The Journal of Experimental Biology 206, 2859-2867

doi:10.1242/jeb.00500



# Geographic variation in energy storage and physiological responses to freezing in the gray treefrogs *Hyla versicolor* and *H. chrysoscelis*

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*Accepted 19 May 2003*

## Summary

**The physiological responses supporting freeze tolerance in anurans are well known, but the evolution of this trait remains little studied. This is the first common-garden study of geographic variation in cryoprotective responses to freezing and the degree of freeze tolerance. We studied the gray treefrogs (*Hyla versicolor* and *H. chrysoscelis*) from sympatric sites in Minnesota, Indiana and Missouri. Patterns in the literature suggest that northern frogs produce more cryoprotectants upon freezing, but we found no geographic variation in cryoprotective responses or degree of freeze tolerance. The concentration of glucose produced upon freezing was higher than previously reported for this species (liver: 475 μmol $g^{-1}$ dry mass). Unfrozen frogs had high levels of glycerol (liver: approx. 150 μmol $g^{-1}$ dry mass), and did not produce more upon freezing. Liver glycogen content (concentration multiplied by liver mass) was highest in frogs from Minnesota and Missouri, and was stored in preference to lipids in Minnesota frogs, possibly to provide energy for the longer northern winters. Minnesota frogs accumulated more ice (53.4±1.8%) after freezing to –2.5°C than Indiana frogs (45.5±3.3%). The two species differed in body size but not in any of the physiological parameters measured. We conclude that these populations show no adaptive variation in freeze tolerance and that comparing published studies may be misleading because of different acclimation and feeding regimes.**

Key words: freeze tolerance, gray treefrog, *Hyla versicolor*, *H. chrysoscelis*, cryoprotection, liver, glucose, glycogen.

## Introduction

Although freeze tolerance in anurans has been known since 1982 (Schmid, 1982), little work has been done on the ecological and evolutionary significance of this remarkable trait. Most work to date has focused on physiological changes that occur on freezing and the various adaptations promoting freeze tolerance (for reviews, see Costanzo and Lee, 1994; Storey and Storey, 1996). Studies that focused on the evolution of freeze tolerance have compared the physiological responses to freezing of freeze-tolerant and freeze-intolerant amphibian species (Costanzo et al., 1993a; Swanson et al., 1996) and have considered the link between dehydration tolerance and freeze tolerance (for a review, see Storey and Storey, 1996). However, some basic issues in the evolution of freeze tolerance have not been addressed. How many times has freeze tolerance evolved? Do amphibians in northern regions tolerate a greater degree of freezing? Our study is the first to consider these issues using a common-garden approach with individuals collected across a broad geographic range. In addition, our model for this study, the gray treefrog species complex (*Hyla versicolor* and *H. chrysoscelis*), allows comparison of the physiological responses to freezing between closely related diploid and tetraploid species.

The gray-treefrog species complex has unique attributes that facilitate the study of evolutionary physiology. First, the tetraploid *Hyla versicolor* has evolved independently at least three times from the diploid *H. chrysoscelis* (Ptacek et al., 1994). Although these two species are often found in sympatry, the phylogenetic lineages within either of these species are generally allopatric (i.e. evolutionary branches do not have overlapping geographic ranges), thus tetraploids are often sympatric with diploids that are not of the diploid lineage from which the tetraploids evolved. There is evidence that sympatric diploid and tetraploid frogs undergo parallel selection for protein alleles (Romano et al., 1987) and that desiccation tolerance varies more among sites than between these two species (Ralin, 1981), so we compared frogs from sites where both species occur in sympatry. Do diploids and tetraploids living in the same environment have the same physiological responses to freezing? Are tetraploids more similar to parental diploids or sympatric diploids?

These two species are a good model for the study of cold tolerance because they are found across a large geographic area. Both species are widely distributed throughout the eastern and southern United States and west to the Great Plains and are sympatric in many places throughout their range. However, in local areas these two species are not necessarily in the same

Table 1. *Summary of published accounts of plasma cryoprotectant concentration during freezing in adult gray treefrogs,* Hyla versicolor *and* H. chrysoscelis

| Population | [Glucose] (mmol $l^{-1}$) | [Glycerol] (mmol $l^{-1}$) | Rearing/collection conditions | Reference |
|---|---|---|---|---|
| *Hyla versicolor* | | | | |
| Hancock Co., IL | 20.1 | 67.1 | Lab reared | Layne, 1999 |
| Hancock Co., IL | 21.8 | 112.5 | Lab reared | Layne and Jones, 2001 |
| Fayette Co., IN | 22.7 | <10 | Lab reared | Layne and Lee, 1989 |
| Hennepin Co., MN | Nil | ~300* | Lab reared | Schmid, 1982 |
| Ontario | 6.8 | 423 | Lab reared | Storey and Storey, 1985 |
| Ontario | 8.3 | 19.3 | Spring collected | Storey and Storey, 1987 |
| *Hyla chrysoscelis* | | | | |
| Butler Co., OH | 24.9 | <0.1 | Lab reared | Costanzo et al., 1992 |
| Minnesota | No mention | Yes* | Fall collected | Schmid, 1986 |

*Measured in muscle extracts and bladder urine. Plasma concentration reported in all other cases.

habitats: at least in Wisconsin, *H. versicolor* is widely distributed but *H. chrysoscelis* is generally limited to regions of grassland and savannah (Jaslow and Vogt, 1997). These species also differ in the northern extent of their range: *H. versicolor* extends farther north into Manitoba, Ontario and New Brunswick (Preston, 1982; McAlpine et al., 1991). Given that *H. versicolor* reaches so much farther north, it is possible that this species is better able to survive northern winters than its diploid parental species.

