A. 1981. Mate choice in the wood frog, *Rana sylvatica*. Evolution 35:707–722.

Costanzo J.P. and R.E. Lee, Jr. 1993. Cryoprotectant production capacity in the freeze-tolerant wood frog, *Rana sylvatica*. Can. J. Zool. 71:71–75.

———. 1994. Biophysical and physiological responses promot-

ing freeze tolerance in vertebrates. News Physiol. Sci. 9:252–256.

Costanzo J.P., R.E. Lee, Jr., and P.H. Lortz. 1993. Glucose concentration regulates freeze tolerance in the wood frog *Rana sylvatica.* J. Exp. Biol. 181:245–255.

Costanzo J.P., R. E. Lee, Jr., and M.F. Wright. 1991. Effect of cooling rate on the survival of frozen wood frogs, *Rana sylvatica.* J. Comp. Physiol. B 161:225–229.

Cummins H. 1920. The role of voice and coloration in spring migrations and sex recognition in frogs. J. Exp. Zool. 30:325–343.

Hemmings S.J. and K.B. Storey. 1994. Alterations in hepatic adrenergic receptor status in *Rana sylvatica* in response to freezing and thawing: implications to the freeze-induced glycemic response. Can. J. Physiol. Pharmacol. 72:1552–1560.

Herreid C.F. and S. Kinney. 1967. Temperature and development of the wood frog, *Rana sylvatica,* in Alaska. Ecology 48:579–590.

Hillman S.S. 1988. Dehydrational effects on brain and cerebrospinal fluid electrolytes in two amphibians. Physiol. Zool. 61:254–259.

Howard R.D. 1980. Mating behaviour and mating success in woodfrogs, *Rana sylvatica.* Anim. Behav. 28:705–716.

Howard R.D. and A.G. Kluge. 1985. Proximate mechanisms of sexual selection in wood frogs. Evolution 39:260–277.

Kessel B. 1965. Breeding dates of *Rana sylvatica* at College, Alaska. Ecology 46:206–208.

Kling K.B., J.P. Costanzo, and R.E. Lee, Jr. 1994. Post-freeze recovery of peripheral nerve function in the freeze-tolerant wood frog, *Rana sylvatica.* J. Comp. Physiol. B 164:316–320.

Layne J.R., Jr. 1992. Postfreeze survival and muscle function in the leopard frog (*Rana pipiens*) and the wood frog (*Rana sylvatica*). J. Therm. Biol. 17:121–124.

———. 1995. Seasonal variation in the cryobiology of *Rana sylvatica* from Pennsylvania. J. Therm. Biol. 20:349–353.

Layne J.R., Jr. and M.C. First. 1991. Resumption of physiological functions in the wood frog (*Rana sylvatica*) after freezing. Am. J. Physiol. 261:R134–R137.

Layne J.R., Jr. and R.E. Lee, Jr. 1995. Adaptations of frogs to survive freezing. Climate Res. 5:53–59.

Layne J.R., Jr., R.E. Lee, Jr., and M.M. Cutwa. 1996. Post-hibernation excretion of glucose in urine of the freeze tolerant frog *Rana sylvatica.* J. Herpetol. 30:85–87.

Lee R.E., Jr., J.P. Costanzo, E.C. Davidson, and J.R. Layne, Jr. 1992. Dynamics of body water during freezing and thawing in a freeze-tolerant frog (*Rana sylvatica*). J. Therm. Biol. 17:263–266.

Licht L.E. 1991. Habitat selection of *Rana pipiens* and *Rana sylvatica* during exposure to warm and cold temperatures. Am. Midland Nat. 125:259–268.

Martof B.S. and R.L. Humphries. 1959. Variation in *Rana sylvatica.* Am. Midland Nat. 61:350–389.

Mazur P. 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol. 247:C125–C142.

Miller L.K. and P.J. Dehlinger. 1969. Neuromuscular function at low temperatures in frogs from cold and warm climates. Comp. Biochem. Physiol. 28:915–921.

Moore J.A. 1942. The role of temperature in speciation of frogs. Biol. Symp. 6:189–213.

Noble G.K. and E.J. Farris. 1929. The method of sex recognition in the wood-frog, *Rana sylvatica* Le Conte. Am. Museum Novitates 363:1–17.

Pérez-Pinón M.A., C.Y. Chan, M. Rosenthal, and T.J. Sick. 1992. Membrane and synaptic activity during anoxia in the isolated turtle cerebellum. Am. J. Physiol. 263:R1057–R1063.

Rubinsky B., S.T.S. Wong, J.-S. Hong, J. Gilbert, M. Roos, and K.B. Storey. 1994. ^{1}H magnetic resonance imaging of freezing and thawing in freeze-tolerant frogs. Am. J. Physiol. 266:R1771–R1777.

Schneider, H. 1988. Peripheral and central mechanisms of vocalization. Pp. 537–558 in B. Fritzsch, M.J. Ryan, W. Wilczynski, T.E. Hetherington, and W. Walkowiak, eds. The Evolution of the Amphibian Auditory System. Wiley, New York.

Seale D.B. 1982. Physical factors influencing oviposition by the woodfrog, *Rana sylvatica,* in Pennsylvania. Copeia 1982: 627–635.

Storey K.B. 1987. Organ-specific metabolism during freezing and thawing in a freeze-tolerant frog. Am. J. Physiol. 253:R292–R297.

Storey K.B. and J.M. Storey. 1984. Biochemical adaptation for freezing tolerance in the wood frog, *Rana sylvatica.* J. Comp. Physiol. B 155:29–36.

———. 1988. Freeze tolerance in animals. Physiol. Rev. 68:27–84.

Strupp M., R. Jund, U. Schneider, and P. Grafe. 1991. Glucose availability and sensitivity to anoxia of isolated rat peroneal nerve. Am. J. Physiol. 261:E389–E394.

Tejedo M. 1993. Do male natterjack toads join larger breeding choruses to increase mating success? Copeia 1993:75–80.

Waldman B. 1982. Adaptive significance of communal oviposition in wood frogs (*Rana sylvatica*). Behav. Ecol. Sociobio. 10:169–174.

Wells K.D. 1977. The social behaviour of anuran amphibians. Anim. Behav. 25:666–693.

Wilczynski W., J.D. Allison, and C.A. Marler. 1993. Sensory pathways linking social and environmental cues to endocrine control regions of amphibian forebrains. Brain Behav. Evol. 42:252–264.