Overwintering Physiology and Hibernacula Microclimates of Blanchard's Cricket Frogs at Their Northwestern Range Boundary

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Blanchard's Cricket Frogs (*Acris crepitans blanchardi*) in the central portion of their range show minimal capacities for freezing tolerance and survive overwinter by using terrestrial hibernacula where they avoid freezing. However, frogs may exhibit greater freeze-tolerance capacity at high latitude range limits, where winter climate is more severe. We studied freezing tolerance, glucose mobilization during freezing, and hibernacula microclimates of cricket frogs in southeastern South Dakota, at the northwestern limit of their range. Cricket frogs from South Dakota generally survived freezing exposure at −1.5 to −2.5°C for 6-h periods (80% survival), but uniformly died when exposed to these same temperatures for 24-h freezing bouts. Hepatic glucose levels and phosphorylase *a* activities increased significantly during freezing, but hepatic glucose levels during freezing remained low, only reaching levels approximating those prior to freezing in freeze-tolerant species. Moreover, muscle glucose and hepatic glycogen levels did not vary with freezing, suggesting little mobilization of glucose from hepatic glycogen stores during freezing, contrasting with patterns in freeze-tolerant frogs. Temperatures in soil cracks and burrows potentially used for hibernacula were variable, with some sites remaining above the freezing point of the body fluids throughout the winter, some sites dropping below the freezing point for only short periods, and some sites dropping below the freezing point for extended periods. These data suggest that cricket frogs in South Dakota, as in other portions of their range, survive overwinter by locating hibernacula that prevent freezing, although their toleration of short freezing bouts may expand the range of suitable hibernacula. These data also suggest that overwinter mortality may be high at the northern range boundary and might limit cricket frogs from expanding their range northward.

ANURANS inhabiting seasonally cold climates survive the winter by either freeze tolerance or freeze avoidance strategies. Most anurans are intolerant of freezing, and typical freeze avoidance strategies include overwintering in aquatic situations or burrowing in the soil to depths below the frostline (Pinder et al., 1992). A few species of frogs that overwinter in shallow terrestrial hibernacula tolerate the freezing of up to 50–70% of their body water (Storey, 1997; Storey and Storey, 2004). Freeze-tolerant frogs accumulate low molecular weight carbohydrates (glucose or glycerol) as cryoprotectants during freezing that assist in limiting freezing to extracellular fluids (Storey and Storey, 2004). Most species, including Chorus Frogs *Pseudacris triseriata* and Spring Peepers *P. crucifer*, which are closely related to cricket frogs (Faivovich et al., 2005; Wiens et al., 2005, 2006), accumulate glucose as a cryoprotectant upon freezing (Storey and Storey, 1986; Churchill and Storey, 1996; Swanson et al., 1996). However, the gray treefrogs *Hyla versicolor* and *H. chrysoscelis* use glycerol as a major cryoprotectant, with glycerol levels increasing during both cold acclimation and freezing, at least in adults (Storey and Storey, 1985, 1986; Costanzo et al., 1992; Layne and Jones, 2001; Irwin and Lee, 2003; Zimmerman et al., 2007). Recent evidence from Wood Frogs (*Rana sylvatica*) suggests that urea also plays a cryoprotectant role in this species (Costanzo and Lee, 2005, 2008; Costanzo et al., 2008).

Blanchard's Cricket Frog (*Acris crepitans blanchardi*) is a small hylid frog inhabiting stream and pond margins in the central United States (Conant and Collins, 1998; Gamble et al., 2008). Blanchard's Cricket Frogs in central portions of their range employ an unusual overwintering strategy, hibernating in moist terrestrial situations that protect frogs from freezing (Irwin et al., 1999). The northern limit of the cricket frog range occurs from southeastern South Dakota across southern Minnesota and Wisconsin to southern Michigan (Conant and Collins, 1998; Kiesow, 2006). Blanchard's Cricket Frog is considered a subspecies of the Northern Cricket Frog, *A. crepitans*, but Gamble et al. (2008) suggest full species status is warranted based on molecular genetic data. Blanchard's Cricket Frog has recently undergone widespread population declines in the northernmost portion of its range, but causes of these declines are not known (Lannoo, 1998; Gray and Brown, 2005; Gray et al., 2005; Lehtinen and Skinner, 2006).