The amount of cryoprotectant produced is directly related to the degree of freeze tolerance, at least in another freeze-tolerant frog, *Rana sylvatica* (Costanzo et al., 1993b). Therefore, we expect frogs from northern populations to produce more cryoprotectant upon freezing. Indeed, published accounts suggest that both gray treefrogs (Table 1) and wood frogs (Storey and Storey, 1988; Costanzo and Lee, 1994) in colder regions produce more cryoprotectant than those from southern portions of the range. However, differences among studies in methodology, especially acclimation regimes, make comparisons across studies difficult and inconclusive (Layne, 1999). Also, no single study has directly compared the physiological responses to freezing of the tetraploid *Hyla versicolor* to its diploid ancestor *H. chrysoscelis*.

Our study is the first to use a common-garden approach to describe geographic variation of freeze tolerance in an amphibian species. This approach allows us to identify differences due to genetic adaptation to the local environment. In addition, our approach allows comparison between species and among phylogenetic lineages in a well-studied species complex.

## Materials and methods

Male gray treefrogs were collected from three populations where *Hyla chrysoscelis* Cope (Hc) and *H. versicolor* Le Conte (Hv) occur in sympatry: Clearwater Co. and adjacent Becker Co. in Minnesota (MN), Phelps Co. in Missouri (MO) and Union Co. in Indiana (IN). All frogs were collected during the breeding season and identified to species by characteristics of the breeding call. They were transported back to Ohio where they were housed in outdoor enclosures at the Miami University Ecology Research Center and fed crickets (size 2.5, Tophat Farms, Portage, MI, USA) twice per week until the frogs stopped feeding with the onset of cold weather. On November 15, the frogs were moved to smaller cages with a soil floor covered by leaf litter and these were held in darkness in a walk-in cold room at 4°C.

To test their physiological responses to freezing, each frog was placed in a 50 ml test tube with a thermocouple adjacent to the frog's ventral surface. After blocking the opening of the tube with foam and connecting the thermocouple to a multichannel chart recorder, the tubes were submerged into a cold bath (RTE-140, Neslab, Portsmouth, NH, USA) at –0.8°C. Once cooled to a temperature of –0.5 to –0.8°C, the frogs were stimulated to freeze by application of aerosol coolant to the outside of the tube. The frogs were held at –0.8°C for 1 h, then cooled to –2.5 at a rate of –0.04°C $h^{-1}$ (a rate observed in wood frogs freezing in nature; J. T. Irwin and J. P. Costanzo, unpublished data) thus reaching the target temperature after 42.5 h. Once at –2.5°C, they were held at this temperature for an additional 4 h to allow equilibrium ice content to be reached.

Upon removal from the cold bath frogs were double-pithed and rapidly dissected in a walk-in cold room at 4°C. Each frog's entire liver was removed, weighed and, after taking a 15 mg subsample for determination of water content, frozen in liquid nitrogen. The right thigh musculature was similarly removed and subsampled, then also frozen in liquid nitrogen. Tissue subsamples were weighed, dried to constant mass at 60°C and reweighed to determine water content. The heart and surrounding blood vessels (sinus venosus, right and left truncus arteriosus) were removed and centrifuged to collect blood from within these structures into heparinized capillary tubes. The heart and the remaining carcass were frozen in liquid nitrogen. Blood retrieved from the heart was preserved in 37% buffered formaldehyde. Measurements of red blood cells (length and

width, $N$=10 cells per frog) under 40× magnification were performed to confirm the species identification originally made through breeding call characteristics (Matson, 1990a). Control frogs (held unfrozen at 0°C) were similarly treated except that blood was sampled directly into hematocrit tubes from the severed truncus arteriosus.

To measure cryoprotectant (glucose and glycerol) concentrations, frog tissues were homogenized in ice-cold 0.6 mol $l^{-1}$ perchloric acid, then neutralized with a half volume of 1 mol $l^{-1}$ $KHCO_3$. Measurements of glycogen required an additional step: a 100 µl sample of the perchloric acid homogenate was incubated (37°C for 3 h) with amyloglucosidase (Sigma Chemical Co., St Louis, MO, USA) in 1 ml of sodium acetate buffer (119 mmol $l^{-1}$ sodium acetate, 77 mmol $l^{-1}$ acetic acid, pH 4.8) to convert all glycogen to glucose. To stop this reaction, the enzyme was destroyed by addition of more 0.6 mol $l^{-1}$ perchloric acid and the solution was again neutralized with 1 mol $l^{-1}$ $KHCO_3$. Measurements of free glucose both in the original extract and after digestion with amyloglucosidase were performed using the glucose oxidase procedure (No. 510, Sigma Chemical Co.). Glycogen was expressed in glucose units and was calculated by subtracting the free tissue glucose from the total glucose after amyloglucosidase digestion. Tissue glycerol was measured using the glycerol phosphate oxidase procedure (No. 337-40A, Sigma Chemical Co.) and lactate using the lactate oxidase procedure (No. 735, Sigma Chemical Co.).

