Overwintering Strategies of *Ascaphus montanus*, the Rocky Mountain Tailed Frog

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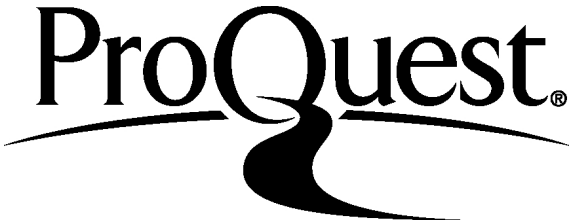

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CHAPTER I

Literature Review

Freeze Biology

Ectotherms have a reduced capacity to control their internal body temperature. This lack of internal control requires organisms to minimize extremes in their body temperatures through external means when environmental conditions are unfavorable (*e.g.*, finding suitable microhabitats). Freezing is a threat to their survival in polar and temperate regions where external temperatures often fall below freezing. Freeze stress has several components. First, there is the physical danger of ice crystals themselves. Ice crystals, even at a microscopic level, are sharp and can rupture membranes, destroying cells (*1*). Second, ice crystals alter the chemical environment. Ice formation outside of the cell removes water from the interstitial fluid as water molecules are incorporated into the crystalline structure, leaving behind solutes and thus creating hyperosmotic conditions that dehydrate cells (*2*). Lastly are physiological effects. External respiration ceases after freezing, leading to anoxic conditions in the cells (*3*). Organisms can respond to freezing stress through three strategies: 1) avoiding it (freeze avoidance); 2) supercooling their tissues (freeze resistance); or 3) tolerating it (freeze tolerance).

Freeze avoidance is the behavioral response of the organism to relocate itself from a microhabitat that may experience subzero temperatures. This removal is sometimes over very large scales, like the north-south migration of Monarch butterflies over an entire continent, or it can be over much smaller scales, such as aquatic organisms choosing the bottom of a pond as opposed to higher strata. The success of freeze avoidance is dependent

on the new microhabitat remaining at temperatures above freezing, and it comes with the energetic cost of moving to a new location.

Animals may avoid freezing at subzero temperatures by lowering the freeze point of an organism's tissues. Through the use of osmolites and anti-freeze proteins, an organism can depress the freezing point of its tissue, providing protection against freezing. Osmolites reduce the melting point of a substance by -1.86°C per osmole and can reduce the supercooling point between 3-6 times. Supercooling is the ability of small volumes of liquid to remain unfrozen below the melting point of the substance.

Antifreeze proteins (AFP) work by lowering the freezing point non-colligatively, (*i.e.* the protection is not based on the concentration of the compound itself; Jorov et al. 2004). However the primary function of this strategy is prevent ice crystal growth. Antifreeze proteins interact with the ice face of existing crystals and prevent additional water molecules from attaching to the ice matrix (*4*). Thus, avoiding ice inoculation and preventing the animal from freezing. In addition, this creates the thermal hysteresis phenomenon of AFPs because the freezing temperature is depressed, however the melting temperature is not, so a disconnect between the two temperatures is observed.

In addition, to supercool, an animal must be able to resist inoculative freezing Supercooling depends on the separation of the internal water from outside ice nucleators. Ice nucleators can be a variety of substances: sand, bacteria, and ice (*5*). Ice nucleation propagates ice quickly by reducing the energy necessary for the water molecules to interact with each other during crystal formation (*6*).

Many fish species found in polar regions use supercooling as a strategy by utilizing AFPs. Polar fish must use AFPs because the water temperature (-2°C) is below the melting point of the fish's fluids. Therefore any ice that may come in contact with the fish, either on

gills or through its digestive tract will propagate ice throughout the fish's body. So fish use AFPs to stop the propagation of ice. AFPs were first described in an Antarctic fish (*Trematomus spp.*) when the osmotic freezing protection was not enough to explain their ability to remain supercooled (*7*). An Antifreeze Glycoprotein (AFGP) was isolated that explained 30% of the freezing protection at concentration 6% w/v (*8*).

Although AFGPs were the first peptides described to have antifreeze properties, there are several more variants from a diverse array of taxa. Anti-Freeze Glycoproteins are distinguished from the other AFPs by Alanine-Alanine-Threonine repeats attached to a disaccharide (*9*). It is not only found in the Antarctic nototheniod fish but in Northern Cod (*Gadus morhoa*) as well (*9*). Type I AFP is characterized by an Alanine rich α-helix and is found in Winter Flounder (*Pseudopleuronectes americanus*) and in Shorthorn Sculpin (*Myoxocephalus scorpius*) (Hew et al. 1985; Sicheri and Yang 1995; Harding et al. 2003). Type II AFP is used by sea raven (*Hemitripterus americanus*) and Pacific herring (*Clupea pallasii*) and is characterized by being much larger (x3) and disulfide-bonded (*9*, *12*, *13*). Type III AFP is found in a diverse array of taxa including the wolf fish (*Anarhichas spp.*) and several genera of Zoarcidae (eel pout; (*9*, *14*, *15*). Type IV AFP is characterized by alanine-rich α-helix bundles and is found in shorthorn sculpin (*Myoxocephalus scorpius*; Deng and Laursen 1998; Harding et al. 2003).

Though AFPs are not uncommon in polar fish none have been reported in amphibians. Supercooling is not thought to be a viable strategy in amphibians due to their moist and thin skins. Thin skin allows ice to propagate across membranes and inoculate the coelom.

Freeze tolerance is the ability to survive the freezing of the interstitial fluid. When this occurs, the body appears to be frozen and dead. The location of ice formation is

controlled by manipulating the concentration of water in a cell through membrane control and cryoprotectants (*17*, *18*). Common cryoprotectants include glucose, urea, glycerol, and alanine (Storey and Storey, 2004). Cryoprotectants prevent freeze damage to a cell by changing the freezing point equilibrium (FPE). This accomplishes several goals: 1) removing water in the cell reduces the probability water will freeze inside the cell; 2) increasing the probability water will freeze outside the cell; and 3) controlling water loss, preventing catastrophic loss of water. Organisms may use varying combinations of cryoprotectants, but all four are typically not found in a single species.

The first freeze tolerant vertebrates described were reptiles, specifically European wall lizard (*Podarcis muralis*). Research demonstrated that these lizards could survive 28% of the total body water frozen (*19, 20*). Moreover, Voituron *et al.* (2002) demonstrated that the European common lizard (*Zootoca vivipara*) was also freeze tolerant, withstanding up to 50% of its water frozen by increasing blood glucose levels. Red-sided garter snakes (*Thamnophis sirtalis*) can survive brief periods of freezing of up to 50% of total body water using glucose and a large free amino acid pool as cryoprotectants (*21*). These adaptations are likely for frost protection during the late fall and early spring, as in the winter the snakes are in underground hibernacula and are not exposed to freezing conditions.

