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RESEARCH ARTICLE

# Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance

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## ABSTRACT

We investigated the ecological physiology and behavior of free-living wood frogs [*Lithobates* (*Rana*) *sylvaticus*] overwintering in Interior Alaska by tracking animals into natural hibernacula, recording microclimate, and determining frog survival in spring. We measured cryoprotectant (glucose) concentrations and identified the presence of antifreeze glycolipids in tissues from subsamples of naturally freezing frogs. We also recorded the behavior of wood frogs preparing to freeze in artificial hibernacula, and tissue glucose concentrations in captive wood frogs frozen in the laboratory to −2.5°C. Wood frogs in natural hibernacula remained frozen for 193±11 consecutive days and experienced average (October–May) temperatures of −6.3°C and average minimum temperatures of –14.6±2.8°C (range −8.9 to −18.1°C) with 100% survival (*N*=18). Mean glucose concentrations were 13-fold higher in muscle, 10-fold higher in heart and 3.3-fold higher in liver in naturally freezing compared with laboratory frozen frogs. Antifreeze glycolipid was present in extracts from muscle and internal organs, but not skin, of frozen frogs. Wood frogs in Interior Alaska survive freezing to extreme limits and durations compared with those described in animals collected in southern Canada or the Midwestern United States. We hypothesize that this enhancement of freeze tolerance in Alaskan wood frogs is due to higher cryoprotectant levels that are produced by repeated freezing and thawing cycles experienced under natural conditions during early autumn.

**KEY WORDS: Freeze tolerance, Wood frog, Cryoprotectant, Antifreeze glycolipid**

## INTRODUCTION

Freeze-tolerant amphibians (those able to survive freezing) freeze at high, sub-zero temperatures to control the rate of extracellular ice formation and permit time to synthesize and distribute cryoprotectants that lessen cellular damage caused by desiccation (Layne et al., 1990; Storey and Storey, 1996). Cryoprotectants can also help increase survival after freezing by preventing intracellular ice formation, stabilizing membranes and macromolecules, and serving as antioxidants, metabolic substrates and metabolic regulators (Storey and Storey, 1996). An additive, protective effect of cryoprotectants is suggested, as loading cells with glucose and urea reduces water loss, stabilizes cells and increases survival after freezing (Costanzo and Lee, 2013). In addition to low molecular mass cryoprotectants, a high molecular mass xylomannan-based antifreeze glycolipid (AFGL) with thermal hysteresis activity is present in certain freeze-tolerant organisms including insects such as the Alaskan beetle, *Upis ceramboides* (Walters et al., 2009), a plant, bittersweet nightshade (*Solanum dulcamara*), and a European frog, *Rana lessonae* (Walters et al., 2011). Most of the AFGL is present on the cell membranes, and therefore its function in these freeze-tolerant species appears to be to prevent the lethal propagation of extracellular ice across the cell membrane into the cytoplasm. AFGL in *R. lessonae* also inhibits potentially damaging recrystallization of ice in the extracellular fluid.

Overwinter conditions of minimum temperature and the duration of sub-zero temperatures vary by location, but most freeze-tolerant amphibians are believed to experience temperatures near 0°C, with only brief periods (<1 week) of below-freezing temperatures (Sinclair et al., 2013; Costanzo and Lee, 2013), although there are few descriptions of field hibernacula microclimate (Costanzo and Lee, 2013). The wood frog, *Lithobates* (*Rana*) *sylvaticus* (LeConte 1825), is a well-studied freeze-tolerant amphibian that uses glucose and urea as cryoprotectants, with urea having an additional role in metabolic suppression (Costanzo and Lee, 2013). Most studies of this species have focused on Midwestern United States and southern Canada populations, which are near the southern limits of the wood frog range. Northward, the wood frog range extends above the Arctic Circle with limits in Alaska close to the Brooks Range and to the Arctic Ocean in western Canada (Martof and Humphries, 1959).

Lower lethal temperatures in wood frogs have been reported as near −7°C (Layne et al., 1998), with a recent account, however, of survival of frogs from Alaska cooled to −16°C in the laboratory (Costanzo et al., 2013). In subarctic Interior Alaska, wood frogs overwinter in the subnivean space covered by duff and leaf litter (Kirton, 1974), where temperatures can remain below freezing for over 6 months with minima near –20°C (Barnes et al., 1996; Sformo et al., 2010). These extreme temperatures combined with previously reported limits to freeze tolerance would suggest that high mortality of wood frogs occurs in Interior Alaska. Our interest was in determining the conditions wood frogs naturally experience while overwintering near the northern limit of their distribution in Alaska, their duration of freezing and rates of survival over two winters, and their behavioral and physiological responses, including measuring levels of cryoprotectant accumulation in tissues and testing for the presence of AFGL.

## RESULTS

### Selection of hibernacula

Free-living wood frogs (*N*=18, body mass 14±1.2 g) prepared for overwintering were found in early September 2000 and 2001 covered with leaves in shallow depressions (forms) 4–10 cm deep within the organic soil located near the edge of spring breeding ponds (Fig. 1). The mean (±s.e.m.) distance of wood frogs from the

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**Fig. 1. A wood frog in a naturally made overwintering form.** Covering leaves have been removed for the photo.

pond edge was 710±821 cm (range 80–2250 cm). When sex was known, females averaged 124 cm and males averaged 1190 cm from the pond (not significant) (Table 1).

Wood frogs filmed after being placed in an indoor soil- and leaf-filled enclosure at −5°C continued to move until just before freezing initiated. Wood frogs burrowed under leaves and created forms by laterally rotating in the soil. If we uncovered wood frogs by removing leaves, they would relocate and create a new, covered form (see supplementary material Movie 1).