After dissection of the tissue samples, each frog carcass was immediately frozen in liquid nitrogen and stored at –80°C. The carcasses were later weighed, dried to constant mass and reweighed. Dried carcasses were pulverized in a coffee grinder, and the lipids were extracted and quantified using a chloroform/methanol procedure (Teitz, 1970).

An additional subsample of frogs was frozen by the above protocol, then measured for ice content. These frogs were rapidly transferred from the cold bath using pre-chilled forceps to 100 ml of distilled water in an insulated calorimeter at room temperature. The temperature change of the water was monitored with a thermocouple connected to a MacLab (AD Instruments, Colorado Springs, CO, USA) data acquisition system and was used to calculate the frog's ice content following the methods of Lee and Lewis (1985) and Layne and Lee (1989). These frogs were dried at 60°C to constant mass to estimate water content of each individual, a requirement for calculation of ice content.

We used analysis of variance (ANOVA; PROC GLM, SAS) to identify which factors (specifically the species, geographic origin and freezing treatment) significantly affected the physiological characteristics including tissue concentrations of metabolites and ice content. The model included all interactions and the data in the figures are presented as least-square means (SAS, LSMEANS). Percent data were arcsine, square-root transformed before analysis. In comparing total liver glycogen content, we performed an analysis of covariance (ANCOVA; PROC GLM, SAS) with body mass (log-transformed) as the covariate. Again, we present least-square means, these being adjusted for body size. An experiment-wise error rate of 0.05 was used in all analyses. Sample size for each group used in the physiological assays was 12, except for the Missouri *H. chrysoscelis* controls ($N$=5) and frozen samples ($N$=6), Missouri *H. chrysoscelis* controls ($N$=3) and frozen samples ($N$=5), and all Minnesota groups ($N$=11 for each species/treatment combination). Ice content was based on $N$=5 for each population/species combination tested. Lipid concentrations were measured on 16 Minnesota frogs, 5 Missouri frogs and 12 Indiana frogs. ANCOVA was not used because mass (log-transformed) was not a significant factor in the analysis of lipid concentration.

The degree of freeze tolerance was assessed as survival of freezing to various temperatures. The freezing protocol for these assessments matched that of the physiological tests, but longer tests were used to reach lower temperatures. Frogs were thawed at 0°C for 24 h, and then allowed to recover on wet filter paper in darkness at 4°C. The frogs were checked throughout recovery for two basic responses: limb retraction (ability to pull in the hindlimb when retracted manually) and righting response (ability to right itself when turned on its dorsum). The time when these responses were first observed was recorded. Frogs were judged to have survived only if they exhibited normal posture and behavior.

All of the experiments presented here were conducted using identical methods during either the winter of 1998–1999 or the winter of 1999–2000. These two years were compared by including year as a variable in the ANOVA model used for the physiological comparisons (PROC GLM, SAS). These two years were never significantly different, thus the data were combined, and this factor was dropped from the regression model. Metabolite concentrations are given in µmol $g^{-1}$ dry tissue mass because tissue water content changed greatly with freezing. All data are presented as least-square means ± S.E.M., except when the data were not corrected for

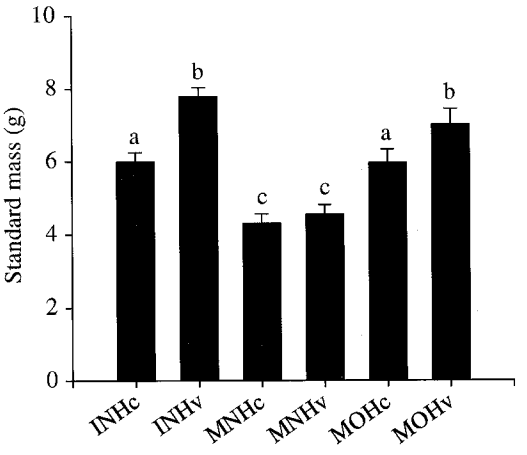


Fig. 1. Differences in standard body mass among the populations and species (both control and frozen frogs are included in each mean). Means not sharing a letter were significantly different (Bonferroni multiple comparison, $\alpha$=0.05). IN, Indiana; MN, Minnesota; MO, Missouri; Hc, *H. chrysoscelis*; Hv, *H. versicolor*.

body mass, in which case they are means ± standard error of the mean (S.E.M.). The discussion below considers the statistical significance of the main effects in the ANOVA model (population, species, freezing treatment).

## Results

As previously shown (Matson, 1990b), *H. versicolor* was generally larger than *H. chrysoscelis* ($F_{1,105}$=17.1, $P$<0.001) and northern frogs were significantly smaller than their southern counterparts ($F_{2,105}$=51.2, $P$<0.001) (Fig. 1). Interestingly, there was also a species-population interaction ($F_{2,105}$=4.7, $P$=0.011) because there was no significant difference in body size between the Minnesota *H. chrysoscelis* and *H. versicolor* (Fig. 1).