The few data on overwintering strategies of Blanchard's Cricket Frogs tested frogs from the central portion of their range (Gray, 1971; Irwin et al., 1999). These studies found that frogs overwintered in terrestrial habitats near the margins of ponds or streams, principally in cracks in the mud or in crayfish burrows. Moreover, cricket frogs did not survive exposure to low oxygen levels in water necessary for overwintering in hypoxic aquatic habitats, as aquatic-wintering species do (e.g., *Rana pipiens*; Irwin et al., 1999). Cricket frogs from the central portion of their range are evidently intolerant of freezing. Gray (1971) found that cricket frogs from central Illinois exposed to −2°C in an aquarium with simulated shoreline died if they remained on the surface, but survived if they were given access to cracks of >12 cm depth. In addition, Irwin et al. (1999) experimentally exposed cricket frogs from southern Ohio to −2.3°C for 24 hours and found that only two of 15 individuals survived this freezing exposure. This is a much lower survival rate than for freezing tolerant anurans, such as Wood Frogs. Interestingly, however, Irwin et al. (1999) found that tissue levels of the cryoprotectant glucose did increase markedly with freezing in cricket frogs, although not to levels present in freeze-tolerant species (Storey and Storey, 1984, 1986; Churchill and Storey, 1996; Swanson et al., 1996), and these elevated glucose levels did not confer freezing tolerance.

The thermal buffering properties of the moist terrestrial hibernacula allow cricket frogs in the central portion of their

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range to avoid freezing. For example, Irwin et al. (1999), in a study in southwestern Ohio, found that soil temperature 2 cm below the surface in a burrow similar to those used by cricket frogs for hibernacula did not drop below the freezing point of the body fluids, despite air temperatures as low as −19.9°C. However, the colder winter climates at high latitude range limits may compromise overwinter survival in amphibians. Southeastern South Dakota lies at the northwestern range limit of Blanchard's Cricket Frog and experiences colder winter climates than more central portions of the species range. For example, average January temperatures from 1900–2008 for southeastern South Dakota and southwestern Ohio are −8.4°C and −1.2°C, respectively (NOAA Climate Division database; http://climate.sdstate.edu/ClimateDivisions/Seasonal.cfm). Because typical cricket frog hibernation sites in southwestern Ohio approached the freezing point of the body fluids (Irwin et al., 1999), the colder climate of South Dakota might require hibernating at deeper sites to avoid freezing, or the development of freezing tolerance mechanisms. Jenkins and Swanson (2005) found that Chorus Frogs (a freeze-tolerant species) in South Dakota show annual variability in their freezing tolerance capacity, and that this variability was associated with glucose mobilization upon freezing, as most frogs from populations mobilizing low levels of glucose died during freezing exposure. Other freezing tolerant anurans show geographic variation in capacity for freezing tolerance that is correlated with severity of the winter climate (Layne and Lee, 1987; Layne, 1999). Perhaps Blanchard's Cricket Frogs from central portions of their range have poorly developed freezing tolerance capacities and freezing-induced glucose mobilization abilities relative to frogs at the northern range limit, but such geographic variation in freezing tolerance is untested in this species.

One possible factor limiting cricket frog distribution in South Dakota is overwinter mortality, which may be greater at the northern boundary of the range than in more southerly portions of the species' range, particularly if freezing tolerance capacities are as poorly developed as in populations from the central portion of their range. Documentation of the overwintering strategy (freeze tolerance vs. freeze avoidance) and the importance of winter mortality in limiting cricket frog populations in South Dakota requires, as a first step, defining hibernation sites used by cricket frogs in South Dakota and delineation of environmental conditions present at these sites, as well as capacity for freezing tolerance. We conducted surveys for cricket frog overwintering sites in the late fall and early winter to determine if frogs at the northern limit of their range use similar overwintering sites to frogs in more southerly locations. We also performed freezing exposure experiments and measured glucose mobilization during freezing on winter acclimated frogs to determine the freezing tolerance and cryoprotectant mobilization capacities of South Dakota cricket frogs. These data will help elucidate whether overwinter mortality due to the cold climates at the northern end of the species' range are a potential factor limiting Blanchard's Cricket Frog distribution along the northern range boundary.