### Freezing conditions and overwinter survival

All 18 free-living wood frogs in natural hibernacula in April 2001 and May 2002 survived winter. We considered that wood frogs began to freeze when soil temperatures were below −1.6°C (lowest observed exotherm in 2011 and 2012) and thawed when temperatures were above −0.16°C, based on the melting point of wood frogs determined previously (Sinclair et al., 2013). Combining data from the two years, the temperature of wood frogs decreased below −1.6°C between 10 and 25 October and first warmed above −0.16°C in spring between 19 April and 9 May; thus, wood frogs were below their freezing point for, on average, 193±11 days (range 175–218 days). Between October and May, average temperatures experienced by individual wood frogs ranged from −3.9 to −8.4°C, with a grand mean of −6.3°C. Minimum temperatures experienced by frogs ranged from −8.9 to −18.1°C, with an average minimum of −14.6±2.8°C. Minimum microhabitat temperatures were usually reached in December even though the lowest air temperatures occurred on 24 January 2001 and 8 February 2002 (Table 1, Figs 2, 3).

Wood frogs freezing in outdoor enclosures in 2011 and 2012 experienced a minimum temperature of −22°C on 22 November 2011 and −17.5°C on 8 December 2012. These wood frogs were frozen for 50 days before being sampled for tissue glucose concentration.

Exotherms following nucleation of ice in wood frogs were observed in animals (all male; body mass 11.9±1.3 g) at an average temperature of −1.12±0.28°C ($N$=15) in 2011 and −1.14±0.34°C ($N$=15) in 2012. The temperature at which exotherms occurred decreased with time, with the regression significant in 2012 ($r^2$=0.21, $F_{1,16}$=9.428, $P$<0.05; Fig. 4) but not in 2011 ($P$>0.10; data not shown). Rates of cooling under natural conditions measured from 0.5°C until nucleation ranged among frogs from 0.35 to 1.60°C $h^{-1}$.

In early October of all years, wood frogs experienced multiple (average of 12, range 10–17) and mostly successive cycles of

**Table 1. Characteristics of natural wood frog hibernacula over two winters**

| Year | Date of freezing | Date of emergence | Total consecutive days below −1.6°C (°C) | Mean form temperature (°C) | Min. form temperature (°C) | Date of min. temperature | Distance to pond in spring (cm) | Sex |
|---|---|---|---|---|---|---|---|---|
| 2000 | 19 Oct. | 29 Apr. | 191 | −6.0±0.02 | −16.0 | 16 Dec. | nr | nr |
| 2000 | 18 Oct. | 25 Apr. | 188 | −7.6±0.01 | −12.3 | 17 Dec. | 175 | nr |
| 2000 | 20 Oct. | 21 Apr. | 182 | −4.9±0.02 | −14.1 | 16 Dec. | 195 | nr |
| 2000 | 17 Oct. | 21 Apr. | 185 | −4.5±0.02 | −9.5 | 16 Dec. | 160 | nr |
| 2000 | 18 Oct. | 25 Apr. | 188 | −3.9±0.01 | −8.9 | 17 Dec. | 1150 | nr |
| 2000 | 20 Oct. | 25 Apr. | 186 | −4.5±0.02 | −10.0 | 3 Feb. | 1540 | nr |
| 2000 | 22 Oct. | 21 Apr. | 180 | −5.1±0.02 | −17.4 | 4 Feb. | 2060 | nr |
| 2000 | 20 Oct. | 25 Apr. | 186 | −7.6±0.01 | −16.7 | 16 Dec. | 2250 | nr |
| 2001 | 12 Oct. | 8 May | 207 | −8.4±0.02 | −18.1 | 8 Dec. | 180 | F |
| 2001 | 11 Oct. | 8 May | 218 | −7.2±0.02 | −16.0 | 20 Dec. | 240 | nr |
| 2001 | 20 Oct. | 3 May | 194 | −6.2±0.01 | −13.5 | 8 Dec. | 140 | M |
| 2001 | 15 Oct. | 6 May | 202 | −7.6±0.02 | −16.7 | 8 Dec. | 90 | F |
| 2001 | 17 Oct. | 3 May | 197 | −5.8±0.02 | −14.7 | 8 Dec. | 120 | F |
| 2001 | 18 Oct. | 9 May | 202 | −5.9±0.02 | −16.0 | 8 Dec. | 80 | F |
| 2001 | 20 Oct. | 2 May | 193 | −6.6±0.01 | −13.5 | 20 Dec. | 120 | F |
| 2001 | 19 Oct. | 29 Apr. | 191 | −6.8±0.01 | −16.0 | 8 Dec. | 150 | F |
| 2001 | 10 Oct. | 9 May | 211 | −7.1±0.02 | −17.4 | 8 Dec. | 2060 | M |
| 2001 | 25 Oct. | 19 Apr. | 175 | −7.6±0.02 | −16.0 | 8 Dec. | 1370 | M |
| Mean | | | 193 | −6.2 | −14.6 | | 710 | |

M, male; F, female; nr, not recorded.

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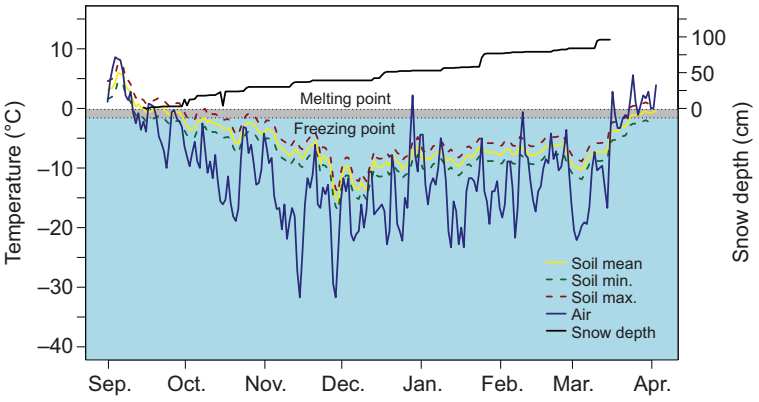


**Fig. 2. Winter hibernacula conditions, 2000 to 2001.** Average daily air temperature, average daily soil temperature (and minimum and maximum soil temperature) and daily snow depth at frog hibernacula (*N*=8) from September 2000 to April 2001. Gray bar represents melting point and freezing point of wood frogs.

freezing soil temperatures during the night and thawing soil temperatures that lasted from 2 to 32 h during the day (Fig. 4).