The populations and species sampled for this study exhibit very little variation in their physiological responses to freezing. Liver glucose increased significantly with freezing ($F_{1,99}$=719.1, $P$<0.001) from baseline levels of approx. 23 μmol $g^{-1}$ dry mass to approx. 460 μmol $g^{-1}$ dry mass (Fig. 2A). In the liver there were no significant differences in glucose concentrations among the populations ($F_{2,99}$=1.10, $P$=0.325) or the species ($F_{1,99}$=3.7, $P$=0.059). In the thigh muscle glucose also increased with freezing ($F_{1,99}$=63.1, $P$<0.001) from approx. 13 μmol $g^{-1}$ dry mass up to approx. 32 μmol $g^{-1}$ dry mass (Fig. 2B). As in the liver, there were no significant differences between the species ($F_{1,99}$=0.1, $P$=0.777) but the populations were significantly different ($F_{2,99}$=4.5, $P$=0.014) because the Missouri *H. chrysoscelis* controls had slightly higher glucose concentrations than the other groups (Fig. 2B). No differences among the populations were present in the frozen frogs.

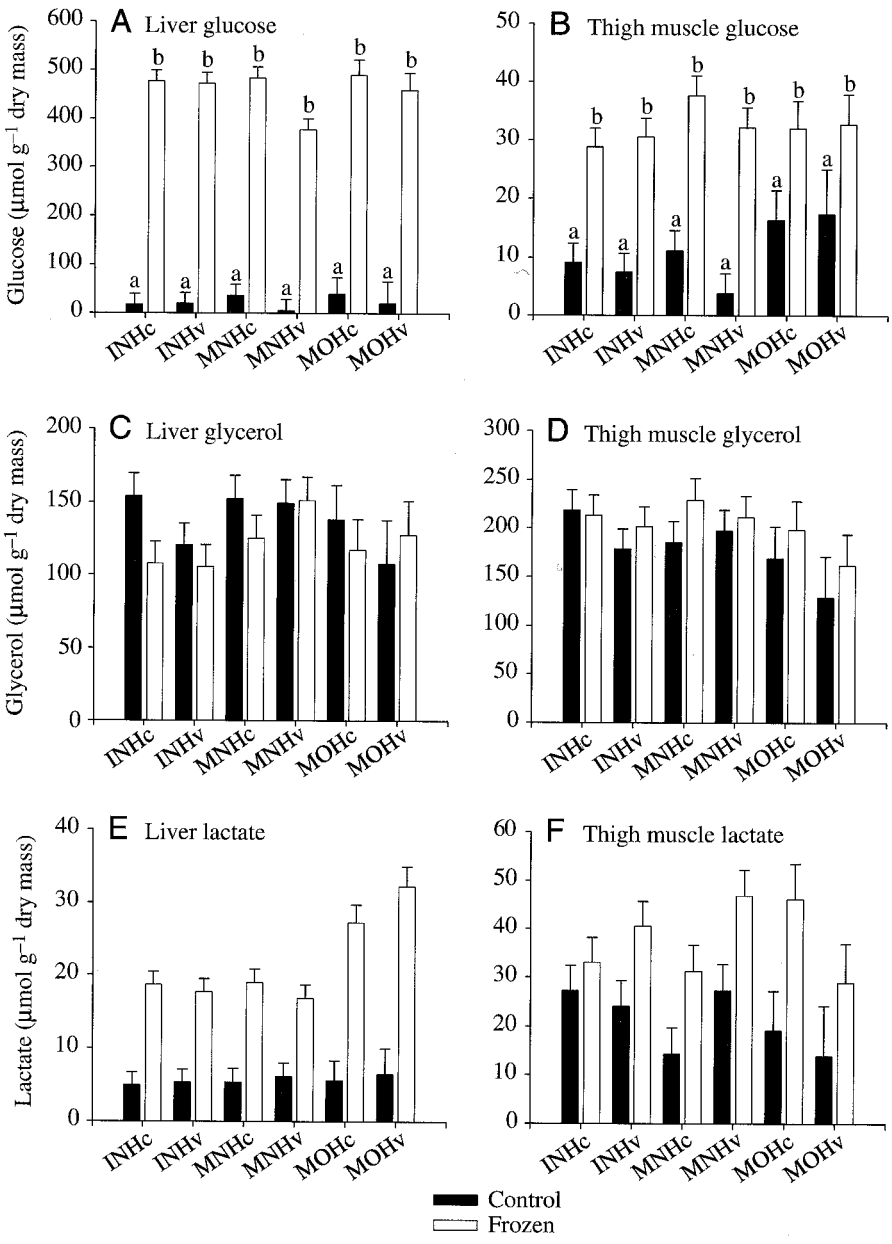


Fig. 2. Glucose (A,B), glycerol (C,D), and lactate (E,F) concentrations in the liver (A,C,E) and thigh muscle (B,D,F) of control (black bars) and frozen (white bars) frogs. Freezing significantly increased tissue glucose concentration (liver: $F$=719, $P$<0.001; thigh muscle: $F$=81.1, $P$<0.001) and lactate concentration (liver: $F$=17.9, $P$>0.001; thigh muscle: $F$=19.8, $P$<0.001). When letters are present, means not sharing a letter were significantly different. Abbreviations as in Fig. 1.

Glycerol followed a different pattern. Glycerol was at very high concentrations in both the frozen frogs and the control frogs. In fact, the overall ANOVA was not significant for glycerol either in liver ($F_{11,99}$=1.3, $P$=0.240) or thigh muscle ($F_{11,99}$=0.91, $P$=0.532). Thus, there were no significant differences between the species or among the populations, nor were there any differences induced by freezing. Glycerol levels were typically 130 μmol $g^{-1}$ dry mass in the liver (Fig. 2C) but about 190 μmol $g^{-1}$ dry mass in the thigh muscle (Fig. 2D).

