1 Wood frog adaptations to overwintering in Alaska: New limits to freezing tolerance.

2 Key words: Freeze tolerance, Wood Frog, Cryoprotectant, antifreeze glycolipid

3 Don Larson*[+#], Luke Middle, Henry Vu[&], Wenhui Zhang[$], Anthony S. Serianni[$], John Duman[&], and Brian M. Barnes*[+]

4 *Corresponding authors: (djlarson@alaska.edu and bmbarnes@alaska.edu)

5 [+]Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, AK, USA, 99775

6 [#]Department of Biology and Wildlife, University of Alaska Fairbanks, Fairbanks, AK, USA, 99775

7 [&] Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA, 46556

8 [$] Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, USA, 46556

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 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 (Oct-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 were13-fold higher in muscle, 10-fold higher in heart, and 3.3-fold in liver in naturally freezing compared to laboratory frozen frogs. Glycolipid antifreeze 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 to those described in animals collected in southern Canada or the U.S. Midwest. 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 fall.

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, since 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, 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 subzero 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*) 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 Midwest 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 antifreeze glycolipids.

RESULTS

Selection of Hibernacula.

Free-living wood frogs (n=18; body mass 14±1.2 g)prepared for overwintering were found in early September 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 average distance of wood frogs from the 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 outdoor soil and leaf filled enclosure at -5°Ccontinued 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 supplemental video).

Freezing conditions and overwinter survival.

All 18 free-living wood frogs in natural hibernacula in April 2001 and 2002 survived winter. We considered that wood frogs began to freeze when soil temperatures were below -1.6°C (see below) and thawed when temperatures were above -0.16°C, based on the melting point of frogs determined by Sinclair *et al*. (2013). Combining data from both years, temperatures of wood frogs decreased below -1.6°C between October 10 and 25 and first warmed above -0.16°C in spring between April 19 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°C to -8.4°C, with a grand mean of -6.3°C. Minimum temperatures experienced by frogs ranged from -8.9°C 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 January 24, 2001 and February 8, 2002 (Table 1 and Fig 2 and 3).

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

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±0.28ºC (n=15) in 2011 and -1.14±0.34ºC (n = 15) in 2012.The temperature at which exotherms occurred decreased with date 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 0.5ºC until nucleation ranged among frogs from 0.35 to 1.60 ºC per hour.

In early October of all years wood frogs experienced multiple (average of 12, range 10-17) and mostly successive cycles of freezing soil temperatures during night and thawing soil temperatures that lasted from 2-32 h during 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±SEM) in liver of 788±98.8 µmol g^{-1} fresh weight, in leg muscle (gracilis major) of 299±32.2 µmol g^{-1} fresh weight, and in heart of 596±50.9 µmol g^{-1} fresh weight. 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 238±40.2 µmol g^{-1} fresh weight, in muscle 23.8±5.6 µmol g^{-1} fresh weight, and in heart 60.5±16.2 µmol g^{-1} fresh weight. 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 hrs ($P>0.10$). 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 average concentrations were 40.2±8.9 µmol g^{-1} fresh weight in the liver, 5.4±1.5 µmol g^{-1} fresh weight in the muscle, 1.9±0.6 µmol g^{-1} fresh weight in the heart. (Fig 5, Table 2).

Antifreeze glycolipid.