Painted turtle (*Chrysemys picta*) hatchlings are freeze tolerant (Packard and Packard, 1995). This species has a large range extending from southern Canada to the Gulf of Mexico, and from the Atlantic to the Pacific Ocean. Like many organisms its ability to survive freezing is associated with latitude (*22*). Hatchlings overwinter in nests which are underground and are too shallow to avoid the frost line. Costanzo et al. (2001) showed that these turtles are able to withstand temperatures below -4°C for over a week. They do this by increasing their glucose levels. Other turtles have been shown to survive freezing as well.

Red-eared slider (*Trachemys scripta elegans*) hatchlings have a similar life history to painted turtles, as the eggs hatch in the fall and the hatchlings overwinter in the nest. They also exhibit freeze tolerance (*21*). The major cryoprotectant used was glucose. Box turtles (*Terrapene carolina*) are also freeze tolerant and at 300 grams, represent one of the largest vertebrates to do so (*23*). Adult turtles hibernate in shallow burrows on the forest floor of the eastern and central United States. Box turtles can survive for over 60 hrs frozen with up to 60% of their total body water frozen.

Amphibians are nearly cosmopolitan and are found in a wide array of environments, from tropical rainforests to the driest deserts. Salamanders generally overwinter underground below the frost line (*24*). Cold adaptation is therefore not well studied in salamanders and only Siberian salamander (*Hynobius keyserlingi*) stands out as freeze tolerant. A Siberian salamander was found in the permafrost and was thawed out. After six months it was sacrificed and the carbon in its body was dated to 96 years earlier (*25*).

It is generally thought that anuran skin, which is highly permeable to water, may make them prone to innoculative freezing (Lee and Costanzo, 1998), in which ice crystals found outside the body initiate ice crystal formation in the body. This innoculative freezing susceptibility prevents the use of supercooling as a strategy. While no frog has been found to use supercooling as an overwintering strategy members of two families have shown at least some freeze tolerance, the family Hylidae, including: spring peeper (*Pseudacris crucifer*), Eastern gray tree frog (*Hyla chrysosceleis*), Cope's gray tree frog (*Hyla versicolor*), Western chorus frog (*Pseudacris maculate*) Southern brown frog (*Litoria ewingi*), and the family Ranidae, including: wood frog (*Lithobates sylvaticus*), the moor frog (*Rana arvalis*), European pool frog (*Pelophylax lessonae*), and European marsh frog (*Pelophylax ridibundus*) (Figure 1) (*26–33*). Most can survive being frozen at -2.5°C for over two weeks,

although the aquatic European pool frog (*P. lessonae*), European marsh frog (*P. ridibundus*) and their hybrid the edible frog (*Pelophylax esculentus*) only survive for a few hours frozen (Voituron et al, 2005).

Few comparative studies have been done on freezing biology. Freeze tolerance in frogs was derived several times throughout anuran history (Figure 3). The preadaptation for freeze tolerance coincide with the adaptations for a more terrestrial lifestyle (*34–36*). The ability to withstand desiccation is the most common preadaptation mentioned because of the role osmotic stress plays in the response to freezing. It is therefore not surprising that the majority of freeze tolerant frogs are terrestrial (Figure 3, *35*). Ranid frogs have evolved freeze tolerance several times in their history. The wood frog evolved freeze tolerance and then separately the moor frog and the Pelophylax complex did as well. Pelophylax is not very good at freezing however. By mapping freeze tolerance on a phylogenetic tree of frogs tested for freeze tolerance one can hypothesize that loss of freeze tolerance has likely happened in some species (Figure 3). The Northern cricket frog (*Acris crepitans*) and the Pacific tree frog (*Hyla regilla*) are similar in habitat and life history to the rest of their family. *Hyla regilla* are less freeze tolerant than there family members but still are able to survive several (8-12) hours frozen(*37*). *Acris crepitans* occupy a range where freezing occurs and have been found to hibernate in terrestrial sites (*38*). *Acris crepitans* does not however have any ability to survive freezing(*39*). What led to this loss is unknown. Surprisingly, bufonid species are especially good at controlling and surviving water loss, yet none in the group have been found to be freeze tolerant.

Ascaphus montanus

Ascaphus spp have been used in locomotion and behavioral studies examining the evolution of frog jumping because *Ascaphus spp.* are basal frog species (Figure 1) (*40–42*).

Ascaphus montanus (Rocky Mountain tailed frog) is a member of the family Ascaphidae along with its sister species *Ascaphus truei* (coastal tailed frog). Ascaphidae forms a monophyletic clade (Amphicoela) with the New Zealand Frogs (Family: Leiopelmatidae; Roelants and Bossuyt 2005). All other frogs are members of Lalagobatrachia which separated from Amphicoela at least 185 mya (*43*).

All members of Amphicoela are generally cryptic and few studies have examined the physiology and metabolism of this group. However, several members of *Leiopelma* are endangered and ecological niche models and habitat use models have been constructed for *Leiopelma hochstetteri* (*44*, *45*). These frogs seem to prefer similar habitat to that of *Ascaphus montanus*; cool, first order streams with coarse subtrates (cobbles and boulders; *40*). In addition, these frogs share many behavioral characteristics. Both walk more than jump and have similar foraging strategies (*46*).

Ascaphus montanus is native to the interior Pacific Northwest (Figure 2; *42*)). *Ascaphus montanus* eggs are generally deposited in paired strings under large boulders in July, emerging as tadpoles in late September through October. *Ascaphus* tadpoles either overwinter in nest sites or emerge and begin to feed on diatoms dependent on the latitude, elevation and climatic conditions the frogs experience (*48*). During the day larvae hide under rocks (Personal Observation) and at night come up to the top to presumably feed on diatoms on the rock face. After several years (2-3) the tadpole metamorphoses into a froglet and becomes reproductively mature after an additional four (males) to five (females) years (*49*). Adults are generally crepuscular (Personal Observation) and may live up to 20 years (*49*).

Little physiological data have been reported on *A. montanus*. Some comparative studies have examined the Critical Thermal Maxima (CTMax). As compared with the Pacific

Tree Frog, *Hyla regilla*, (29.6 °C) the CTMax of *A. montanus* (27.6°C) was significantly lower (*50*). Tadpoles also have a low CTMax. Where 53% of second year tadpoles and 75% of 1 year tadpoles died at 18 °C (*51*). Other studies have examined desiccation tolerance. An adult expired after losing only 28% of body mass to desiccation, which is proportionally less mass than 28 of 30 species of frog compared (Claussen 1973; Hillman et al. 2000). No data have been collected on the basal metabolic rates of *A. montanus.* Nor have data been collected on critical thermal minimums. However, *A. montanus* have been observed jumping, swimming and copulating at low temperatures (2°C, Personal Observation) and have been found in streams that were supercooled to -1°C (*54*).