A significant amount of lactate, a by-product of anaerobic metabolism, was accumulated during freezing in both the liver ($F_{1,99}$=160.9, $P$<0.001; Fig. 2E) and thigh muscle ($F_{1,99}$=19.8, $P$<0.001; Fig. 2F). The liver lactate concentrations rose from typically 5–6 μmol $g^{-1}$ dry mass up to 18 μmol $g^{-1}$ dry mass (but were higher in the Missouri animals, see below). Concentrations in the thigh muscle increased from 21 to 38 μmol $g^{-1}$ dry mass. There was also a significant effect of population on the accumulation of lactate in liver ($F_{2,99}$=7.5,

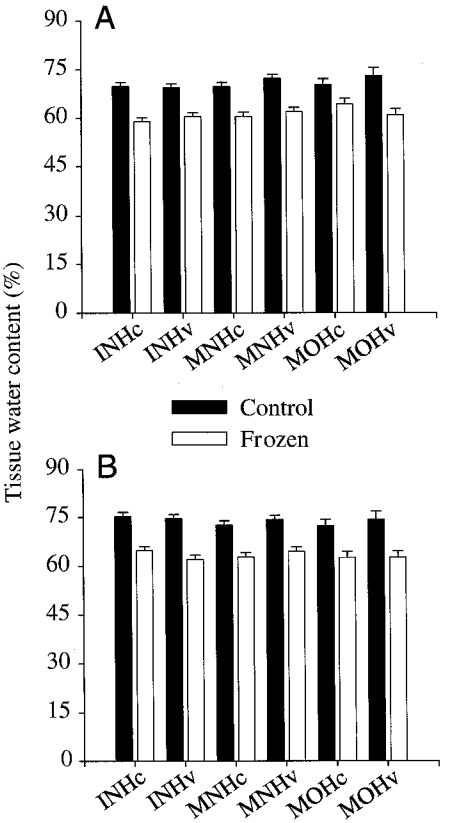


Fig. 3. Liver (A) and thigh muscle (B) tissue water content of control (black bars) and frozen (white bars) frogs. Liver water content always fell significantly with freezing, but there were no differences between species or among the populations within the control or frozen groups. Abbreviations as in Fig. 1.

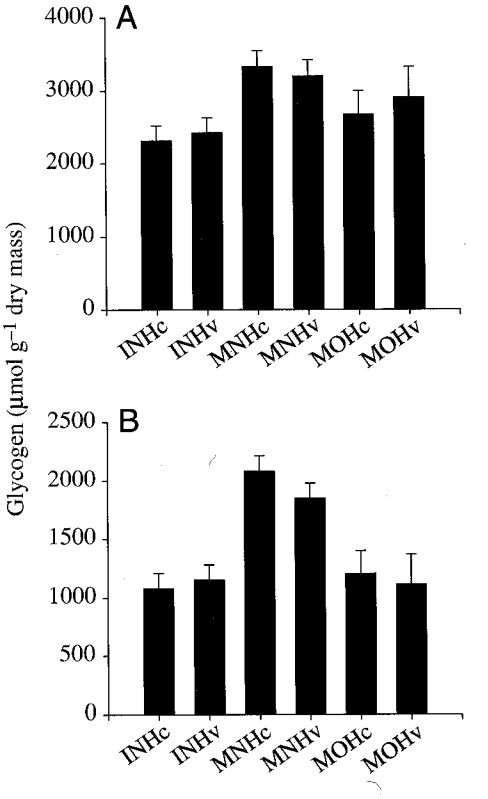


Fig. 4. Liver (A) and thigh muscle (B) glycogen concentration (in glucose units) for control frogs of the various species and populations sampled. There were no significant differences among the control frogs in liver concentration but MN frogs had higher muscle concentration. Abbreviations as in Fig. 1.

$P$=0.001), probably because the Missouri animals accumulated more lactate during freezing, especially the Missouri *H. versicolor*. No population differences were observed in the thigh muscle, which was probably due to the high variability of lactate concentration in this tissue.

Tissue water content was significantly reduced by freezing. This was true for liver ($F_{1,99}$=148.5, $P$<0.001; Fig. 3A) and thigh muscle ($F_{1,99}$=156.6, $P$<0.001; Fig. 3B) as water was drawn from the tissues into growing ice crystals (Lee et al., 1992). In liver, water content fell from 71% to 61% (least-square means) and in thigh muscle it fell from 74% to 63% (least-square means). The liver water content did not differ between the species ($F_{1,99}$=0.8, $P$=0.378) or among the populations ($F_{2,99}$=2.8, $P$=0.069). The same was true of thigh muscle ($F_{1,99}$=0.2, $P$=0.653 for species; $F_{2,99}$=0.7, $P$=0.516 for population).

Liver glycogen, the proposed source for glucose and glycerol (Storey and Storey, 1985), was measurably reduced by freezing ($F_{1,99}$=12.2, $P$<0.001) from 2810 to 2283 μmol $g^{-1}$ dry mass (least-square mean, all frogs included) (not shown). There were no significant differences among the populations of control frogs (Fig. 4A). In the thigh muscle there was a significant difference among the populations ($F_{2,99}$=69.0, $P$<0.001; Fig. 4B). The Minnesota frogs had nearly twice the muscle glycogen concentration (2019 μmol $g^{-1}$ dry mass) of their Indiana (1031 μmol $g^{-1}$ dry mass) and Missouri (1025 μmol $g^{-1}$ dry mass) counterparts.