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 or antifreeze glycolipid (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 antifreeze protein, and therefore perhaps resulted from an antifreeze glycolipid. 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 Figure 6 (A, full spectrum; B, 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 Figure 6C. While the saccharide regions in Figures 6B and 6C do not match with regard to relative signal intensities, there is good correspondence between the two spectra with regard to signal positions, 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 ßMannose and ßXylose residues in 1→4-linkage. In addition, in the wood frog sample, signals are observed near 1.5 ppm (Figure 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 Figure 6. In addition, signals consistent with the presence of protein did not 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 antifreeze glycolipid in wood frogs. We found that both freeze tolerance endurance and minimum temperatures experienced by Alaska wood frogs are more extreme than previously established. We also demonstrate wood frogs freezing under natural conditions accumulate much higher tissue concentrations of glucose compared to 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 (1974) also observed wood frogs relocating after disturbance in early fall. Overwintering wood frogs were found close to breeding ponds (0.8-2.2 m from pond's edge) with females tending to overwinter closer to ponds than males. This finding, although not statistically significant, supports 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 (supplemental video). In comparison, the Couch's Spadefoot Toad (*Scaphiopus couchii*) uses its clawed hind legs to burrow into sandy substrate (Mayhew, 1965). Wood frogs, without claws, may rotate instead of dig since 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).

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*. (2013) 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 to76 consecutive hours during each freezing incident for a total of only 11-13 days frozen over the course of the winter.

Until recently, lower lethal temperatures of wood frogs were reported as approximately -7°C (Layne *et al*. 1998). Costanzo *et al*. (2013) extended this limit to -16°C for Alaskan frogs, 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 wood frogs that survived freezing, and we believe that they were alive when sampled. In2001 and 2002, animals surviving to spring under natural conditions experienced at least -8.9°C with the average minimum temperature of-14.6±2.8°C (Table 1). While hibernacula temperatures remained relatively stable over the winter, air temperatures fluctuated greatly and reached minima of -36.8°C in 2001 and -40.7°Cin 2002 (Fig 2 and 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 (5cm below the surface; Environmental Data Center Team, Toolik Field Station, 2013)measured on the North Slope of Alaska 250 km north of the wood frog distribution limits usually are not lower than hibernaculum 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 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 (Constanzo *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 and Table 2). Despite being cooled at rates of 0.05 and 0.5ºC/h, slower than the rates observed under natural conditions (as high as 1.6 ºC/h), 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 as compared to those in corresponding tissues in wood frogs that froze outdoors (Table 2). Wood frogs from Ontario, Canada and the U.S. Midwest frozen in the laboratory had glucose concentrations that were 8-62% in liver and 3-10% in heart of those 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 fall, approximately 3.5 fold the concentrations in liver and muscle measured per gram of frog compared to 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 (Constanzo *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 that includes multiple freeze thaw cycles. We hypothesize that it is the pattern of freezing under natural conditions that 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 ºC most nights, and exotherms, indicative of the initiation of freezing, occurred shortly followed 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 occurred (Fig 4) could be explained by the increase in overall solute concentration that occurs in frogs as they accumulated glucose, since increasing osmolarity decreases the supercooling point in fluids (Zachariessen, 1985). The decrease in exotherm temperatures of frogs over time was statistically significant in only one of the two years we measured, however.

If each exotherm results in a stimulus for conversion of stored glycogen to glucose and if glucose accumulates in tissues due to 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 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 Storey1988) 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 over48 h of freezing 5 days after thaw in plasma, brain, liver, heart and muscle of Alaskan frogs compared to values of 2-30% (average 9%) in Ohio frogs (Constanzo *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 stimulus 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 due to the presence of antifreeze glycolipids 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 antifreeze proteins. However, trypsin treatment did not affect the TH activity (Table 3), suggesting that the TH is not dependent on a protein. In contrast, treatment of the R1 organ sample with endo ß-(1→4) xylanase eliminated the TH, as was the case with the antifreeze glycolipid 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 ßMannose and ßXylose residues in 1→4-linkage (Walters *et al*, 2009, 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 are 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 that is lethal in most cells of freeze tolerant animals. Limitations to 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 antifreeze glycolipid. Further, the low temperatures wood frogs experience should

minimize rates of metabolism so that waste accumulation and hypoxia do not constrain freeze tolerance. Kirton (1974) observed that juvenile wood frogs that did not survive overwintering were desiccated at the beginning of spring. We observed similar desiccation due to sublimation when holding frozen wood frogs in a laboratory setting (Larson and Barnes, 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 created 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 and Meshcheryakova, 2010), are comparable to capabilities of North American wood frogs. Whether the extremes in freezing tolerance demonstrated here in northern compared to more southern populations of wood frogs are due to differences in glycogen concentrations and acclimatization and patterns of temperature change during freezing or due to differences in their genetics, and thereby represent evolutionary change, awaits further study.