MATERIALS AND METHODS

Overwintering hibernacula.—During late October to November of 2005 through 2007, just prior to the period when frogs enter hibernation, we observed large numbers of cricket frogs congregating at several sites along the James and Big Sioux rivers in southeastern South Dakota. We did not observe frogs in large numbers at these sites during summer surveys, or during spring (March and early April) when data loggers were retrieved, which suggests that frogs were congregating at these sites for hibernation. During these late fall surveys, we observed frogs near, or emerging from, numerous deep cracks in mud (up to 20–25 cm in depth) and burrows in the riverbanks, suggesting that these cracks and burrows served as hibernacula. We deployed a number of data loggers (iButton Model 1921Z, Maxim, Sunnyvale, CA; 14 mm diameter, 5 mm thick), wrapped in self-adhesive cellophane to inhibit moisture damage, at four sites along the James River (43°10′37″N, 97°36′56″W; 43°08′40″N, 97°34′39″W; 43°08′57″N, 97°35′18″W; 43°08′39″N, 97°32′18″W) and two sites along the Big Sioux River (42°48′54″N, 96°35′47″W; 42°48′37″N, 96°35′44″W). When we observed frogs emerging from a crack or burrow in the shoreline, we measured depth and then placed data loggers (12 in 2005, 16 in 2006, and 13 in 2007) into the mud at the bottom of the crack or burrow. During excavation of our 2005–2006 data loggers along the James River in early April 2006 (see below), we observed a cricket frog emerging from the soil approximately 15 cm from where a data logger had been placed. This suggests that data loggers were placed in appropriate locations to record hibernacula conditions.

We marked locations of the data loggers at each site by flagging, and attached one data logger (iButton Model 1921G, Maxim, Sunnyvale, CA) to the stem of one flag at each study site, roughly 0.4 m above a prospective hibernaculum, to record unshaded ambient air temperature. The data loggers we used in this study are accurate to 0.25°C. We programmed data loggers to take temperature readings every two hours for the duration of the winter period (November through March or early April). We recovered data loggers from prospective hibernation sites in March or early April, depending on spring water levels, and retrieved nine of 12 data loggers in 2006 and ten of 13 data loggers in 2008. After extensive flooding during the spring and summer of 2007, we recovered only one data logger from those deployed during the winter of 2006–2007. We calibrated data loggers upon retrieval against a thermometer traceable to the U.S. National Institute of Standards and Technology and corrected data logger temperature recordings for any differences (generally no more than 0.5 to 1°C) in subsequent analyses.

Freezing tolerance studies.—To test freezing-tolerance capacity for Blanchard's Cricket Frogs from South Dakota, we acclimated frogs to winter conditions according to the protocol of Swanson et al. (1996). We collected frogs for freezing experiments in late October or early November, just prior to entrance into hibernation, from the same study sites along the James and Big Sioux rivers where data loggers were deployed in prospective hibernacula. After collection, we placed frogs in plastic terraria lined with moist paper towels and covered frogs with leaf litter. For two weeks after collection, we kept frogs in an incubator at 10°C in complete darkness without food to allow frogs to digest any material within their digestive tracts. Then we reduced the temperature to 2°C, where it was kept until frogs were used in freezing trials during December–February. We checked frogs every 2–3 days over the winter period for adequate terrarium

moisture, and replaced the moist paper towels every two weeks to avoid mold growth.

We tested freezing survival of frogs following 6-h and 24-h freezing bouts. The 24-h freezing bouts are routinely tolerated by the closely related Chorus Frog in South Dakota but not by freeze-intolerant species, such as Woodhouse's Toad *Bufo woodhousii* and Great Plains Toad *B. cognatus* (Swanson et al., 1996). We incorporated the 6-h freezing survival tests based on our prospective hibernaculum temperature data from the 2005–2006 winter, where temperatures for four prospective hibernacula dropped below the freezing point of the body fluids for only short periods, ranging from 2–12 h (Fig. 1). We chose 6 h for our freezing tolerance experiments to determine if frogs could minimally survive freezing bouts at the lower end of this range. For freezing survival experiments, we placed frogs in a freezing chamber consisting of a foam-lined glass jar immersed in a circulating water/ethylene glycol bath (Forma Scientific Inc., Model 2095, Marietta, OH). We monitored body temperature (T_b) throughout the trials with a thermistor thermometer (YSI Model 43 TB and YSI Model 427 thermistor probe, Yellow Springs, OH), with the probe secured to the frog's abdomen with masking tape. We recorded T_b every 15 seconds over the course of the freezing trial. We also continuously monitored the bath temperature with a thermistor thermometer (YSI Model 4600). When both frog and freezing chamber had come to equilibrium around −1.0°C, we nucleated freezing by placing an ice crystal against the frog's hindlimb. This invariably produced a freezing exotherm, which verified freezing. Following freezing, we slowly reduced the temperature of the bath to between −1.5 and −2.5°C (bath cooling rate of approximately 1.0°C/hr) for the remainder of the freezing bout.