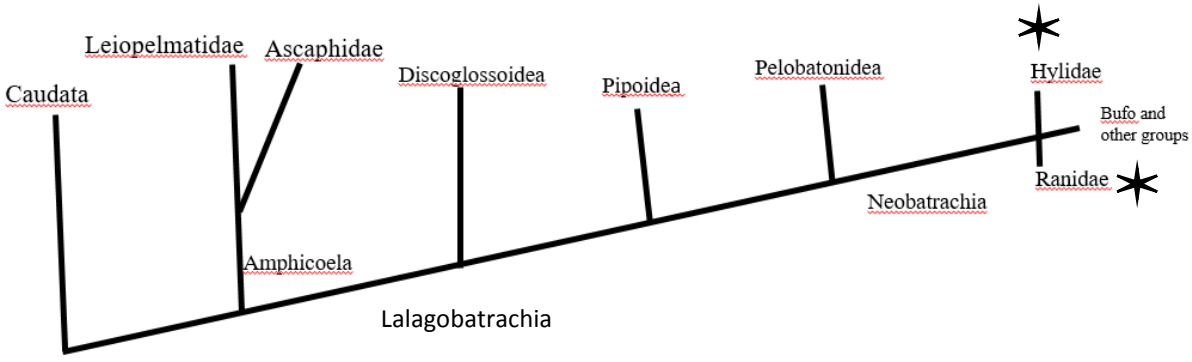

Figure 1: Simplified phylogeny of Anurans with freeze tolerance. Phylogeny is modified from Roelants and Bossuyt, 2006 with freeze tolerance represented by snowflakes with groups that have exhibited freeze tolerance.

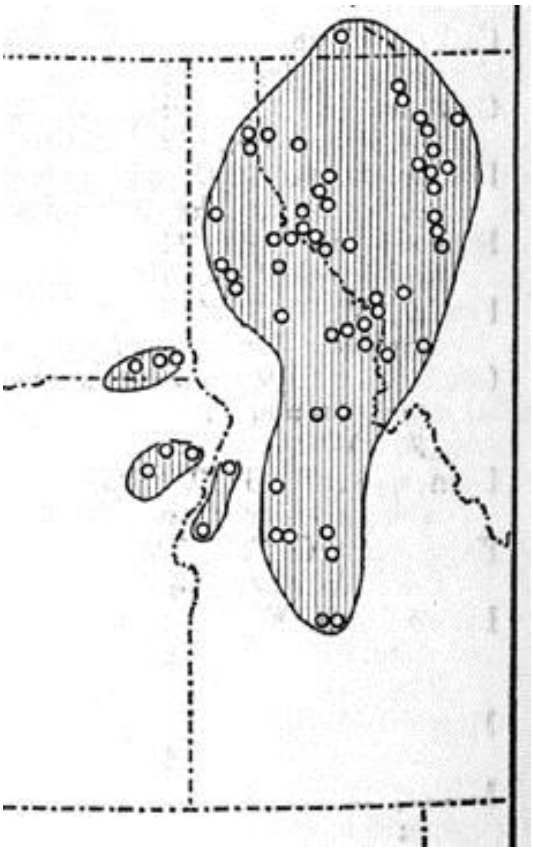

Figure 2: Range Map of *Ascaphus montanus.* Modified from Metter 1972.

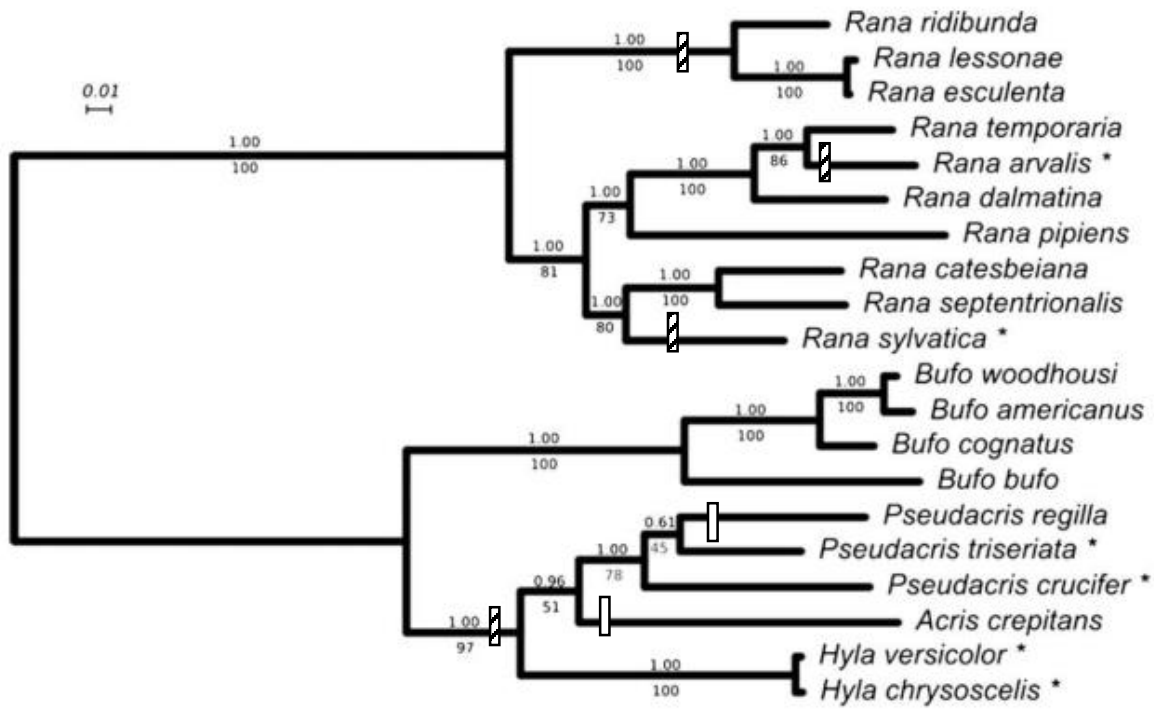

Figure 3: Phylogeny of species tested for freeze tolerance. from Voituron et al 2009 based on Bayesian inference and partial 12S, 16S rDNA, valine and cytochrome b. Freeze tolerant species are marked with stars. Freeze tolerance appearance is marked with dashed hash marks when it evolved and blank hash marks represent losses of freeze tolerance.

CHAPTER II

EXPERIMENT

Introduction

As ectotherms, body temperature of amphibians rarely varies from ambient, and as such, they must be able to minimize potential harmful effects of extreme environmental temperature. Responses to temperature may be most profound for amphibians living in temperate and polar regions during winter. Most amphibians behaviorally avoid low winter temperatures by overwintering in thermally buffered habitats. For instance, amphibians frequently hibernate on the bottom of bodies of water where temperatures rarely fall below 4°C, while some terrestrial amphibians overwinter underground below the frost line (*24*, *56*). In contrast, a small subset of terrestrial amphibian species overwinter in microhabitats such as leaf litter that are exposed to subzero temperatures, and are able to survive by being freeze tolerant (*26–29*, *57*). Freeze tolerance occurs only in a few highly derived species of terrestrial amphibians, with little record of this strategy being used in aquatic or semi-aquatic species (*35*)

The biggest threat with low temperature is potential for freezing and the stresses associated with ice formation. Intracellular ice formation is lethal to all but a few cell types. Therefore to survive freezing, ice formation must be maintained extracellularly. However, ice formation outside of the cell removes water from the interstitial fluid as water molecules are incorporated into the crystalline structure, leaving behind solutes and thus creating hyperosmotic conditions that dehydrate cells (*2*). Osmotic cellular dehydration is considered the primary cause of freeze-induced damage (*2*). In addition, oxygen is excluded and circulation is stopped upon freezing, thus cells of freeze tolerant organisms must be able to

resist anoxic conditions. Lastly, ice crystals are sharp at a microscope level and can rupture membranes, destroying cells (*22*).