MATERIALS AND METHODS

Field and Laboratory Studies.

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 º N, 147.8 º W), (n=8, 10, respectively). We attached radio transmitters (model V1G102A with 10 cm whip antenna, Sirtrack, Havelock North, New Zealand; weight 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 4 additional wood frogs (2 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, 20x20x20cm) 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 minutes 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 1x2.4x2.4m 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 20x20x20cm wired cage and placed a temperature logger (Tidbit, Onset Computer, Bourne, MA, USA) in contact with each frog. Temperatures were recorded every 30 seconds. Frozen wood frogs were collected on December 12th, 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 2 cameras for 18 hours 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 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 temperatures. 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 per hour from 1°C 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 hours (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 N 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 antifreeze glycolipids and NMR spectroscopy

Tissues from 11 naturally frozen frogs were collected in spring 2012and shipped frozen on dry ice to the University of Notre Dame where they were held at -80^{o}C until processed. The frogs were thawed and dissected with the tissues and organs separated and pooled into three groups: skeletal muscle (1.8 grams), skin (4.8 grams), and internal organs (remaining tissues and heart, liver, lungs, etc., but not bone; 18.0 grams). These were cut into small pieces with scissors and homogenized in 50 mMTris-HCl buffer (pH7.4) at an 8:1

ratio of volume of buffer to wet mass of tissue. The homogenized tissues were sonicated (Heat Systems-Ultrasonics, Inc. Farmingdale, NY, USA, W-385 sonicator) using the sonicator horn and three 30-second intervals (power level 3). The samples were centrifuged (10,000 g for 20 minutes at 4^{o}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 to solubilize lipophilic membrane bound molecules. This sample was centrifuged at 10,000g for 20 minutes at 4^{o}C, and the supernatant dialyzed (3,500 MW cut-off, Spectrapor) for 24 hr at 4^{o}C. This is 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 antifreeze proteins 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 Mill-Q water for 48 hr to remove the glycerol, freeze dried, and re-dissolved in a small volume of Mill-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 thermal hysteresis (TH, melting point and freezing point differ) indicative of the presence of antifreeze proteins and/or glycolipid. Subsamples that exhibited TH were treated with proteomics grade trypsin (Sigma, porcine pancreas) according to the manufacturer's directions. Loss of TH after trypsin treatment would indicate that TH resulted from antifreeze proteins. Because previously investigated antifreeze glycolipids contained xylose and were inactivated by xylanase (Walters *et al*,

2009; 2011), subsamples with TH were also treated with endo ß-(1→4) xylanase (Sigma, St. Louis, MO, USA from *Thermomyces languginosus*) in 50mM sodium citrate buffer, pH=5.0.

Samples with TH suspected of containing AFGLs were lyophilized then dissolved in 200 mL of 20 mM 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 HOD 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 values are reported as ±S.E.M. Significance of statistical analyses was accepted at $P<0.05$.

List of symbols and abbreviations

AFGL- antifreeze glycolipid

TH- thermal hysteresis

Competing Interests

No competing interest to declare

Author Contributions

D. Larson, L. Middle and B. Barnes conceived the study, designed the experiments. D. Larson, L. Middle and B. Barnes collected and analyzed the data. H. Vu, W. Zhang, A. Serianni, and J. Duman purified the AFGL and conducted and analyzed all NMR results. D. Larson, J. Duman, A. Serianni, and B. Barnes 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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Figure 1 **Wood frog in a naturally made overwintering form; covering leaves removed for photo.**

Figure 2 **Average daily air temperature, average daily soil temperature, daily variance (±SEM), and daily snow depth at frog hibernacula (n=8) from September 2000 to April 2001**