Liver glycogen concentration, expressed on a per gram basis, is not the best indicator of glycogen availability. We calculated total liver glycogen content, rather than concentration, by multiplying liver glycogen concentration by intact liver mass. Body mass (log-transformed) was included as a covariate in this analysis because it strongly influenced liver glycogen content ($F_{1,50}$=9.8, $P$=0.009) through effects on liver size. Total liver glycogen was significantly reduced with freezing from 389 to 260 μmol ($P$<0.001; least-square mean, all frogs included) as it was mobilized to produce glucose. The populations differed significantly in total liver glycogen content ($F_{2,50}$=9.1, $P$<0.001) with control Minnesota and Missouri frogs having significantly higher liver glycogen contents than control Indiana frogs (Fig. 5A).

Accumulation of high levels of glycogen in control northern frogs came at the expense of lipid storage. The populations differed significantly in carcass lipid content ($F_{2,30}$=7.2, $P$<0.001) with the Minnesota frogs having the lowest concentration (Fig. 5B). To demonstrate that this is not simply because Minnesota frogs were smaller, we calculated a ratio of

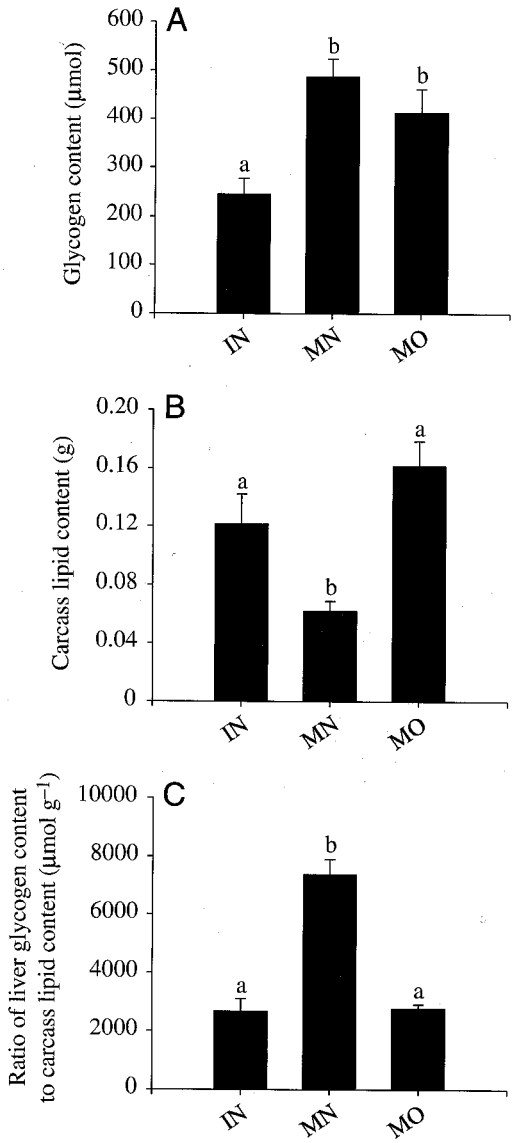


Fig. 5. (A) Total liver glycogen in control frogs estimated by multiplying liver glycogen concentration ( μmol $g^{-1}$ dry mass) by intact liver mass. (B) Total carcass lipid content. (C) Ratio of liver glycogen to carcass lipid content. In all cases means not sharing a letter were significantly different (Bonferroni multiple comparison, $\alpha$=0.05). IN, Indiana; MN, Minnesota; MO, Missouri.

total liver glycogen to total carcass lipid content (Fig. 5C). Also, a significant negative correlation exists between liver glycogen concentration and carcass lipid content ($F_{1,32}$=6.0, $P$=0.020, $r^2$=16%) in control frogs. Thus, there is an apparent trade-off between glycogen and lipid storage.

The amount of ice that accumulated during a freeze to –2.5°C was measured on Minnesota *H. chrysoscelis* and *H. versicolor*, and Indiana *H. chrysoscelis* (Fig. 6). The only significant effect in this comparison was population ($F_{1,14}$=7.8, $P$=0.016), with Minnesota frogs accumulating more ice than the Indiana frogs.

There were no major differences among the groups in the minimum temperature survived. There may have been slightly higher survival at –5.5 and –6.5°C in the Minnesota frogs but sample sizes were too low to achieve statistical significance (Table 2). Measurements of recovery parameters were based only on survivors, thus sample size was low and a statistical analysis not possible. On average, frogs frozen to –5.5 and –6.5°C took twice as long to recover limb-retraction ability (approx. 110 h) than those frozen to –3.5 and –4.5°C (approx. 50 h), and a similar pattern was present in recovery of the righting response. There were no consistent differences between the species or among the populations in the time to recover limb retraction or the righting response.

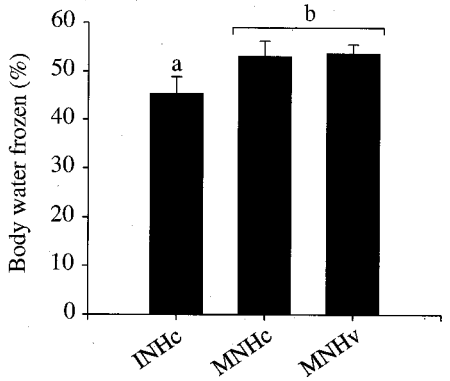


Fig. 6. The ice content of frogs from several populations/species after freezing to –2.5°C. Minnesota frogs accumulated more ice than the Indiana frogs. IN, Indiana; MN, Minnesota; MO, Missouri; Hc, *H. chrysoscelis*; Hv, *H. versicolor*.