### Glucose concentrations

Glucose concentrations in tissues of free-living wood frogs sampled while frozen in April 2001 and 2002 and in wood frogs held in outdoor enclosures and sampled frozen in December 2011 and 2012 were not significantly different (all comparisons, $P>0.20$), and therefore values for each tissue were combined over years. Naturally frozen wood frogs had glucose concentrations (mean ± s.e.m.) in liver of 788±98.8 μmol $g^{-1}$ fresh mass, in leg muscle (gracilis major) of 299±32.2 μmol $g^{-1}$ fresh mass and in heart of 596±50.9 μmol $g^{-1}$ fresh mass. These tissue glucose concentrations were significantly higher than corresponding values in liver ($F_{2,53}$=25.4, $P<0.0001$), heart ($F_{2,53}$=25.4, $P<0.0001$) and leg muscle ($F_{2,53}$=25.4, $P<0.0001$) measured in laboratory frozen wood frogs. Laboratory frozen wood frogs had glucose concentrations in liver of 238±40.2 μmol $g^{-1}$ fresh mass, in muscle of 23.8±5.6 μmol $g^{-1}$ fresh mass and in heart of 60.5±16.2 μmol $g^{-1}$ fresh mass. There were no significant differences between mean glucose concentrations in tissues from laboratory frozen wood frogs held at −2.5°C for durations of 24, 30, 74 and 144 h ($P>0.10$). Both laboratory and naturally frozen wood frogs had significantly higher ($P<0.0001$) glucose concentrations in corresponding tissues than in unfrozen, control wood frogs, where mean concentrations were 40.2±8.9 μmol $g^{-1}$ fresh mass in the liver, 5.4±1.5 μmol $g^{-1}$ fresh mass in the muscle and 1.9±0.6 μmol $g^{-1}$ fresh mass in the heart (Fig. 5, Table 2).

### AFGL

R1 samples (containing solute that was in solution and/or weakly bound to the cell membranes, see Materials and methods for details) extracted from both the skeletal muscle and organ fractions showed thermal hysteresis (TH) activity, as did the R2 (more strongly bound membrane-associated) organ samples, indicating the presence of either antifreeze protein (AFP) or AFGL (Table 3). In contrast, the skin had minimal TH. Most of the TH activity was extracted with the initial R1 buffer, but a lesser amount of activity was present in the organ R2 sample as well, indicating that at least some of the activity was associated with the cell membrane.

Overnight treatment of the muscle R1 sample with trypsin did not reduce the level of TH (Table 3), suggesting that the activity was not due to an AFP, and therefore perhaps resulted from an AFGL. Also, elimination of TH in the organ R1 sample by xylanase treatment (Table 3) indicated that AFGL was likely responsible for the TH. This was confirmed by the 600 MHz $^1$H NMR spectrum of the R1 sample from frog muscle shown in Fig. 6A (full spectrum) and Fig. 6B (expanded region containing saccharide signals). For comparison, the same expanded region of the $^1$H NMR spectrum of the AFGL isolated from the freeze-tolerant beetle *Upis ceramboides* is shown in Fig. 6C. While the saccharide regions in Fig. 6B,C do not match with regard to relative signal intensity, there is good correspondence between the two spectra with regard to signal position, as illustrated for the downfield anomeric proton signals $H1_M$ and $H1_X$, and the up field $H5b_X$ and $H2_X$ signals. These data indicate that the frog sample is chemically similar to the *U. ceramboides* AFGL; namely, both are composed of β-mannose and β-xylose residues in 1→4-linkage. In addition, in the wood frog sample, signals are observed near 1.5 ppm (Fig. 6A), indicating the presence of $CH_2$ groups and suggesting the possibility that the sample contains a lipid component as proposed for the *U. ceramboides* AFGL. NMR spectra of muscle R2 and organ R1 and R2 samples (not shown) were similar to that of the muscle R1 sample shown in Fig. 6. In addition, signals consistent with the presence of protein did not

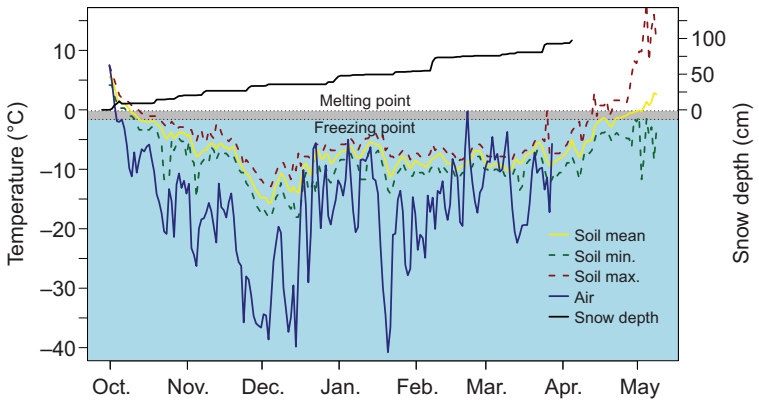


**Fig. 3. Winter hibernacula conditions, 2001 to 2002.** Average daily air temperature, average daily soil temperature (and minimum and maximum soil temperature) and daily snow depth at frog hibernacula (*N*=10) from October 2001 to May 2002.