Figure 3 **Daily snow depth, average daily air temperature, average daily soil temperature, minimum and maximum daily temperature among frog hibernacula (n=10) from October 2001 to May 2002**

Figure 4 **Wood frog temperatures in a frog form recorded every 30 seconds from September 24 to October 27, 2012. Melting point is -0.16 (dashed line) (Sinclair et al., 2013) and freezing point (solid line) regression line $P<0.05$, $r^2=0.21$, $F_{1,16}=9.428$. Circles indicate observed exotherms**

Figure 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 and error bars are ±SEM. Body mass did not affect glucose concentrations (ANCOVA p>0.65) and concentrations are expressed per gram wet weight. 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 ($F_{2,53}= 15.4$ $P<0.0001$)]**

Figure 6 **High-resolution ^{1}H NMR spectra of AFGL isolated from wood frog skeletal muscle (A and B) and from *Upis ceramboides* (C; data taken from Walters *et al*., 2009). The full spectrum of the frog AFGL is shown in (A), and an expanded region of (A) containing the saccharide signals is shown in (B). Signal assignments were made according to Walters *et al*., 2009. Subscripts X and M refer to the hydrogen atoms found in the ß-Mannose and ß-Xylose rings, respectively.**

475 **TABLES**

Table 1. Characteristics of natural wood frog hibernacula over 2 winters. nr indicates not recorded.

Year	Date of Freezing	Date of Emergence	Total consecutive days below -1.6(°C)	Mean form temperature Mean±SEM (°C)	Minimum form temperature (°C)	Date of minimum temperature	Distance to pond in spring (cm)	Sex
2000	19 Oct	29 April	191	-6.0±0.02	-16.0	16 Dec	nr	nr
2000	18 Oct	25 April	188	-7.6±0.01	-12.3	17 Dec	175	nr
2000	20 Oct	21 April	182	-4.9±0.02	-14.1	16 Dec	195	nr
2000	17 Oct	21 April	185	-4.5±0.02	-9.5	16 Dec	160	nr
2000	18 Oct	25 April	188	-3.9±0.01	-8.9	17 Dec	1150	nr
2000	20 Oct	25 April	186	-4.5±0.02	-10.0	3 Feb	1540	nr
2000	22 Oct	21 April	180	-5.1±0.02	-17.4	4 Feb	2060	nr
2000	20 Oct	25 April	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 April	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 April	175	-7.6±0.02	-16.0	8 Dec	1370	M
mean			193	-6.2	-14.6		710	

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Table 2. Glucose concentrations (µmol g^{-1} fresh weight) in liver, heart, and thigh muscle in unfrozen (control), linearly laboratory frozen, and naturally frozen wood frogs from Alaska, USA, Ohio, USA, and Ontario, Canada

Treatment	Number Collected	Collected from	Liver µmol g^{-1} fresh weight	Heart µmol g^{-1} fresh weight	Thigh Muscle µmol g^{-1} fresh weight
Control *	7	AK, USA	40.2± 8.9	1.9±0.6	5.4±1.5
Linearly *	15	AK, USA	238±40.2	60.5±16.2	23.8±5.6
Naturally Frozen*	19	AK, USA	788±98.8	596±50.9	299±32.2
Linearly Frozen+	6	ON, CAN	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

* This study +Storey and Storey, 1984 ^Irwin *et al.*, 2002 #Costanzo *et al.*, 2013

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482 **Table 3.**Thermal hysteresis (TH, freezing point minus melting point) of various wood frog muscle, organ and skin samples. R1

483 sample contains soluble and/or weakly membrane bound TH factors. R2 contains more strongly membrane bound TH factors.