Freeze tolerant organisms typically produce high concentrations of molecules termed cryoprotectants. Common cryoprotectants include glucose, urea, glycerol, and alanine (Storey and Storey, 2004). Cryoprotectants prevent freeze damage to a cell by changing the freezing point equilibrium (FPE). In addition to controlling the dehydration of cells, the FPE dictates the amount of ice that is produced and how large the crystals grow. The array of cryoprotectants differs among species. *Lithobates sylvatica* (wood frog) uses glucose (411±60 mmol l^{-1}plasma, a 400 fold increase) and urea (56.5 ± 3.2 μmol g^{-1} liver, no increase due to freezing) as cryoprotectants whereas *Hyla versicolor* utilizes glycerol (16.3 ± 6.8 μmol ml^{-1} plasma, 160 fold increase) and glucose (25.9 ± 11.6 μmol ml^{-1} plasma, a 25 fold increase) (*58*).

Ascaphus montanus is a semi-aquatic frog that overwinters in cold, shallow, and swift mountain streams in the western Rocky Mountains. It is a cryophile that is well adapted to the cold environment and summer animals willdie at temperatures above 20°C (Clausen, 1973). In winter, *A. montanus* may encounter freezing conditions in the mountain streams in which it resides. Due to their high flow rate, these streams can become supercooled to -1°C (*54*). That temperature is below the normal freeze point of vertebrates (*59*). *Ascaphus montanus* is likely at risk of freezing in these streams not only because of the low temperature of the stream but also because they likely physically contact ice and ice nucleating agents such as sand, and mineral crystals in the environment. *A. montanus* has high site fidelity and resides within a neighborhood of only 350m which makes it unlikely that they migrate to avoid these conditions (*49, 60*). In conjunction with these risks *A.*

montanus is long-lived (20+ year lifespan) which requires a high survivorship (*61*). Therefore, *A. montanus* must use some strategy to respond.

A. montanus has been used in locomotion and behavioral studies examining the evolution of frog jumping (*40*, *41*), because it is thought to retain ancestral morphological and behavioral characters as the sister group to all other frogs Lalagobatrachia. These groups separated nearly 185 mya (*43*). The identification of freeze tolerance in *A. montanus* would demonstrate that this phenomenon is not restricted to Ranidae and Hylidae in Neobatrachia, but could occur throughout the anuran phylogeny. In addition, due to *A. montanus*' cold-adapted biology it could be the first frog found to primarily utilize supercooling as a strategy.

The purpose of this study is to determine the potential overwintering strategies of *A. montanus*. Ice formation is stochastic. Therefore, we examined not only the supercooling point and resistance to ice inoculation but the ability to remain supercooled, as well. We also examined the ability to survive freezing. After determining the ability to survive freezing we quantified spectrophotometrically the production of several well described cryoprotectants, including glucose, glycerol and urea.

Materials and Methods

Freeze tolerance/supercooling

Collection

Ascaphus montanus individuals were collected in Northern Idaho from May-June 2013 and June 2014, and transported to the Southern Illinois University Edwardsville (SIUE) campus within a week of capture (Essner et al. 2012). Individuals were housed in an enclosure consisting of two large cattle tanks with a continuously operating pump and water

chiller on an aquarium timer set to maintain a temperature of 10°C (Essner et al. 2014). Frogs were fed with fortified live crickets *ad libitum*.

Acclimation

To mimic seasonal conditions, water temperature of the enclosure was lowered from 10° C to 5°C, stepwise over a seven day period. Animals were held at this temperature for one month during which live crickets were fed to the animals *ad libitum* until the frogs ceased feeding. After one month at 5 °C the temperature was dropped to 2°C in a stepwise fashion over 4 days while the photoperiod was reduced from 12L/12D to an 8L/16D to mimic conditions at collecting sites near Sandpoint, Idaho (48.2667° N, 116.5667° W), in the Fall/Winter. Frogs were held at these conditions for three months prior to the beginning of the experiment.

Experiment

In May 2014, frogs were removed from their enclosure, blotted dry, weighed and placed in 50 ml Falcon tubes. Thermocouples were inserted into the tubes and held adjacent to the frogs with foam plugs. Tubes containing frogs were partially submerged in a temperature controlled alcohol bath and cooled from 2 °C at 0.2 °C h^{-1} until they spontaneously froze (i.e. supercooling point; n=3). Two additional groups were cooled at the above rate until reaching, and being held at -1 °C. Frogs maintained at -1 °C were either held at this temperature for seven days to determine length of time they could remain supercooled (n=4) or had 500 mg of ice placed on their skin to determine how well they could resist innoculative freezing (n=4). This was quantified by recording the length of time until an exotherm was recorded. An exotherm is the increase in temperature from the heat fusion being released during the crystallization of ice. After the last frog froze in the supercooling point determination trial, or after being held at -1 °C for seven days, all frogs were warmed at

0.2 °C h^{-1} until reaching 2°C. They were then removed from the falcon tube and placed in an incubator at 2°C with access to water. Survival was determined by assessing righting reflex 24 h after removal from the alcohol bath.

Metabolite Analysis

Collection/acclimations

To determine cryoprotectant concentrations in response to freezing, frogs were collected in Northern Idaho in June of 2014, brought to and held at the SIUE campus as described above. These frogs were subjected to the same acclimation regime as described above, however the acclimation procedure was initiated in November.

Experiment

On March 2015, frogs (n=3) were removed from their enclosure, blotted dry, weighed and placed in 50 ml Falcon tubes. Thermocouples were inserted into the tubes and held adjacent to the frogs with foam plugs. Tubes containing frogs were partially submerged in a temperature controlled alcohol bath and cooled from 2 °C at 0.2 °C h^{-1} until reaching, and being held at -1 °C. Frogs maintained at -1 °C either froze spontaneously or had a small ice chip placed on their back to inoculate freezing after 3 days. After being held at -1 °C for seven days, frogs were removed, double pithed, and the gracilis and gastrocnemius muscles, heart, and liver were removed on ice. Water content of the liver was determined after a portion of the tissue was blotted dry, weighed and dried in an oven at 60 °C. The remaining portions of the liver and all other tissues were immediately frozen in liquid nitrogen and placed in cold storage (-80°C). Control frogs (n=3) were subjected to the same acclimatization procedure as experimental frogs but were held unfrozen at 2 C° until being processed in the same manner as described above.