After freezing bouts, we returned frogs to the incubator for thawing on moist paper towels at 2°C. We tested frogs for freezing survival at one, three, and seven days following freezing by testing righting response and leg retraction reflexes and assessing normal posture and locomotion. We considered frogs exhibiting positive responses to all survival criteria to have successfully survived the freezing bout.

On a separate set of winter-acclimated frogs, we measured liver and mixed leg muscle glucose and glycogen concentrations and glycogen phosphorylase activities on frozen (6-h and 24-h freezing bouts) and unfrozen control frogs. At the termination of freezing bouts, we double-pithed and quickly excised liver and mixed leg muscles over ice. Unfrozen control frogs were double-pithed directly from the incubator before excising tissues. We flash-froze tissues in liquid nitrogen and stored them at −80°C until later glucose, glycogen, and phosphorylase assays. We used a colorimetric glucose oxidase assay kit (WAKO Chemicals, Richmond, VA) for measurement of glucose and glycogen (as glucosyl units) and a spectrophotometric assay coupled to the reduction of NADP for measurement of glycogen phosphorylase activity. Because glycogen phosphorylase exists in active and inactive forms, we assayed both active (phosphorylase *a*) and total (after conversion of inactive to active form by addition of ADP to the assay medium; Swanson et al., 1996) phosphorylase activity and calculated the percentage of phosphorylase in the active form. We conducted assays according to the procedures outlined in Swanson et al. (1996), Edwards et al. (2000), and Jenkins and Swanson (2005). For comparisons of mean tissue glucose and glycogen levels, phosphorylase activities, and percent active phosphorylase, we used one-way ANOVA with Tukey's test to identify significant differences among groups. If parametric assumptions of equal variance or normal distribution were not met, we first tried $\log_{10}$ or ln-transforming data before ANOVA. If parametric assumptions were still not met, we used Kruskal-Wallis test with Dunn's *post hoc* test for these comparisons. We employed arcsin-square root transformation for percent active phosphorylase data prior to statistical comparisons.

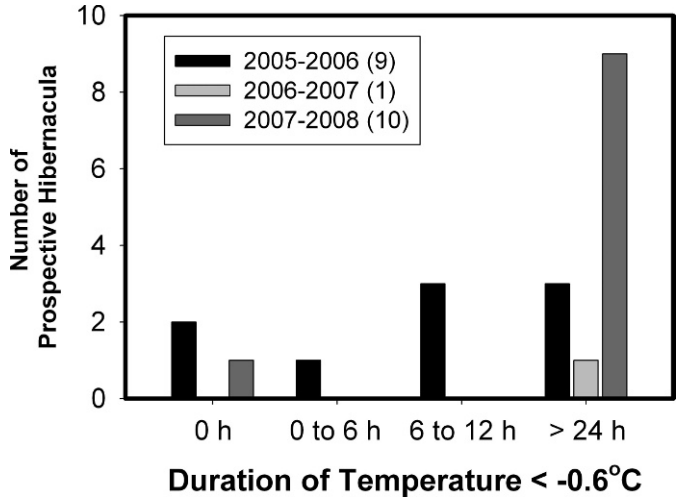

Fig. 1. Number of prospective hibernacula experiencing maximum durations of 0 h, 0 to 6 h, 6 to 12 h, and >24 h below the freezing point of cricket frog body fluids (−0.6°C) during the different winters of the study (no prospective hibernacula exhibited maximum durations below the freezing point of the body fluids of 12 to 24 h). The four duration intervals plotted represent temperature exposures that are likely survivable (0 h and 0 to 6 h), potentially survivable (6 to 12 h), and likely fatal (>24 h) to cricket frogs based on our freezing tolerance tests. All data loggers from the >24 h group experienced durations of at least 42 hours below −0.6°C. Numbers included in parenthesis in the figure legend are the total number of temperature data loggers recovered during each winter.

RESULTS

Overwintering hibernacula.—Air temperatures varied substantially within and among the three winters, with 2005–2006 having above-average temperatures, 2006–2007 having nearly average temperatures and 2007–2008 having below average temperatures. For example, average daily winter (December–February) temperature (data from South Dakota Office of Climatology website; http://climate.sdstate.edu/climate_site/climate_page.htm) for Yankton, South Dakota, which is near the James River study sites, was −3.5°C in 2005–2006, −5.9°C in 2006–2007, and −8.3°C in 2007–2008, compared to a long-term (1971–2000) average of −5.7°C. Despite this variation among years, air temperatures at all study sites dipped below −25°C for short periods every winter.