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Sample	**Thermal Hysteresis (^{o}C)**
A. Muscle R1	0.61
B. A (above) + trypsin	0.62
C. Muscle R2	0.02
D. Organs R1	1.29
E. D (above) diluted 1/1 with citrate buffer	0.88
F. D (above) diluted 1/1 with citrate buffer + xylanase	0.08
G. Organs R2	0.31
H. Skin R1	0.10
I. Skin R2	0.04

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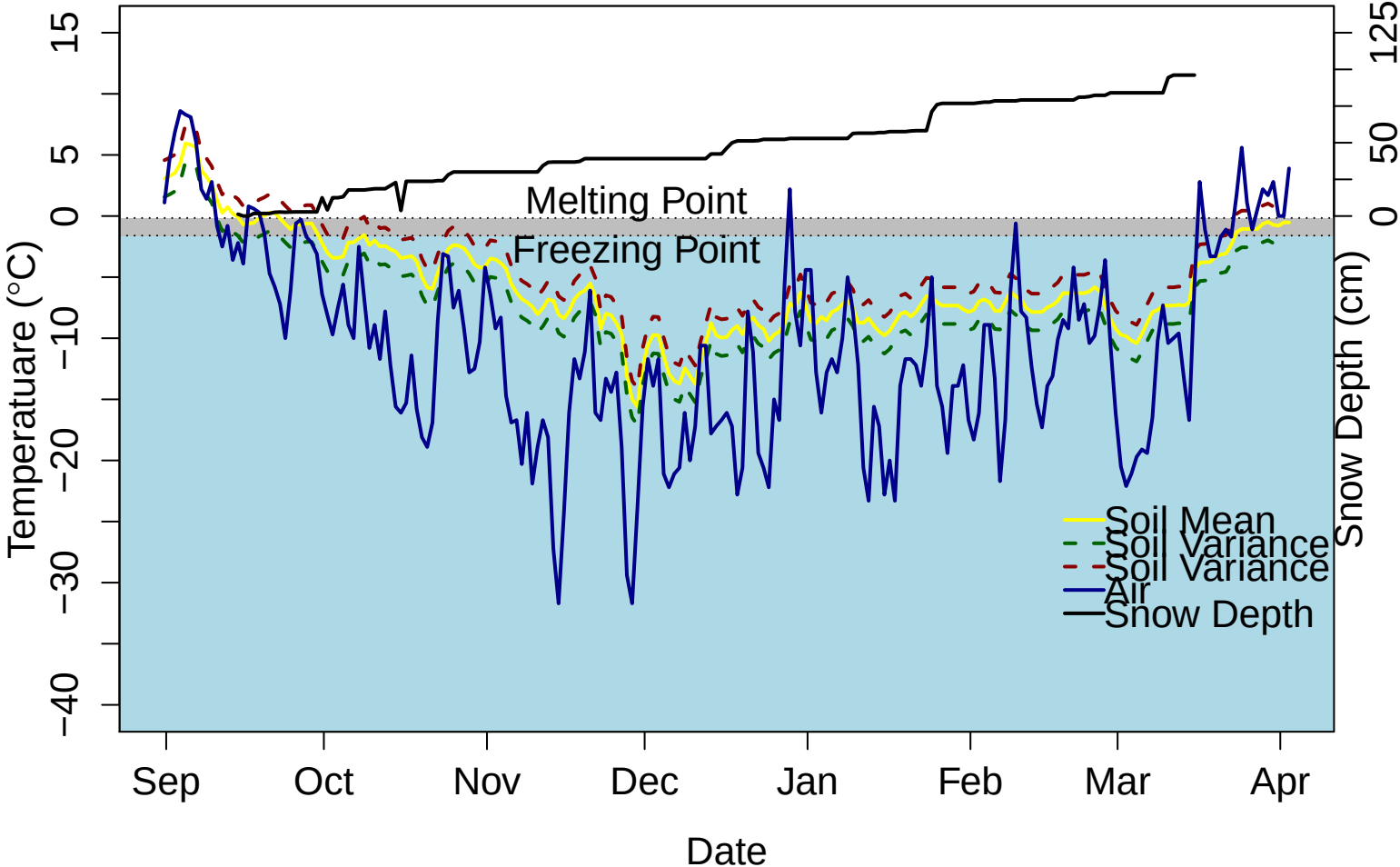
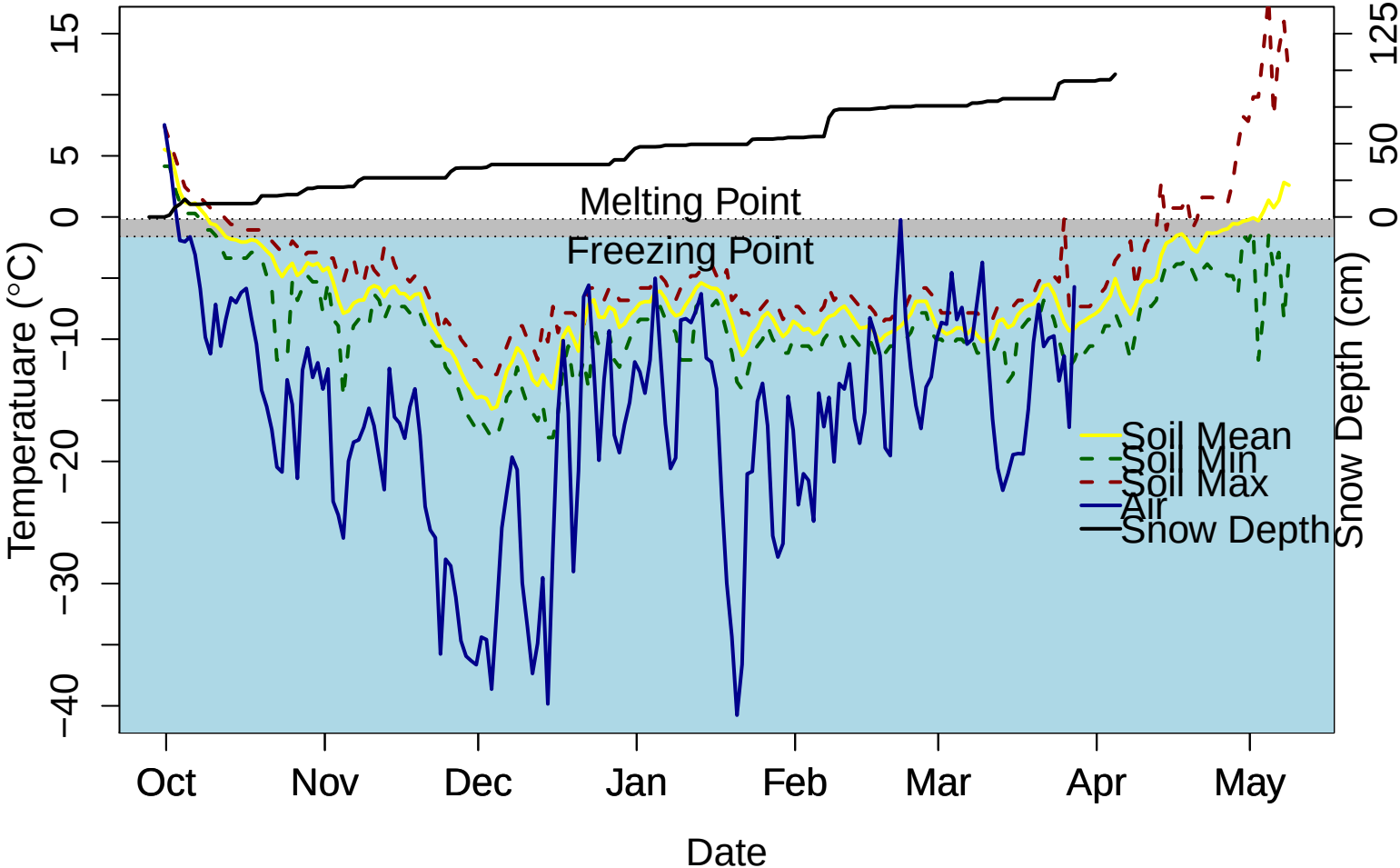
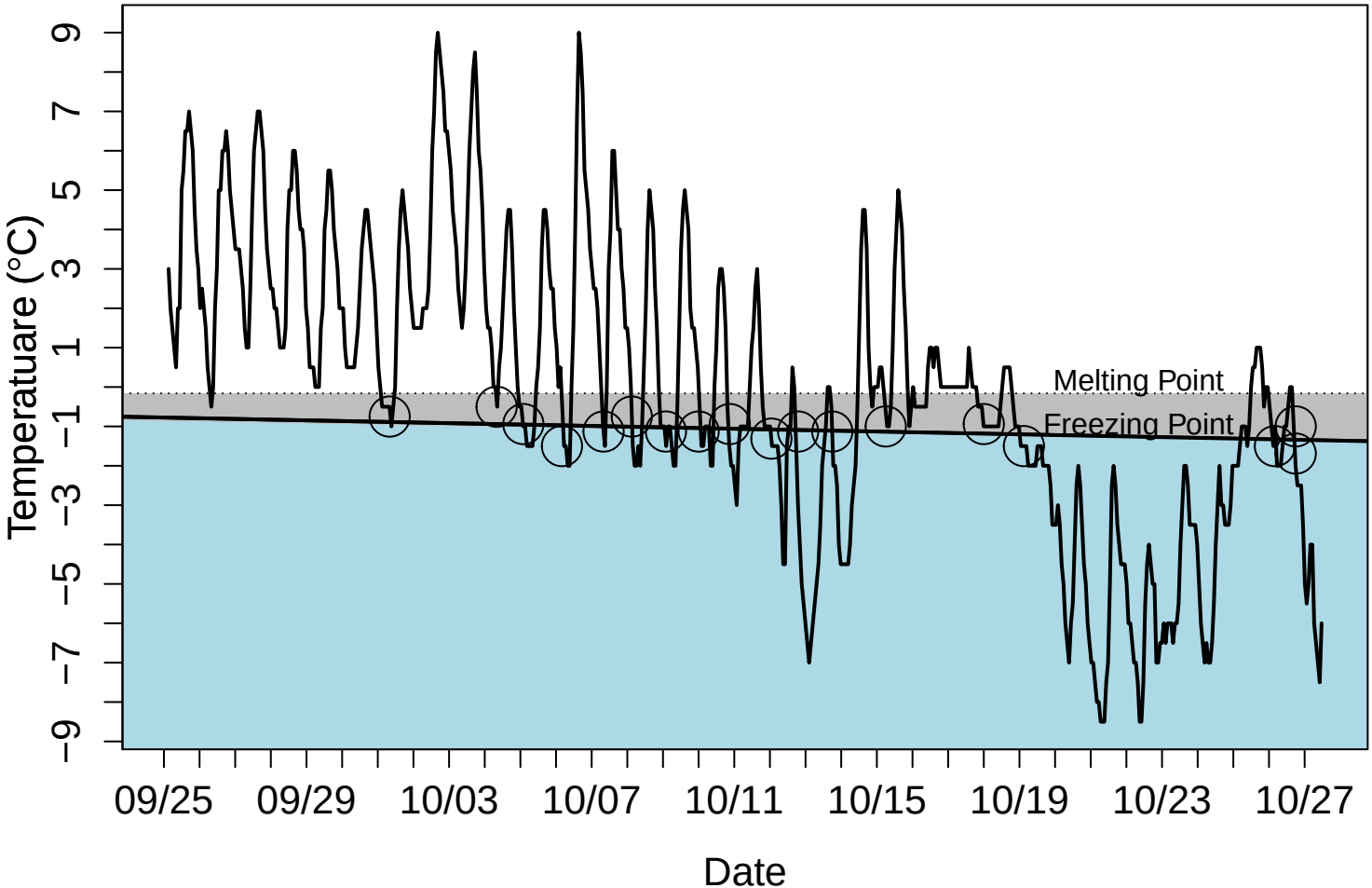