Metabolite Analysis

Tissues were homogenized and deproteinated in chilled 6% perchloric acid and centrifuged for 6 min at 20900g at 4°C prior to neutralizing the supernatant with an equal part 6% KOH. The supernatant was spectrophotometrically tested for glucose, urea, and glycerol. Assay kits were used for urea and glucose (Sigma Aldrich GAK-20, MAK006). Glycerol was quantified using Sigma-Aldrich Free Glycerol Reagent (F6428) and standards were created from Glycerol Standard Solution (G7793). Statistical analysis included ANOVA followed by a Tukey's HSD *post-hoc* test and Welch's t-tests where appropriate

Results

Frogs that were inoculated had an average time to freeze of 16.25 ± 4.50 minutes with no time going above 30 minutes (Table 1). No frog survived freezing from inoculation (Table 1). The supercooling point average was -5.02 ± 0.14°C with a point no higher than -4.80°C (Table 2). No frog survived freezing from supercooling point determination (SCP; Table 2). Frogs held at -1°C froze on average 72.9 ± 22.1 hours ranging from 48.2 – 139 hours (Table 3). However, two of the four frogs survived freezing, days after an exotherm was recorded (Table 3). Survival after freezing in the SCP determination trial was not expected due to the low temperature and high rate of crystallization, thus preventing the ability to produce cryoprotectants (*64*). At low temperatures ice forms too quickly for the animals respond. Frogs do not generally respond to freezing until ice has inoculated them (*65*). During all trails frogs produced mucous, either in response to desiccation or temperature.

Glucose concentration did not significantly increase with freezing (Figure 4). Urea concentrations and glycerol levels in the liver were below the detection limit for the assays. Water content in the liver increased significantly after freezing by 40% (Figure 5).

Table 1: Mass, time until freezing (min) and survival of winter acclimated *A. montanus* adults that were cooled from 2 to -1°C at 0.2°C h^{-1}.

Frog	Mass	Time until Freezing (minutes)	Survival
1	5.92g	13	No
2	5.88g	24	No
3	3.53g	23	No
4	2.94g	5	No

Table 2: Mass, temperature at freezing and survival of winter acclimated *A. montanus* adults that were cooled from 2°C at 0.2°C h^{-1}.

Frog	Mass	Supercooling Point	Survival
1	3.57g	-4.99°C	No
2	2.70g	-5.27°C	No
3	3.33g	-4.80°C	No

Table 3: Mass, time until freezing (hours) and survival of winter acclimated *A. montanus* adults that were cooled from 2 to -1°C at 0.2°C h^{-1}.

Frog	Mass	Time until Freezing (Hours)	Survival
1	5.44g	48.2	Yes
2	3.16g	48.2	No
3	6.36g	56.0	Yes
4	3.50g	139.0	No

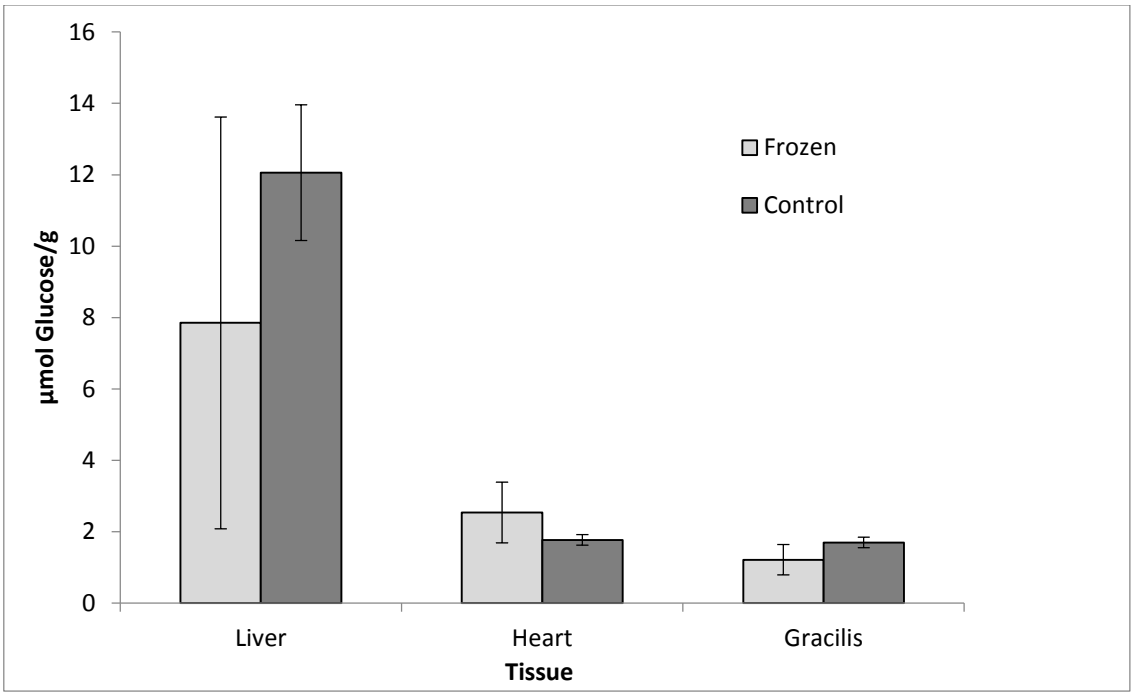

Figure 4: Mean ± SEM of glucose (µmols gram tissue^{-1}) of the liver, heart and gracilis muscle in *Ascaphus montanus* adults (n=3) that were cooled from 2 °C at 0.2 °C h^{-1} until reaching, and being held at -1 °C.. Differences among groups was significant ($p = 0.049$) but there was no significant difference between frozen and control ($p > 0.80$).