Prospective hibernacula temperatures exhibited much less variation, but nevertheless showed substantial variation with respect to the freezing point of the body fluids of frogs of −0.6°C (Fig. 1). In the 2005–2006 winter, two prospective hibernacula did not drop below −0.6°C, four dropped below −0.6°C, but only for a maximum period of 12 hours (lowest recorded temperature = −1.89°C), and three dropped below the freezing point of body fluids for extended periods, ranging from 42–122 h (Fig. 2). The one prospective

hibernaculum for which we were able to recover a data logger deployed during the winter of 2006–2007 exhibited temperatures below the freezing point of body fluids for several extended periods of up to 9.2 days. Finally, during 2007–2008, temperatures in one prospective hibernaculum did not drop below the freezing point of body fluids, but all other prospective hibernacula exhibited temperatures below −0.6°C for extended periods, with maximum periods below the freezing point of the body fluids ranging from 52 h to 14 d, and temperatures dropping below −5°C in several hibernacula.

Freezing tolerance and glucose mobilization.—Blanchard's Cricket Frogs from southeastern South Dakota showed generally poor freezing survival. Frogs uniformly died when exposed to 24-h freezing bouts at −1.5 to −2.5°C (0 survivors out of 12 exposed to freezing bouts). However, 80% (eight survivors out of ten exposed to freezing bouts) of frogs survived a 6-h freezing bout at the same temperatures.

Mean hepatic glucose levels were significantly elevated in frozen frogs, with 6-h frozen frogs showing the highest glucose levels (6.6-times control levels) and 24-h frozen frogs also with significantly elevated glucose levels (3.5-fold) relative to unfrozen controls (Table 1). Hepatic glycogen levels did not differ significantly with freezing. Hepatic glycogen phosphorylase *a* activity was significantly elevated in 24-h frozen frogs relative to unfrozen controls (27-fold), but no other significant differences in active-form or total phosphorylase in liver were apparent (Table 1). Percent active phosphorylase values in liver were also higher in 24-h frozen frogs than in controls, but other groups did not differ significantly. Hepatic total phosphorylase activity did not differ significantly among treatments (Table 1). In addition, no differences in muscle glucose, glycogen, phosphorylase activities, or percent active phosphorylase occurred among treatments (Table 1).

DISCUSSION

Blanchard's Cricket Frogs from southeastern South Dakota, the northwestern limit of their range, were not tolerant of freezing exposure, as all frogs exposed to 24-h freezing bouts died. This contrasts with freeze-tolerant anuran species, which regularly tolerate such freezing exposure (Storey and Storey, 1988, 2004; Swanson et al., 1996). Contrary to our predictions, cricket frogs from South Dakota were not more tolerant of freezing than cricket frogs from the central portion of their range in southwestern Ohio, despite the colder winter climate in South Dakota. Irwin et al. (1999) found that two of 15 cricket frogs tested survived 24-h freezing bouts in the southwestern Ohio population, although these authors suggested that freezing tolerance is not a tenable strategy for overwinter survival in this species. Thus, these data provide no evidence for greater freezing tolerance at the northern range limit in Blanchard's Cricket Frogs.

Consistent with the absence of freezing tolerance, South Dakota cricket frogs showed little capacity for glucose mobilization during freezing. For most freeze-tolerant anurans, including closely related Chorus Frogs and Spring Peepers, glucose is a major cryoprotectant and tissue glucose levels increase markedly upon freezing, generally along with reductions in hepatic glycogen stores (Storey, 1990; Churchill and Storey, 1996; Swanson et al., 1996; Storey and Storey, 2004). Hepatic glucose levels did increase with freezing in cricket frogs in this study, but only to levels in the range of, or slightly greater than, those in unfrozen control individuals of freeze-tolerant frogs (Storey and Storey, 1984, 1985; Storey, 1987; Churchill and Storey,