Table 2. *Survival of gray treefrogs of both species and from several populations to various freezing temperatures*

| | Temperature (°C) | | | | |
|---|---|---|---|---|---|
| Population/Species | –3.5 | –4.5 | –5.5 | –6.5 | –7.5 |
| Indiana | | | | | |
| *H. chrysoscelis* | 3/3 | 6/6 | 6/12 | 0/9 | |
| *H. versicolor* | 3/3 | 6/6 | 2/10 | 3/9 | 0/1 |
| Minnesota | | | | | |
| *H. chrysoscelis* | | | 4/5 | 1/4 | |
| *H. versicolor* | | | 5/5 | 2/7 | |
| Missouri | | | | | |
| *H. chrysoscelis* | | | 1/3 | | |
| *H. versicolor* | | | 2/3 | | |

Frogs were not used in more than one experiment.

## Discussion

This study provided new insights into the use of cryoprotectants in the gray treefrog species group. Because this study was the first to compare the two species of gray treefrogs and also the first to include several geographic locations in a single study, we have been able to dispel some misconceptions

regarding anuran freeze tolerance that have arisen from the comparison of published studies. Layne (1999) pointed out that interpopulational comparisons have been hampered by different methods for cold-conditioning of frogs, an observation based on his finding that Illinois *H. versicolor* had a higher degree of freeze tolerance than reported in previous studies of the same species from a similar climate in Indiana. To eliminate this problem, we raised gray treefrogs from several populations together, thus allowing comparisons of geographic and interspecific variation in freeze tolerance. The following discussion compares our experimental groups in terms of cryoprotective responses, liver glycogen concentration (the source for cryoprotectants), and their degree of freeze tolerance and recovery from freezing.

In contrast to other studies, we found that gray treefrogs produce as much glucose as other freeze-tolerant amphibians. Indeed, glucose concentrations were as high as those reported in Ontario wood frogs *R. sylvatica* (Storey and Storey, 1984; Storey, 1987). Given its high concentration, glucose is likely to play a cryoprotective role, as it does in the wood frog (Costanzo et al., 1993b). Why have other studies not reported high glucose concentrations upon freezing? First, most studies of gray treefrogs have only measured glucose concentrations in the plasma, not the liver. The liver is likely the site of glucose synthesis (Storey and Storey, 1985), and we found very high concentrations of glucose in this organ. The only other study to measure liver glucose in frozen gray treefrogs (Storey and Storey, 1985) found only 16.6 μmol $g^{-1}$ dry mass in the single adult male sampled whereas we typically saw 180 μmol $g^{-1}$ dry mass. We also found more glucose in the thigh muscle: 12.2 μmol $g^{-1}$ fresh mass in our study *versus* approx. 5.6 μmol $g^{-1}$ fresh mass in Storey and Storey (1985). Liver glycogen concentration and laboratory acclimation regimes may account for this difference (see below).

In our study, glycerol was present in high concentrations before freezing, and not further elevated by freezing. This is in contrast to other studies where glycerol was very low initially and production stimulated only upon freezing (e.g. Storey and Storey, 1985). The hypothesis of Layne and Jones (2001) that longer, cooler acclimation periods may stimulate glycerol production is consistent with our data, since our frogs were acclimated naturally outdoors until moved to 4°C on November 15. The environmental conditions during this period such as drought stress or natural changes in photoperiod may have stimulated glycerol production (as happens in some insects; Rojas et al., 1986). The cues initiating glycerol production in the gray treefrogs require more study.

Until now, there have been no reports of *H. chrysoscelis* using glycerol as a cryoprotectant (other than a brief mention without any supporting data by Schmid (1986). Only Costanzo et al. (1992) measured glycerol in *H. chrysoscelis* and they found no detectable amounts, but these measurements were made on summer animals following a short-term cold acclimation. Given our results using animals from a population in Indiana close to that studied by Costanzo et al. (1992), as well populations from Minnesota and Missouri, it is clear that *H. chrysoscelis* can produce substantial quantities of glycerol as a cryoprotectant, just as *H. versicolor* does. In fact, the two species did not differ in glycerol production or, indeed, in any of their physiological responses to freezing.

The concentrations of glycerol that we measured were substantially higher than those reported previously from Indiana and Illinois (Layne and Lee, 1989; Layne, 1999; Layne and Jones, 2001). Our results are more similar to those of treefrogs studied in Ontario and Minnesota (Schmid, 1982; Storey and Storey, 1985). This strengthens the argument by Layne (1999) that interpopulation comparisons are plagued by methodological differences. All of the species and populations we studied responded to freezing in essentially the same way, thus there are no genetically based differences in freeze tolerance due to ploidy or geographic location.