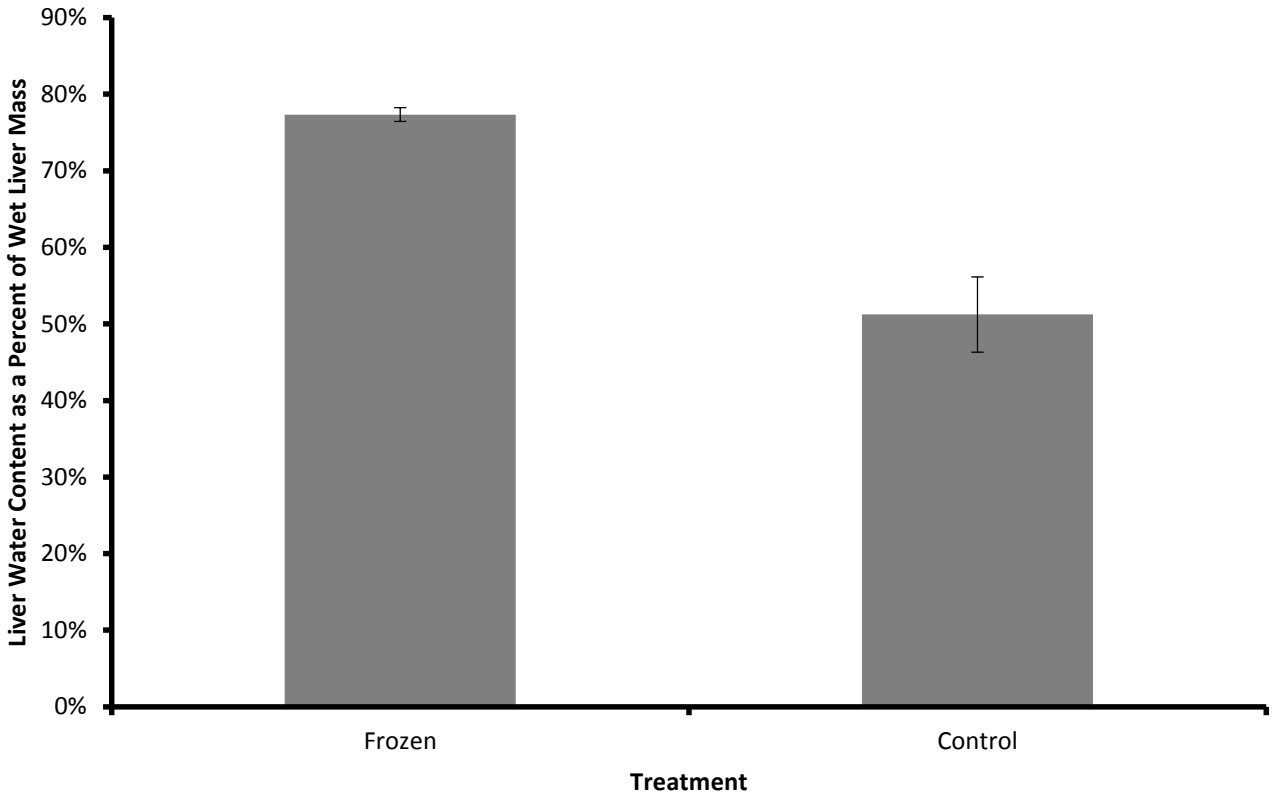

Figure 5: Mean ± SEM of water content of the liver as a percent of mass by treatment. Frozen *Ascaphus montanus* adults (n=3) were cooled from 2 °C at 0.2 °C h^{-1} until reaching, and being held at -1 °C. Water content increased significantly in frozen individuals compared to control individuals ($p = 0.006$)

Discussion

The purpose of this study was to determine the overwintering strategies of *A. montanus*. Therefore, we examined the supercooling ability of *A. montanus*, as well as their ability to survive freezing. Amphibians have complex life histories with differing stresses at each stage and individuals living in polar and temperate regions have the additional stress of winter. Wood frogs only live at most six years and most only mate once (*66*); whereas, *A. montanus* may not be reproductively mature until 8 years old and adults have been found in the wild at ages exceeding 15 years (*49*). Such longevity must necessitate high survivorship (*61*). Therefore, whatever the risk from freezing, *A. montanus* seems to be well-adapted.

Strategies for overwintering: Supercooling

Based on the results of this study, *Ascaphus montanus* likely uses a complex strategy utilizing both freeze resistance and freeze avoidance to survive the winters employing the low supercooling point (SCP), ability to remain supercooled and lack of chill coma (temperature at which an organism ceases movement) at freezing temperatures, allowing it to move to find a better microhabitat. The inability of *A. montanus* of to resist ice inoculation is not uncommon. This inability has been shown across anuran taxa and is likely due to the thin skin of frogs (Layne et al. 1990; Layne 1991, Swanson 1996). When compared to a species of similar size *A. montanus* had a much lower SCP at -5.02 ± 0.14°C. The Spring Peeper, *Pseudacris crucifer*, is around the same size as *A. montanus* (~ 3-5g) but has a SCP of only -1.98°C (*67*). Admittedly, the frogs that were tested for a supercooling point in the present study were relatively small. Smaller organisms are known to have lower SCPs because ice formation is a stochastic event and smaller organisms have less water and therefore are more likely to avoid ice formation (*68*). This does not explain the magnitude of the difference in SCPs as frogs that are much larger such as *Lithobates pipens*

and *L. sylvatica* have SCPs of approximately the same magnitude of *Pseudacris crucifer* (*67*). Another small frog that may use supercooling as a strategy are juvenile spadefoot toads, *Spea bombifrons* (*69*). Their SCP was -4.3 ± 0.7 °C. They were comparable to individuals in this study at 2.84 ± 1.08 g. However, they did not survive the freeze that followed. *Spea bombifrons* uses supercooling to avoid burrowing to excessive depth in order to save energy only burrowing lower when necessary to avoid freezing conditions (*69*).

Additionally, most frogs when encountering cold temperatures go into chill coma while *A. montanus* is responsive to stimuli until -3°C. (Personal Observation). The streams in which these frogs reside are between 2 and -1°C in the winter without anchor ice. (Bull and Carter, 1996) The lack of chill coma at freezing temperatures allows *A. montanus* to move once temperatures become dangerously low. The ability of *A. montanus* to remain supercooled for extended periods (72.9 ± 22.1 hrs) allows for the use of supercooling as a strategy to move to a more thermally equitable habitat (freeze avoidance). Although, *A. montanus* has a small home range (350m;(*49*) it has been found to move when temperatures are above its ideal range (*70*). However, the distance these frogs moved was not described.

Therefore it is possible *A. montanus*' low supercooling point may mean individuals avoid freezing through supercooling, using their basally elevated glucose as a cryoprotectant and their small size to prevent spontaneous freezing until they find a microhabitat above 0°C. The freeze tolerance described may represent an artifact of the elevated glucose levels and ability to function at low temperatures. .

Strategies for overwintering: Freeze Tolerance

It could be freeze tolerance in these animals is an adaptation to respond to unpredictable cold temperatures while foraging. The temperature can suddenly drop and the

frog would then freeze overnight and after when if it warms the frog can find refuge in the stream. *Ascaphus montanus* adults did not have high concentrations of measured cryoprotectants prior to or immediately after freezing. The concentrations of glucose in *A. montanus* are comparable to *L. sylvatica* that were injected with saline prior to freezing (*71*). However no individuals survived the -5 °C freeze that followed injection (*71*). Most frog species in response to freezing increase the concentration of glucose and other cryoprotectants. Two frogs that showed less freeze tolerance than *A. montanus*, *Pelophylax esculenta* and *Pelophylax lessonae*, significantly increased their glucose concentrations in response to freezing (41.9 ± 0.2 μmol g^{-1} and 73.7 ± 0.7 μmol g^{-1} respectively) and survived for less than 15 h (*31*). Like *A. montanus*, *Pelophylax ridibundus* did not significantly increase its glucose concentration (8.1 ± 0.2 μmol g^{-1}) in response to freezing and it survived only 20h after freezing (*31, 36*). The control glucoses concentration of *A. montanus* was approximately the same as *Lithobates pipens* frozen concentration(10.0 ± 0.8 μmol g^{-1}), although *L. pipens* is not freeze tolerant (*72*).