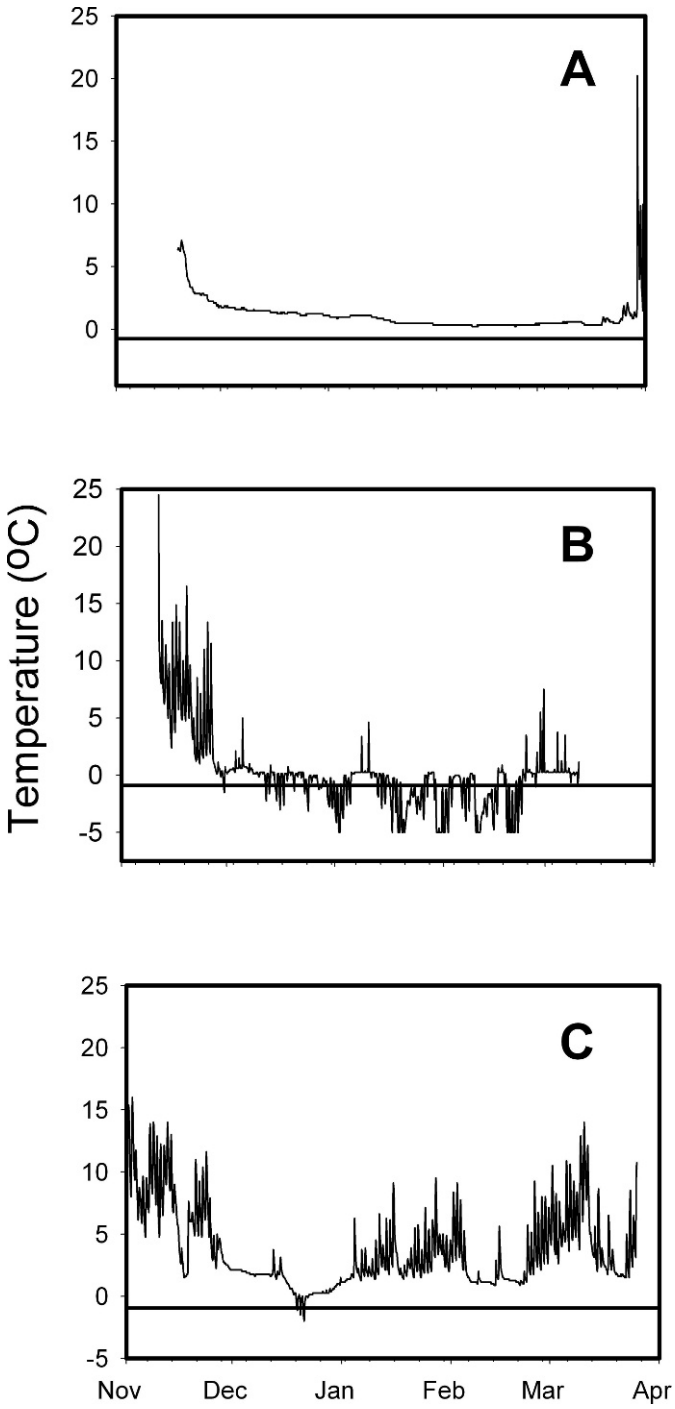

Fig. 2. Winter temperatures in prospective hibernacula, exhibiting the different temperature trends to which frogs may be exposed. (A) Temperatures within the prospective hibernaculum did not drop below the freezing point of the body fluids (−0.6°C, denoted by the heavy line) for the entire winter period (Big Sioux River 2005–2006). (B) Temperatures within the prospective hibernaculum dropped below the freezing point of the body fluids for multiple extended periods over the winter (James River 2007–2008). (C) Temperatures within the prospective hibernaculum dropped below the freezing point of the body fluids, but only for a few hours on a few nights during the winter period (James River 2007–2008).

Table 1. Mean (± SD) Hepatic and Leg Muscle Glucose (μmol·g wet mass^{-1}), Glycogen (μmol glucosyl units·g wet mass^{-1}), and Glycogen Phosphorylase (active form [*a*] and total) Activity (μmol·min^{-1}·g wet mass^{-1}) in Unfrozen Control and 6-h and 24-h Frozen Frogs. Sample sizes for each group are given in parenthesis. Superscripts A, B, and C denote significant differences among groups with different letters.