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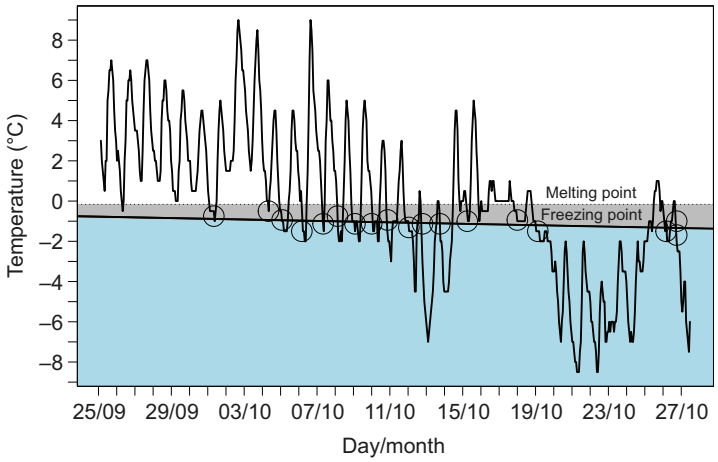


**Fig. 4. Wood frog temperatures in a frog form recorded every 30 s from 24 September to 27 October 2012.** The melting point of frogs is −0.16°C (dotted line) (Sinclair et al., 2013). Freezing point (solid line) regression line: $P<0.05$, $r^2=0.21$, $F_{1,16}=9.428$. Circles indicate observed exotherms.

appear in the NMR spectra, adding further evidence of the absence of AFP in the sample.

## DISCUSSION

Our study is the first to examine the ecological physiology, biochemistry and behavior of freeze-tolerant wood frogs overwintering under natural conditions. We describe movements of wood frogs preparing to overwinter, the locations and microclimates of their hibernacula, and tissue cryoprotectant concentrations in free-living wood frogs near the northern limits of their species' distribution in Interior Alaska. This study is also the first to report the presence of AFGL in wood frogs. We found that both freeze tolerance endurance and minimum temperatures experienced by Alaskan wood frogs are more extreme than previously established. We also demonstrate that wood frogs freezing under natural conditions accumulate much higher tissue concentrations of glucose compared with levels measured in captive wood frogs frozen under standard laboratory protocols.

In Interior Alaska, wood frogs overwintered on the forest floor within mixed spruce and birch woods. Wood frogs were located within soil in small forms 4–10 cm below the top of the leaf litter, covered with decaying leaves and branches. Disturbed wood frogs relocated to a new form; Kirton also observed wood frogs relocating after disturbance in early autumn (Kirton, 1974). Overwintering wood frogs were found close to breeding ponds (0.8–2.2 m from the pond's edge) with females tending to overwinter closer to ponds than males. This finding, although not statistically significant, contrasts with previous reports of wood frogs in which males were located in spring closer to breeding sites than were females (Regosin et al., 2003).

In artificial enclosures, wood frogs moved underneath the leaf litter and pressed the soil down by rotating their body laterally to create a form within the dense, moist soil (supplementary material Movie 1). In comparison, the Couch's spadefoot toad (*Scaphiopus couchii*) uses its clawed hindlegs to burrow into sandy substrate (Mayhew, 1965). Wood frogs, without claws, may rotate instead of dig as the soil likely requires less effort for the wood frog to compact than displace. Wood frogs were active at sub-zero temperatures and capable of movement until ice nucleation was initiated. This species is characterized by tolerance to cold, showing rapid embryonic development at low temperatures (Moore, 1939).

The duration of freezing survival in wood frogs in Interior Alaska was much longer than that reported from other studies, with temperatures within wood frog hibernacula remaining below the freezing point for up to 218 days, over 7 months, with 100% survival (Table 1). In contrast, a laboratory study with Alaskan wood frogs placed a limit of 2 months for freezing endurance with 50% survival (Costanzo et al., 2013). To our knowledge, no other study has measured temperatures of free-living wood frogs in their hibernacula; however, in the warmer climate of Ontario, Canada, Sinclair et al. recorded winter temperatures in the subnivean space where wood frogs had been observed and concluded that conditions would result in wood frogs being frozen for up to 76 consecutive hours during each freezing incident for a total of only 11–13 days frozen over the course of the winter (Sinclair et al., 2013).

Until recently, lower lethal temperatures of wood frogs were reported as approximately −7°C (Layne et al., 1998). Costanzo et al. extended this limit to −16°C for Alaskan frogs (Costanzo et al., 2013), and here we further extend this to −18.1°C, the minimum temperature experienced by wood frogs overwintering under natural conditions (all of which survived). It is likely that wood frogs can survive still lower temperatures, at least to −20°C, that regularly occur below the snow in Interior Alaska (Sformo et al., 2010). Wood frogs selected for glucose determinations in 2011 were exposed to a minimum temperature of −22°C, and although these were not examined for survival, tissue glucose concentrations were the same as those of wood frogs that survived freezing, and we believe that they were alive when sampled. In 2001 and 2002, animals surviving to spring under natural conditions experienced at least −8.9°C, with a mean minimum temperature of −14.6±2.8°C (Table 1). While

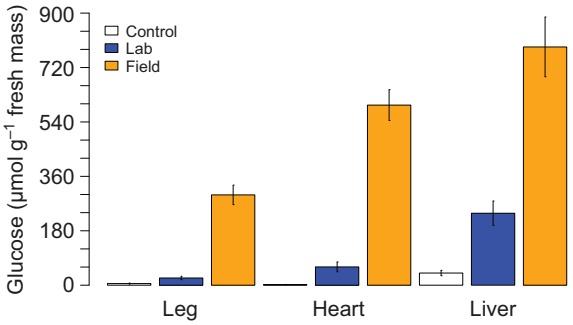


**Fig. 5. Leg muscle, heart and liver tissue glucose concentrations in unfrozen control, laboratory frozen and naturally frozen wood frogs (*N*=7, 15, 34, respectively).** Concentration is represented as means ± s.e.m. Body mass did not affect glucose concentration (ANCOVA $P>0.65$) and concentrations are expressed per gram wet mass. All values vary significantly from each other (liver $F_{2,53}=25.4$ $P<0.0001$; heart $F_{2,53}=36.4$ $P<0.0001$; and leg muscle $F_{2,53}=15.4$ $P<0.0001$).