The difference between cryoprotectant levels only becomes more pronounced with freeze tolerant anurans. *L. sylvatica* glucose concentration (194 ± 16 μmol g^{-1}) in the liver is an order of magnitude above *A. montanus* (*73*). *P. triseriata* frozen for just 24h upregulated its liver glucose concentration over 3 fold (26.6 ± 4.6 μmol g^{-1} to 89.3 ± 18.3 μmol g^{-1}(*74*). These data indicate that if a frog is to be freeze tolerant it must increase its cryoprotectant load extensively. In that light, it seems unlikely that *A. montanus* utilizes only freeze tolerance as a strategy to survive.

This study shows that *Ascaphus montanus* adults have some ability to survive freezing and may have a novel overwintering strategy, suggesting that the ability to survive freezing may be found throughout the Anura. Most frogs previously studied relied on either

finding microhabitats buffered from freezing conditions or survived freezing. It seems *A. montanus* may do both. Harding and Quinn (2015) in their review of *Ascaphus spp.* literature mentioned gaps in knowledge of CTMax at various life history stages but did not include CTMin. This is vital to understand at least half of the frog's life. Winter last from November to May, six months out of the year these frogs may experience dangerous conditions but it is unknown what is dangerous if there is no data on CTmin.

I hypothesize that these frogs use their low supercooling point and ability to remain active at low temperatures to avoid freezing by moving to suitable microhabitat when necessary. By better understanding the physical environment these frogs live in we can predict the strategies that are more likely to be used.

Future work should focus on three factors: stream conditions, behavior, and physiology. To quantify the thermal gradient of the streams over time, researchers should begin by placing I-buttons along the stream in different habitats (e.g., riffles, seeps and pools). In addition the frog's movement patterns throughout the season could be quantified through the use of radio telemetry. Collaring frogs and then setting up static base radio stations along the stream would give detailed movement patterns at time intervals that have not been described. Combined with the I-button data this would give detailed microhabitat data which is essential when looking at the ecophysiology of organism (*75–77*).

Physiological experiments should attempt to repeat this study with more individuals, as well as check for increases in the free amino acid pool. In addition it would be helpful to examine glycogen levels before and after freezing to determine if glycogen was being mobilized into glucose. Other areas to examine are the genome for proteins associated with either freeze tolerance or supercooling, such as aquaporins, AFP, and ice nucleating proteins.

The mucus produced by the frogs should be tested for antifreeze proteins. In addition, all experiments should be repeated on the tadpoles as they are obligate stream users.

Snow cover acts as an insulator keeping the temperatures beneath the snow relatively warm compared to ambient. Climate change has already reduced snow cover (*78*). What effect this has on streams is still uncertain but Bull and Brown (1996) found ice in exposed portions of the stream. Although there was no anchor ice, *A. montanus*' inability to resist ice inoculation does not bode well for this species.

LITERATURE CITED

1. B. Storey, Biochemistry below 0C. *HSRS Brazilian J. Med. Biol. Res.* **29**, 283–307 (1996).
2. P. Mazur, Freezing of living cells: mechanisms and implications. *Am. J. Physiol.* **247**, C125–42 (1984).
3. J. A. MacDonald, K. B. Storey, Protein phosphatase type-1 from skeletal muscle of the freeze-tolerant wood frog. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **131**, 27–36 (2002).
4. A. Jorov, B. S. Zhorov, D. S. C. Yang, Theoretical study of interaction of winter flounder antifreeze protein with ice. *Protein Sci.* **13**, 1524–37 (2004).
5. M. R. Lee, R. E. Lee, J. M. Strong-Gunderson, S. R. Minges, Isolation of ice-nucleating active bacteria from the freeze-tolerant frog, Rana sylvatica. *Cryobiology*. **32**, 358–365 (1995).
6. K. E. Zachariassen, E. Kristiansen, Ice nucleation and antinucleation in nature. *Cryobiology*. **41**, 257–79 (2000).
7. G. N. Somero, A. L. Devries, Temperature Tolerances of Some Antarctic Fishes. *Science (80-.).* **156**, 257–258 (1967).
8. A. L. Devries, D. E. Wohlschlag, Freezing Resistance in Some Antarctic Fishes. *Science (80-.).* **163**, 1073–1075 (1969).
9. M. M. Harding, P. I. Anderberg, A. D. J. Haymet, "Antifreeze" glycoproteins from polar fish. *Eur. J. Biochem.* **270**, 1381–1392 (2003).
10. F. Sicheri, D. S. C. Yang, Ice-binding structure and mechanism of an antifreeze protein from winter flounder. *Nature*. **375** (1995), pp. 427–431.
11. C. L. HEW, S. JOSHI, N.-C. WANG, M.-H. KAO, V. S. ANANTHANARAYANAN, Structures of shorthorn sculpin antifreeze polypeptides. *Eur. J. Biochem.* **151**, 167–172 (1985).
12. J. A. Raymond, Freezing Resistance in Some Northern Populations of Pacific Herring, Clupea-Harengus-Pallasi. *Can. J. Fish. Aquat. Sci.* **46**, 2104–2107 (1989).
13. D. Slaughter, G. L. Fletcher, V. S. Ananthanarayanan, C. L. Hew, Antifreeze proteins from the sea raven, Hemitripterus americanus. Further evidence for diversity among fish polypeptide antifreezes. *J. Biol. Chem.* **256**, 2022–6 (1981).
14. J. D. Schrag, C.-H. C. Cheng, M. Panico, H. R. Morris, A. L. Deries, Primary and secondary structure of antifreeze peptides from arctic and antartic zoarcid fishes. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **915**, 357–370 (1987).
15. N. R. Le François, S. G. Lamarre, H. Tveiten, P. U. Blier, J. Bailey, Sperm cryoconservation in Anarhichas sp., endangered cold-water aquaculture species with internal fertilization. *Aquac. Int.* **16**, 273–279 (2007).

16. G. Deng, R. A. Laursen, Isolation and characterization of an antifreeze protein from the longhorn sculpin, Myoxocephalus octodecimspinosis. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **1388**, 305–314 (1998).

17. P. A. King, M. N. Rosholt, K. B. Storey, Adaptations of plasma membrane glucose transport faciliate cryoprotectant distribution in freeze-tolerant frogs. *Am. J. Physiol.* **265**, R1036–R1042 (1993).

18. J. J. P. Costanzo, M. C. F. do Amaral, A. J. Rosendale, R. E. Lee, Hibernation physiology, freezing adaptation and extreme freeze tolerance in a northern population of the wood frog. *J. Exp. Biol.* **216**, 3461–3473 (2013).

19. R. Weigmann, Die Wirkung starker Abkühlung auf Amphibien und Reptilien. *Z Wiss Zool*. **134**, 641–692 (1929).

20. D. L. Claussen, M. D. Townsley, R. G. Bausch, Supercooling and freeze-tolerance in the European wall lizard, Podarcis muralis, with a revisional history of the discovery of freeze-tolerance in vertebrates. *J. Comp. Physiol. B.* **160**, 137–143 (1990).

21. T. A. Churchill, K. B. Storey, Responses to Freezing Exposure of Hatchling Turtles. *J. Exp. Biol.* **233**, 221–233 (1992).