	Control	6-h frozen	24-h frozen	*F*-statistic[1]	*P*-value
Liver glucose	2.5 ± 1.6 (5)^{C}	16.50 ± 3.55 (8)^{A}	8.8 ± 5.4 (8)^{B}	18.71	<0.001
Liver glycogen	157.1 ± 48.8 (6)	256.0 ± 131.1 (8)	113.9 ± 159.6 (8)	2.57	0.103
Liver phosphorylase *a*	0.1 ± 0.1 (6)^{B}	1.0 ± 0.8 (7)^{A,B}	2.7 ± 2.6 (6)^{A}	H = 11.29	0.004
Liver total phosphorylase	3.4 ± 1.0 (6)	5.9 ± 1.4 (7)	5.7 ± 3.5 (6)	2.39	0.123
Liver % active phosphorylase	2.9 ± 1.9 (6)^{B}	17.3 ± 15.6 (7)^{A,B}	43.7 ± 22.0 (6)^{A}	H = 10.82	0.004
Muscle glucose	1.0 ± 0.5 (6)	0.79 ± 0.48 (8)	1.3 ± 1.2 (8)	0.71	0.504
Muscle glycogen	6.9 ± 5.3 (6)	16.81 ± 9.7 (8)	16.8 ± 22.0 (8)	0.97	0.397
Muscle phosphorylase *a*	0.2 ± 0.1 (6)	0.2 ± 0.1 (7)	1.1 ± 2.1 (6)	0.97	0.401
Muscle total phosphorylase	15.0 ± 7.2 (6)	11.86 ± 2.8 (7)	12.4 ± 6.3 (6)	0.57	0.579
Muscle % active phosphorylase	1.7 ± 1.1 (6)	2.0 ± 1.0 (7)	8.5 ± 13.2 (6)	0.88	0.434

[1] The *H* statistic for Kruskal-Wallis tests is provided when parametric assumptions of equal variance or normally distributed data were not met.

1996; Swanson et al., 1996). Hepatic glycogen is the likely source for these elevated glucose concentrations, although freezing-induced dehydration probably accounts for part of the increased levels of tissue glucose. However, we failed to detect any effect of freezing on hepatic glycogen levels, probably because the high inter-individual variability in hepatic glycogen obscured any minor differences associated with freezing. Mean post-freeze hepatic glucose levels for frogs in this study (8.8 to 16.5 μmol·g wet mass^{-1}) were also lower than those for freezing-exposed cricket frogs from southwestern Ohio (107.5 μmol·g wet mass^{-1}; Irwin et al., 1999), which is consistent with the poor capacity for freezing tolerance in South Dakota populations.

Hepatic activity of glycogen phosphorylase *a* increased significantly after 24-h freezing exposure in cricket frogs in this study, but not after 6-h freezing exposure. This increase may be related to freezing-induced dehydration, with some increase in activity directly due to freeze-concentration within tissues. Elevated hepatic phosphorylase activity is also associated with dehydration stress in anurans generally (Churchill and Storey, 1994), although increases in phosphorylase *a* activity with dehydration do not occur universally in anurans (Edwards et al., 2004). The higher proportion of hepatic phosphorylase activity in the active form in 24-h frozen cricket frogs suggests a regulated conversion of the enzyme from inactive to active form, associated with freezing-induced dehydration. An alternative possibility is that regulatory control over phosphorylase activity was compromised in 24-h frozen cricket frogs because these individuals were likely dead, but a nonsignificant trend toward increasing phosphorylase *a* activity was also present in 6-h frozen frogs, which were likely still alive. The general pattern of hepatic phosphorylase activation promoting glucose mobilization with freezing in freeze-tolerant anurans includes an immediate increase in phosphorylase *a* activity followed by a slower increase in total phosphorylase activity (Storey and Storey, 1985; Storey, 1990; Churchill and Storey, 1996; but see Swanson et al., 1996; Edwards et al., 2000). Hepatic total phosphorylase activity did not increase with freezing in cricket frogs, despite the increase in phosphorylase *a* activity. In addition, hepatic phosphorylase *a* activity in cricket frogs, even after the freezing-induced increase, was several-fold lower than hepatic phosphorylase *a* activity in freeze-tolerant frogs following freezing (Storey and Storey, 1984; Storey, 1987; Churchill and Storey, 1996; Swanson et al., 1996; Jenkins and Swanson, 2005), suggesting relatively low capacity for glucose mobilization. Finally, glucose levels in leg muscle did not increase nor did hepatic glycogen levels decrease with freezing in frogs in this study, suggesting a lack of glucose mobilization from hepatic stores for delivery to other tissues as a cryoprotectant following freezing, which is contrary to general patterns in freeze-tolerant frogs (Storey, 1990; Storey and Storey, 2004). Consequently, the elevated hepatic phosphorylase *a* activity and the relatively minor increase in hepatic glucose levels with freezing were ineffective in promoting cryoprotectant mobilization and freezing tolerance in cricket frogs. This pattern is similar to that for other freezing-intolerant anurans (Storey and Storey, 1986; Swanson et al., 1996; Steiner et al., 2000; Voituron et al., 2005).