The Journal of Experimental Biology (2014) doi:10.1242/jeb.101931

**Table 2. Glucose concentrations in liver, heart and thigh muscle in unfrozen and frozen wood frogs**

| Treatment | No. collected | Collection site | Glucose concentration (μmol $g^{-1}$ fresh mass) | | |
|---|---|---|---|---|---|
| | | | Liver | Heart | Thigh muscle |
| Control* | 7 | AK, USA | 40.2±8.9 | 1.9±0.6 | 5.4±1.5 |
| Linearly frozen* | 15 | AK, USA | 238±40.2 | 60.5±16.2 | 23.8±5.6 |
| Naturally frozen* | 34 | AK, USA | 788±98.8 | 596±50.9 | 299±32.2 |
| Linearly frozen‡ | 6 | ON, Canada | 387.8±44.8 | 198.3±27.3 | 26.5±2.7 |
| Linearly frozen§ | 3 | OH, USA | 63.7±14.1 | – | 9.7± 2.3 |
| Linearly frozen¶ | 4 | OH, USA | 261.2±55 | 174.4±26.6 | 37.6±3.5 |
| Linearly frozen¶ | 8 | AK, USA | 194.3±16 | 163±7.6 | 62.0±2.8 |

Data were obtained from unfrozen (control), linearly laboratory frozen and naturally frozen wood frogs from Alaska, USA, Ohio, USA, and Ontario, Canada.
*Present study; ‡Storey and Storey, 1984; §Irwin et al., 2003; ¶Costanzo et al., 2013.

hibernacula temperatures remained relatively stable over the winter, air temperatures fluctuated greatly and reached minima of −36.8°C in 2001 and −40.7°C in 2002 (Figs 2, 3). Relative warmth and stability in hibernacula temperatures were the result of the insulation created by air trapped in overlying leaves and snow cover. In both years, snow depth increased over the winter, resulting in all but two minimum temperatures occurring in December, although the lowest air temperatures occurred later. Soil temperatures [5 cm below the surface; Toolik Field Station (Environmental Data Center Team, 2013)] measured on the North Slope of Alaska, 250 km north of the wood frog distribution limits usually are not lower than hibernacula temperatures in Interior Alaska, suggesting that minimum temperatures in winter do not limit the northern range of wood frogs. Their northern range in Alaska may instead be limited by other abiotic conditions such as the geographical barrier of the Brooks Range or prolonged low water temperatures in breeding ponds that may prevent complete metamorphosis of tadpoles in summer (Martof and Humphries, 1959; Herreid and Kinney, 1967).

Enhanced tolerance to freezing has been previously demonstrated in Alaskan wood frogs in a preliminary field study (Middle and Barnes, 2001) and recently in the laboratory (Costanzo et al., 2013), although the results presented here extend limits in both minimum temperature and especially duration of freezing. The physiological basis of this profound cold tolerance may lie in the high levels of glucose accumulation in tissues, effects of additional cryoprotectants such as urea (Costanzo et al., 2013), the presence of AFGL, or likely a combination of these and other factors that create protection from extracellular ice formation and accompanying desiccation.

Glucose concentrations in liver, heart and leg muscle from naturally freezing wood frogs were much higher than levels measured in corresponding tissues from wood frogs frozen in the laboratory in this and in other studies (Fig. 5, Table 2). Despite being cooled at rates of 0.05 and 0.5°C $h^{-1}$, slower than the rates observed under natural conditions (as high as 1.6°C $h^{-1}$), Alaskan wood frogs frozen in the laboratory accumulated glucose to levels that were only 22–40% in liver, 6–31% in heart and 7–20% in thigh muscle compared with those in corresponding tissues in wood frogs that froze outdoors (Table 2). Wood frogs from Ontario, Canada, and the Midwestern United States frozen in the laboratory had glucose concentrations that were 8–62% in liver and 3–10% in heart of those

**Table 3. Thermal hysteresis of various wood frog muscle, organ and skin samples**

| Sample | TH (°C) |
|---|---|
| Muscle R1 | 0.61 |
| Muscle R1 + trypsin | 0.62 |
| Muscle R2 | 0.02 |
| Organ R1 | 1.29 |
| Organ R1 diluted 1:1 with citrate buffer | 0.88 |
| Organ R1 diluted 1:1 with citrate buffer + xylanase | 0.08 |
| Organ R2 | 0.31 |
| Skin R1 | 0.10 |
| Skin R2 | 0.04 |

Thermal hysteresis (TH, freezing point minus melting point). R1 sample contains soluble and/or weakly membrane-bound TH factors. R2 contains more strongly membrane-bound TH factors.