Temperatuare (°C)
15
5
0
−10
−20
−30
−40
Snow Depth (cm)
125
50
0
Melting Point
Freezing Point
Soil Mean
Soil Variance
Soil Variance
Air
Snow Depth
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
Date


Temperatuare (°C)
15
5
0
−10
−20
−30
−40
Snow Depth (cm)
125
50
0
Melting Point
Freezing Point
Soil Mean
Soil Min
Soil Max
Air
Snow Depth
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Date


Temperatuare (°C)
9
7
5
3
1
−1
−3
−5
−7
−9
Melting Point
Freezing Point
09/25
09/29
10/03
10/07
10/11
10/15
10/19
10/23
10/27
Date

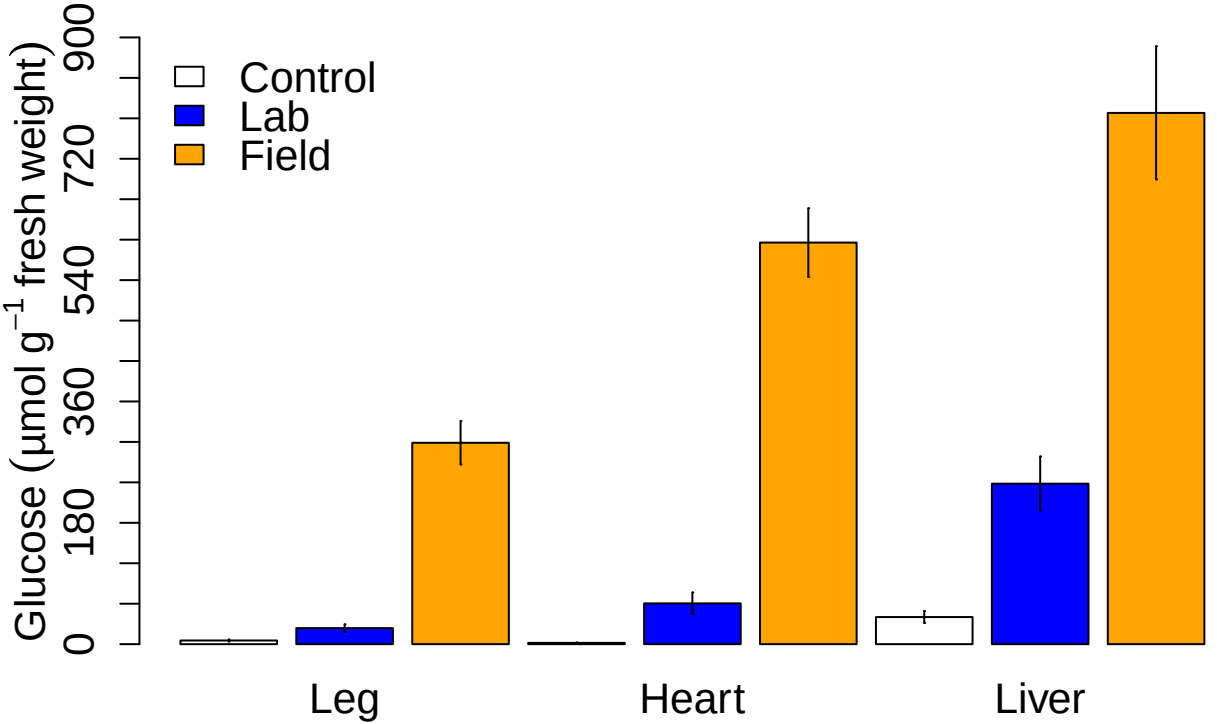

Glucose (µmol g^{-1} fresh weight)
0
180
360
540
720
900
Control
Lab
Field
Leg
Heart
Liver

Figure 6