We observed South Dakota cricket frogs emerging from cracks and burrows in the mud of sloping stream banks in the fall, just prior to entry into hibernation, suggesting that frogs used these sites for hibernacula. Such sites are similar to hibernacula sites described from more central portions of the species' range, where hibernacula include mud cracks and crayfish burrows (Gray, 1971; Irwin et al., 1999). Temperatures within most of these prospective hibernacula (13 out of 20 total monitored) in southeastern South Dakota regularly dropped below the freezing point of the frogs' body fluids for longer than 24 h, and often for extended periods of up to two weeks, and we found several data loggers encased in frozen soil when we tried to retrieve them in early spring. Thus, prospective hibernacula temperatures in South Dakota were generally lower than those in similar prospective hibernacula from southwestern Ohio, where thermal buffering provided by the moist soil prevented temperatures from dropping below the freezing point of body fluids (Irwin et al., 1999). Results from our freezing experiments suggest that cricket frogs would not survive the winter if hibernating in the majority of prospective hibernacula in southeastern South Dakota. However, three prospective hibernacula (two from the warm winter of 2005–2006 and one from the cold winter of 2007–2008) were more thermally protected and did not drop below the freezing point of the body fluids, so these prospective hibernacula would likely permit overwinter survival of cricket frogs. Four additional prospective hibernacula during the winter of 2005–2006 dropped below the freezing point

of the body fluids, but only for a maximum of 12 h, with a minimum temperature of −1.89°C. The general tolerance of 6-h freezing bouts by cricket frogs in this study suggests that frogs may have also been able to overwinter successfully in these prospective hibernacula.

These data are consistent with the conclusions of Irwin et al. (1999) that overwinter survival in Blanchard's Cricket Frogs is dependent on using hibernacula with appropriate physical microclimate characteristics to buffer frogs from temperatures that drop below the freezing point of the body fluids for extended periods. The preponderance of prospective hibernacula that drop below these temperatures for extended periods in southeastern South Dakota suggests that behavioral selection of appropriate hibernacula microclimates is critical to overwinter survival for cricket frogs at the northwestern limit of their range. Blanchard's Cricket Frogs are capable of microhabitat selection (Smith et al., 2003), preferring terrestrial habitats to aquatic habitats under simulated winter conditions (Irwin et al., 1999) and selecting cracks in simulated mud banks when exposed to declining temperatures (Gray, 1971). However, whether frogs are capable of identifying those sites with appropriate microclimates to support overwinter survival and what factors might inform such choices are unknown and will require further study.

The absence of freezing tolerance capacity coupled with the extended periods below the freezing point of body fluids documented in this study suggest that overwinter mortality of cricket frogs at our study sites may be high and that hibernacula microhabitat temperatures may act to limit the northern distribution of this species. Further studies analyzing detailed physical microhabitat characteristics of hibernacula sites compared with random sites in the northern portion of the cricket frog range (in South Dakota and elsewhere) for comparison with potential hibernacula sites beyond the current range of the species will help to determine if temperatures within potential hibernacula to the north of the current range limits drop too low for effective overwinter survival in these frogs. In addition, such studies would identify critical microclimate features necessary for providing the thermally buffered hibernacula necessary for successful overwintering for cricket frogs. Finally, recent global climate change has resulted in warmer winters in the northern prairie region (Swanson and Palmer, 2009), and these warmer winters might allow hibernacula microclimates, both within and north of the current cricket frog distribution, to become suitable for successful overwintering. This could potentially allow Blanchard's Cricket Frogs to expand their range northward in South Dakota and elsewhere along their current northern range boundary, given available suitable habitat for breeding and overwintering. Such an expansion could have positive effects on populations of this species, which are currently undergoing their most substantial population declines in the northern portion of their range (Lannoo, 1998; Gray and Brown, 2005; Gray et al., 2005; Lehtinen and Skinner, 2006). Alternatively, if winter precipitation increases with climate change, this could result in flooding of potential hibernation sites, which could increase overwinter mortality of cricket frogs because of their intolerance to submergence and the hypoxic conditions that often accompany aquatic overwintering in northern climates (Irwin et al., 1999). Consequently, predicting effects of climate change of cricket frog populations is complex and will require further study to resolve these issues.

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