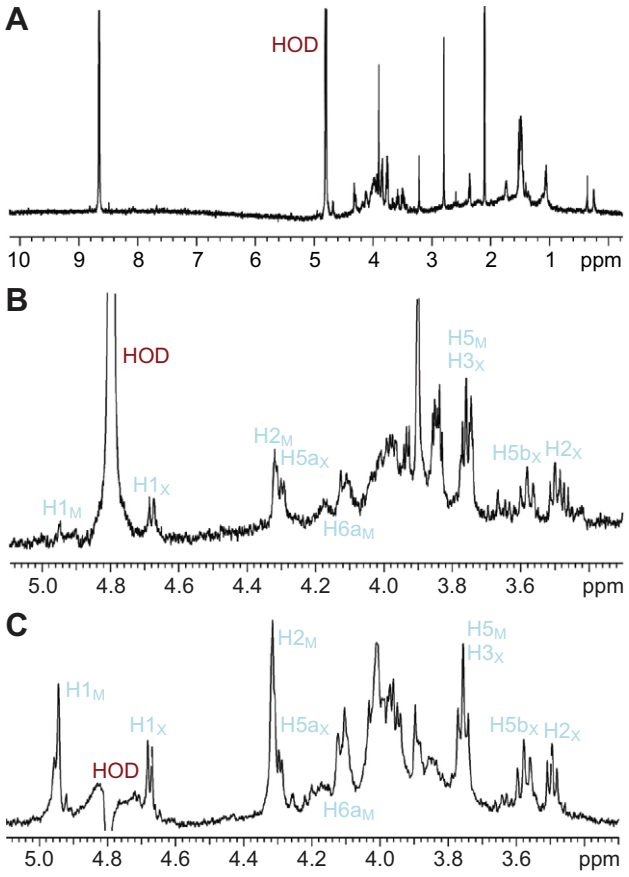


**Fig. 6. High-resolution $^1$H NMR spectra of antifreeze glycoprotein (AFGL) isolated from wood frog skeletal muscle and from *Upis ceramboides*.** (A) The full $^1$H NMR spectrum of the wood frog AFGL. (B) An expanded region of A containing the saccharide signals. (C) $^1$H NMR spectrum from *U. ceramboides* [data taken from Walters et al. (Walters et al., 2009)]. Signal assignments were made as described elsewhere (Walters et al., 2009). Subscripts X and M refer to the hydrogen atoms found in the β-mannose and β-xylose rings, respectively.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.101931

measured in naturally freezing Alaskan frogs (Table 2). Alaskan wood frogs may accumulate these high levels of glucose in their tissues by initially storing more glycogen as a source, releasing more glucose when freezing, or through repeated episodes of freezing-stimulated release of glucose, coupled with decreased rates of glucose uptake or loss during thaw.

Wood frogs collected in Interior Alaska indeed accumulate very high levels of glycogen in the autumn, ~3.5-fold the concentrations in liver and muscle measured per gram of frog compared with wood frogs collected in Ohio (Costanzo et al., 2013). Despite these large differences in the relative amount of glycogen stored, Alaskan and Ohio wood frogs were similar, however, in how much glucose they mobilized into liver, heart and muscle 48 h after freezing is initiated, when freezing occurs via a linear decrease in temperature (Costanzo et al., 2013). This result suggests that it is not just the large stores of glycogen that account for the high levels of mobilized glucose in naturally frozen Alaskan frogs but also the pattern of freezing, which includes multiple freeze–thaw cycles.

We hypothesize that it is the pattern of freezing under natural conditions, which includes multiple freezing and thawing cycles, that causes the high concentrations of glucose that accumulate in tissues of Alaskan wood frogs, and that these high glucose concentrations contribute to the enhanced tolerance to cold that we have demonstrated. Beginning in early October of each year, soil temperatures in wood frog hibernacula decreased below −0.5°C most nights, and exotherms, indicative of the initiation of freezing, occurred followed shortly by thawing conditions during most days that lasted for 12.2 h, on average. Wood frogs overwintering under natural conditions experienced as many as 17 mostly successive freezing and thawing episodes before temperatures decreased and remained below freezing until spring.

The decrease in the temperature at which successive exotherms appeared (Fig. 4) could be explained by the increase in overall solute concentration that occurs in frogs as they accumulated glucose, as increasing osmolarity decreases the supercooling point in fluids (Zachariassen, 1985). The decrease in exotherm temperatures of frogs over time was statistically significant in only one of the two years from which measurements were acquired, however.

If each exotherm results in a stimulus for conversion of stored glycogen to glucose and if glucose accumulates in tissues as a result of low rates of loss or re-synthesis into glycogen during thaw at low temperatures, then successive freeze–thaw cycles in wood frogs should result in higher and higher tissue concentrations of glucose. Inoculative nucleation of freezing detected in the skin of wood frogs is a required stimulus for the breakdown of liver glycogen stored into glucose, which is then distributed throughout the body (Storey and Storey, 1986), and consecutive 2 day cycles of freezing and thawing resulted in higher glucose levels than in controls (Storey and Storey, 1988) although not to the levels shown in naturally freezing frogs in this study. Also, glucose is indeed retained in tissues at higher levels after thaw in Alaskan relative to southern populations of wood frogs. Successive freeze–thaw cycles lead to accumulation of tissue glucose concentrations because glucose synthesis following freezing is faster than reconversion of glucose to glycogen following thaw (Storey and Storey, 1986). Glucose levels remained at 20–50% (average 30%) of maximal values reached over 48 h of freezing 5 days after thaw in plasma, brain, liver, heart and muscle of Alaskan frogs compared with values of 2–30% (average 9%) in Ohio frogs (Costanzo et al., 2013). Levels of distributed glucose may change little during the daily intervals of slightly above freezing temperatures experienced by free-living frogs in Alaska that lasted only about 12 h before another stimulus for glucose release occurred at night. Whether wood frogs from southern populations experiencing successive freezing and thawing stimuli can accumulate as high a level of glucose in tissues as Alaskan frogs do and whether this would enhance their tolerance to freezing is not known.