What accounts for the differences seen between our work and the previous studies? Why did we see higher glucose and glycerol production? These differences are likely to stem from differences in glycogen availability. Unfortunately, only one previous study of gray treefrogs included measurements of glycogen in the liver and muscle. This work focused mostly on juveniles, which typically have low glycogen concentrations (Storey and Storey, 1985). The one adult measured (a male collected in the fall and housed in the laboratory for one month) had 342 μmol glycogen $g^{-1}$ dry mass, less than the 600–1000 μmol $g^{-1}$ dry mass we measured here. Our data are more similar to the more extensive samples made on wood frogs from Ontario, which typically have 700–1000 μmol $g^{-1}$ dry mass. These wood frogs also produce levels of glucose similar to those that we found in the gray treefrogs (Storey and Storey, 1985; Storey, 1987). Thus, gray treefrogs with large hepatic glycogen reserves produce glucose upon freezing much like the wood frog does.

The differences in glycogen concentration probably stem from differences in the acclimation regime. Frogs accumulate glycogen with the onset of cold weather (Pasanen and Koskela, 1974; Smith, 1950). However, this requires that the appropriate cue, low temperature, is present and that food is still available from which glycogen reserves can be created (Blier and Guderley, 1986). In the previous studies of gray treefrogs, all of the animals were 'step-acclimated'. That is, the frogs were moved through one or more abrupt steps of progressively colder temperatures and shorter photoperiods. However, upon the first drop in temperature, food was withheld (e.g. Storey and Storey, 1985; Layne and Lee, 1989; Costanzo et al., 1992; Layne, 1999). Thus, although the cue for glycogen accumulation was present, the frogs no longer had a food source available from which to produce glycogen. The result was lower tissue glycogen content. In contrast, the frogs used in our experiments were raised outdoors where they experienced the natural changes in seasonal temperature, precipitation and day length. During this time, we continued to feed the frogs and they ate readily on warm days, even in late October. Thus, they had a greater opportunity to accumulate glycogen. The amount of glycogen accumulated was most similar to those of wood frogs collected in Ontario during the

late fall and used shortly afterward for experiments (Storey and Storey, 1986), an acclimation regime very similar to the one we used here. Thus, acclimation and feeding regimes have produced apparent geographic variation that is not based on local adaptation.

While the amount of glycogen influences cryoprotective responses when comparing between studies, once there is adequate glycogen for a maximal cryoprotective response, the addition of more glycogen does not improve glucose or glycerol production. This is illustrated by the Minnesota frogs in this study. Although they had larger glycogen reserves available (Fig. 5A), these frogs did not produce more glucose (Fig. 2A,B) or glycerol (Fig. 2C,D) than the other populations. Thus, the higher glycogen reserves in northern frogs (and the corresponding drop in lipid storage; Fig. 5) may be an adaptation to provide energy for the extended northern winter (see review in Pasanen and Koskela, 1974) and/or to enhance survival of repeated freeze/thaw cycles (Storey, 1987), rather than to enhance cryoprotective responses to freezing. The similarity in glycogen content in the two species (rather than similarity within a genetic lineage), provides additional support that local selection drives parallel evolution for physiological traits in these two species (Romano et al., 1987).

The degree of freeze tolerance did not vary greatly among the populations and species when the frogs were raised in a common environment (Table 1). There was a significantly greater accumulation of lactate in the liver of Missouri frogs (Fig. 2C), but even this difference was slight and the highest lactate concentrations were still on a par with those observed in the study by Storey and Storey (1985) of Ontario gray treefrogs. The minimum temperatures survived by gray treefrogs in this study were closer to those of Ontario frogs (as were the physiological responses to freezing discussed above) than previous studies of gray treefrogs in the Midwest (Layne and Lee, 1989; Costanzo et al., 1992; Layne, 1999). The lack of variation and overall high degree of freeze tolerance may be related to the high concentrations of cryoprotectants produced by these frogs. There do not seem to be any genetic differences among the populations that limit or enhance freeze tolerance. This may suggest that (1) gray treefrogs raised under these conditions are at the physiological limit of freeze tolerance and/or (2) gray treefrogs from different geographic areas do not experience great differences in freezing temperatures in nature and thus do not require a higher degree of freeze tolerance in northern locales as originally predicted. Both of these hypotheses require further investigation.

In summary, there is little adaptive variation in the cryoprotective responses and survival of freezing between *H. chrysoscelis* and *H. versicolor* and among gray treefrogs collected from Indiana, Minnesota and Missouri. We must consider, however, that the common-garden approach taken in our experiments may have eliminated variation in temperature, photoperiod, food availability, or other factors that frogs from these populations may have experienced in nature. How these factors contribute to the degree of natural freeze tolerance exhibited by gray treefrogs in nature, and also the extent and duration of freezing conditions that frogs experience in nature, remain to be explored.

The authors thank Carl Gerhardt, William Schmid, and Jon Ross for help in locating collecting sites in Missouri and Minnesota. Jackie Litzgus, Casey Tucker, Sean Walker and Leah Irwin helped collect frogs. Jackie Litzgus and Shala Hankison made substantial contributions to feeding and caring for the frogs. Jon Costanzo assisted with the bioassay protocols, except for the glycogen protocol, which was kindly provided by Catherine Bevier. This work was supported by NSF IBN-0090204 to R.E.L., a Sigma Xi Grant-in-aid to J.T.I., and Miami University's summer workshop program. J.T.I. was supported by a P.D.F. from NSERC Canada (held at McGill University) and the David Burpee Endowment (Bucknell University) during preparation of the manuscript. This research was approved by Miami University's Animal Care and Use Committee.

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