Freezing tolerance in wood frogs may also be enhanced because of the presence of AFGL in their membranes and tissues. Ice-purified extracts derived from homogenized samples of skeletal muscle and internal organs of naturally overwintering wood frogs demonstrated a level of TH activity that is usually associated with AFP. However, trypsin treatment did not affect the TH activity (Table 3), suggesting that TH is not dependent on a protein. In contrast, treatment of the R1 organ sample with endo β-(1→4) xylanase eliminated TH, as was the case with the AFGL from the freeze-tolerant Alaskan beetle *U. ceramboides* (Walters et al., 2009). Also, NMR spectra of wood frog antifreeze showed signals with similar positions to saccharides of AFGL from *R. lessonae*, as well as from various insects and a plant, indicating a backbone consisting of β-mannose and β-xylose residues in 1→4-linkage (Walters et al., 2009; Walters et al., 2011). While lipid signals were also present in the NMR spectra, perhaps indicating the presence of fatty acids that anchor the AFGL in membranes, the NMR spectra did not exhibit amino acid signals consistent with protein. Consequently, wood frogs appear to have an AFGL similar to those described in other species. While the function(s) of the AFGLs is not known, they may inhibit damaging recrystallization of ice in the extracellular fluid where ice is present and prevent propagation of extracellular ice across the cell membrane and into the cytoplasm, which is lethal in most cells of freeze-tolerant animals.

Limitations to the duration of freeze tolerance and minimum freezing temperature include extracellular recrystallization, metabolic demand, waste accumulation, intracellular ice formation and desiccation (Knight and Duman, 1986; Storey and Storey, 1988; Layne et al., 1998). Wood frogs overwintering in Interior Alaska must prevent intracellular ice formation and limit extracellular recrystallization for over 6 months; they may accomplish this despite very low temperatures by accumulating high levels of intracellular cryoprotectants and production of AFGL. Further, the low temperatures wood frogs experience should minimize rates of metabolism so that waste accumulation and hypoxia do not constrain freeze tolerance. Kirton observed that juvenile wood frogs that did not survive overwintering were desiccated at the beginning of spring (Kirton, 1974). We observed similar desiccation due to sublimation when holding frozen wood frogs in a laboratory setting (D.J.L. and B.M.B., unpublished). Wood frogs frozen in moist environments, such as wet moss, are able to maintain a greater volume of body water than wood frogs frozen in dry environments (Churchill and Storey, 1993). Forms constructed under leaves should create a moist environment for overwintering, and therefore wood frogs may hibernate underneath leaf litter to minimize rates of water loss, as well as to buffer the extremes and variability of air temperatures.

Our results demonstrate that Alaskan wood frogs can survive being frozen for up to 7 months with minimum temperatures below −18°C. Only the Siberian salamanders *Salamandrella schrenckii* and *S. keyserlingii*, which endure 4–5 months frozen with survival of individuals to −35°C (Berman et al., 1984; Berman et al., 2010), are comparable to the capabilities of North American wood frogs. Whether the extremes in freezing tolerance demonstrated here in northern compared with more southern populations of wood frogs are due to differences in glycogen concentrations and acclimatization and patterns of temperature change during freezing,

or are due to differences in their genetics, and thereby represent evolutionary change, awaits further study.

## MATERIALS AND METHODS

### Field and laboratory studies

This study was conducted with the approval of the Institutional Animal Care and Use Committee of the University of Alaska, Fairbanks (protocol no. 259022-3) and permits issued by the Alaskan Department of Fish and Game.

We studied wood frogs over the course of four winters. Initially, we collected adult wood frogs by hand in September 2000 and 2001 by searching open fields near known breeding ponds in birch and spruce boreal forest around the Fairbanks North Star Borough (64.8°N, 147.8°W; *N*=8 and 10, respectively). We attached radio transmitters (model V1G102A with 10 cm whip antenna, Sirtrack, Havelock North, New Zealand; mass 0.95 g) with cyanoacrylate glue to the back of individual wood frogs that weighed at least 12 g. Tagged wood frogs were held overnight and released the following day at their collection sites. Using radio receivers and Yagi antennas (Telonics Inc., Mesa, AZ, USA), we re-located wood frogs daily until they stopped moving. In late September 2000 and 2001, we located four additional wood frogs (two each year) within their hibernacula by raking the leaf litter near the edge of breeding ponds. A temperature logger probe (Hobo Pro, Onset Corp., Bourne, MA, USA) was positioned between each wood frog's ventrum and the surrounding soil, and a wire-mesh cage (1 cm squares, 20×20×20 cm) was placed over each wood frog to prevent disturbed wood frogs from relocating. Air temperature was recorded with a temperature logger probe placed 2 m above the duff layer located near overwintering frogs. Temperatures were recorded every 5 min until wood frogs emerged from hibernation the next spring. Snow depth was taken from daily recordings for the nearby Fairbanks International Airport (5.5 km from the study site). Beginning in early April, we assessed wood frogs for movement each day. Wood frogs were considered thawed and alive when they moved from the small depressions in the duff within which they overwintered. Four wood frogs were collected in early April 2001 and double pithed before thawing; tissues from these wood frogs were collected for glucose determinations.

In 2011 and 2012, we also collected 15 male wood frogs each year from July to August in the Fairbanks North Star Borough. Each wood frog was swabbed and determined to be negative for chytrid fungus with qPCR (Pisces Molecular, LLC, Boulder, CO, USA) and transferred to 1×2.4×2.4 m outdoor enclosures in the Biological Reserve (64.8°N, 147.8°W) at the University of Alaska Fairbanks. The enclosures were located in a birch and spruce forest with conditions similar to the natural habitat of wood frog overwintering locations. Pools of water were present in the enclosures, and wood frogs were fed crickets and wingless fruit flies daily until temperatures decreased below freezing in mid-September. We surrounded each dormant wood frog with a 20×20×20 cm wire-mesh cage and placed a temperature logger (Tidbit, Onset Computer, Bourne, MA, USA) in contact with each frog. Temperatures were recorded every 30 s. Frozen wood frogs were collected on 12 December 2011 and 2012, double pithed, and their tissues collected for glucose determinations.

We filled a plastic pool-container (121 cm diameter) with 10 cm soil and 5–10 cm of leaf litter and placed the pool in an environmental chamber held at −5°C. In early September 2012, two naturally acclimated wood frogs from our outdoor enclosure were released into the leaf litter and filmed with two cameras for 18 h as they became frozen.

We collected 22 adult male wood frogs in August 2001 and we acclimated them for 1 week in a refrigerator set at 5°C. Wood frogs that were to be frozen were placed in 50 ml plastic containers with a type T thermocouple placed against their ventrum. A thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, OH, USA) recorded temperature. We cooled wood frogs in their containers in an alcohol–water bath (Neslab ULT-80, Waltham, MA, USA) at a constant rate of 0.5°C $h^{-1}$ from 1 to −2.5°C. Wood frogs were nucleated with ice at −1°C and an exotherm indicating freezing was observed. Wood frogs were held at −2.5°C for 24, 30, 74 and 144 h (*N*=3, 3, 6, 3). Frozen and unfrozen, control (*N*=7) wood frogs were pithed and tissues were collected for glucose determinations.

Liver, leg and heart tissue were dissected from each wood frog. Tissue samples (50 mg) were homogenized with 0.6 mol $l^{-1}$ ice-cold perchloric acid and centrifuged. Extracts were neutralized and assayed in triplicate for glucose concentrations with a YSI-2000 analyzer, comparing with a standard solution (YSI, Inc., Yellow Springs, OH, USA).

### Screening and isolation for AFGL and NMR spectroscopy

Tissues from 11 naturally frozen frogs were collected in spring 2012 and shipped frozen on dry ice to the University of Notre Dame, where they were held at −80°C until processed. The frogs were thawed and dissected, with the tissues and organs separated and pooled into three groups: skeletal muscle (1.8 g), skin (4.8 g) and internal organs (remaining tissues and heart, liver, lungs, etc., but not bone; 18.0 g). These were cut into small pieces with scissors and homogenized in 50 mmol $l^{-1}$ Tris-HCl buffer (pH 7.4) at an 8:1 ratio of volume of buffer to wet mass of tissue. The homogenized tissues were sonicated (W-385 sonicator, Heat Systems-Ultrasonic Inc., Farmingdale, NY, USA) using the sonicator horn and three, 30 s intervals (power level 3). The samples were centrifuged (10,000 ***g*** for 20 min at 4°C) and the supernatant (identified as the 'R1' sample) and pellet separated. The pellet was extracted with the urea-based buffer from the Bio-Rad Ready Prep sequential extraction kit (Bio-Rad, Hercules, CA, USA) to solubilize lipophilic membrane-bound molecules. This sample was centrifuged at 10,000 ***g*** for 20 min at 4°C, and the supernatant dialyzed (3500 MW cut-off, Spectrapor) for 24 h at 4°C. This was identified as the 'R2' sample. The osmolality of both samples was adjusted to 200 mOsm with glycerol and subjected to multiple rounds of ice-affinity purification (Walters et al., 2009), a technique that utilizes the unique ability of thermal hysteresis producing AFP and glycolipids to bind to ice rather than to be excluded from the ice crystal lattice as ice formation proceeds. Following this, the samples were dialyzed against Milli-Q water for 48 h to remove the glycerol, freeze dried, and re-dissolved in a small volume of Milli-Q water.

Freezing and melting points of the resulting samples were measured using a nanoliter osmometer (Nickell et al., 2013) to determine whether the samples displayed TH (melting point and freezing point differ), indicative of the presence of AFP and/or AFGL. Subsamples that exhibited TH were treated with proteomics-grade trypsin (porcine pancreas, Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Loss of TH after trypsin treatment would indicate that TH resulted from AFP. Because previously investigated AFGLs contained xylose and were inactivated by xylanase (Walters et al., 2009; Walters et al., 2011), subsamples with TH were also treated with endo β-(1→4) xylanase (from *Thermomyces languginosus*; Sigma) in 50 mmol $l^{-1}$ sodium citrate buffer, pH 5.0.

Samples with TH suspected of containing AFGLs were lyophilized then dissolved in 200 ml of 20 mmol $l^{-1}$ aqueous ($^2H_2O$) sodium phosphate buffer at pH 7.5 (meter reading), and the resulting solution was transferred to a 5 mm symmetrical Shigemi NMR microtube with susceptibility matched to $^2H_2O$. High-resolution 1D $^1H$ NMR spectra were obtained at 40°C on a Varian UNITYPlus 600 MHz FT-NMR spectrometer equipped with a 5 mm $^1H$-$^{19}F/^{15}N$-$^{31}P$ AutoX dual broadband probe. $^1H$ NMR spectra were collected with 1500 transients, 7670 Hz spectral windows and ~3.0 s recycle times. Exponential line-broadening of 0.5 Hz was applied to free induction decays prior to Fourier transformation. The final digital resolution of transformed spectra was 0.03 Hz/point. Spectra were referenced internally to the residual $^2H_2O$ signal at 4.800 ppm.

### Statistical inferences

Sample means were compared using Student's *t*-test and analysis of variance (ANOVA) and analysis of co-variance (ANCOVA) followed by Tukey multiple comparisons tests. Linear regressions were calculated for exotherms. Mean (±s.e.m.) values are reported. Significance of statistical analyses was accepted at $P<0.05$.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

D.J.L., L.M. and B.M.B. conceived the study and designed the experiments. D.J.L., L.M. and B.M.B. collected and analyzed the data. H.V., W.Z., A.S.S. and J.D. purified the AFGL and conducted and analyzed all NMR results. D.J.L., J.D., A.S.S. and B.M.B. wrote the paper. All authors contributed substantially to developing the manuscript and take full responsibility for the content of the paper.

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### Supplementary material

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