22. J. M. Storey, K. B. Storey, *Cold hardiness and freeze tolerance* (2004).

23. J. P. Costanzo, D. L. Claussen, Natural freeze tolerance in the terrestrial turtle, Terrapene carolina. *J. exp. Zool.* **254**, 228–232 (1990).

24. R. a Grizzell, The Hibernation Site of Three Snakes and a Salamander. *Copeia*. **1949**, 231–232 (1949).

25. N. Shcherbak, N. Kovalyukh, Age of a Living Amphibian (Hynobius keyserlingi) Dug out from Ice. *Doklady*. **211**, 359–360 (1973).

26. J. R. Layne, J. Kefauver, Freeze tolerance and postfreeze recovery in the frog Pseudacris crucifer. *Copeia*. **1997**, 260–264 (1997).

27. J. R. Layne, R. E. Lee, Seasonal variation in freeze tolerance and ice content of the tree frog Hyla versicolor. *J. Exp. Zool.* **249**, 133–137 (1989).

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

29. D. . MacArthur, J. W. . Dandy, Physiological Aspects of Overwintering in the Boreal Chorus Frog (Pseudacris triseriata maculata). *Comp. Biochem. Physiol.* **72**, 137–141 (1982).

30. K. B. Storey, Freeze tolerance in the frog,Rana sylvatica. *Experientia*. **40** (1984), pp. 1261–1262.

31. Y. Voituron, P. Joly, M. Eugene, H. Barre, Freezing tolerance of the European water frogs: the good, the bad, and the ugly. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, 1563–1570 (2005).

32. R. L. Essner, D. J. Suffian, P. J. Bishop, S. M. Reilly, Landing in basal frogs: evidence of saltational patterns in the evolution of anuran locomotion. *Naturwissenschaften*. **97**, 935–939 (2010).

33. K. Rexer-Huber, P. J. Bishop, D. A. Wharton, Field ecology of freezing: Linking microhabitat use with freezing tolerance in *L itoria ewingii*. *Austral Ecol.* **40**, 933–940 (2015).

34. D. L. Swanson, B. M. Graves, K. L. Koster, Freezing tolerance/intolerance and cryoprotectant synthesis in terrestrially overwintering anurans in the Great Plains, USA. *J. Comp. Physiol. B.* **166**, 110–119 (1996).

35. Y. Voituron, H. Barre, H. Ramlov, C. J. Douady, Freeze tolerance evolution among anurans: Frequency and timing of appearance. *Cryobiology*. **58**, 241–247 (2009).

36. Y. Voituron, N. Mouquet, C. de Mazancourt, J. Clobert, To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. *Am. Nat.* **160**, 255–270 (2002).

37. S. A. Croes, R. E. Thomas, Freeze Tolerance and Cryoprotectant Synthesis of the Pacific Tree Frog Hyla regilla. *Copeia*. **2000**, 863–868 (2000).

38. R. H. Gray, Fall Activity and Overwintering of the Cricket Frog (Acris crepitans) in Central Illinois. *Copeia*. **1971**, 748–750 (1971).

39. J. T. Irwin, J. P. Costanzo, R. E. Lee, Jr., Terrestrial hibernation in the northern cricket frog, *Acris crepitans*. *Can. J. Zool.* **77**, 1240–1246 (1999).

40. R. L. Essner, D. J. Suffian, P. J. Bishop, S. M. Reilly, Landing in basal frogs: evidence of saltational patterns in the evolution of anuran locomotion. *Naturwissenschaften*. **97**, 935–939 (2010).

41. S. M. Reilly, R. L. Essner Jr., S. Wren, L. Easton, Bisho, Movement patterns in leiopelmatid frogs: Insights into the locomotor repertoire of basal anurans. *Elsevier B.V.* **121**, 43–53 (2015).

42. C. Gissi, D. San Mauro, G. Pesole, R. Zardoya, Mitochondrial phylogeny of Anura (Amphibia): a case study of congruent phylogenetic reconstruction using amino acid and nucleotide characters. *Gene*. **366**, 228–37 (2006).

43. K. Roelants, F. Bossuyt, Archaeobatrachian paraphyly and pangaean diversification of crown-group frogs. *Syst. Biol.* **54**, 111–26 (2005).

44. A. Fouquet, G. F. Ficetola, A. Haigh, N. Gemmell, Using ecological niche modelling to infer past, present and future environmental suitability for Leiopelma hochstetteri, an endangered New Zealand native frog. *Biol. Conserv.* **143**, 1375–1384 (2010).

45. E. Nájera-Hillman *et al.*, Habitat-use model for the New Zealand endemic frog Leiopelma hochstetteri. *Endanger. Species Res.* **9**, 23–31 (2009).

46. S. M. Reilly, R. L. Essner Jr., S. Wren, L. Easton, Bisho, Movement patterns in leiopelmatid frogs: Insights into the locomotor repertoire of basal anurans. *Elsevier B.V.* **121**, 43–53 (2015).

47. D. E. Metter, A Morphological and Ecological Comparison of two Populations of the Ascaphus truei Stejneger ’. *Copeia*. **1964**, 181–195 (1964).

48. M. P. Hayes, T. Quinn, “Review and Synthesis of Literature on Tailed Frogs (genus Ascaphus) with Special Reference to Managed Landscapes” (Olympia, WA, 2015).

49. C. H. Daugherty, A. L. Sheldon, Age-determination, growth, and life history of a Montana population of the tailed frog (Ascaphus truei). *Herpetologica*. **38**, 461–468 (1982).

50. D. L. Claussen, The Thermal Relations Of The Tailed Frog , Ascaphus truei , And The Pacific Treefrog , Hyla regilla. *Comp. Biochem. Physiol.* **44A**, 137–153 (1973).

51. B. R. Hossack *et al.*, Population-level thermal performance of a cold-water ectotherm is linked to ontogeny and local environmental heterogeneity. *Freshw. Biol.* **58**, 2215–2225 (2013).

52. D. L. Claussen, The Water Relations of the Tailed Frog , Ascaphus truei , and the Pacific Treefrog , Hyla regilla. *Comp. Biochem. Physiol.* **44A**, 155–171 (1973).

53. S. S. Hillman *et al.*, Correlation of Ventricle Mass and Dehydration Tolerance in Amphibians. *Herpetol. ’ Leag.* **56**, 413–420 (2000).

54. E. L. Bull, H. A. Brown, Winter Observations of Tailed Frogs in Northeastern Oregon. *Northwest. Nat.* **77**, 45–47 (1996).

55. D. E. Metter, *Catalogue of American amphibians and reptiles* (American Society of Ichthyologists and Herpetologists, New York, 1972).

56. A. R. Emery, A. H. Berst, K. Kodaira, Under-ice observations of wintering sites of leopard frogs. *Copeia*. **1972**, 123–126 (1972).

57. K. B. Storey, J. M. Storey, Freeze Tolerance and Intolerance as Strategies of Winter Survival in Terrestrially-Hibernating Amphibians. *Comp. Biochem. Physiol.* **83**, 613–617 (1986).

58. J. M. Storey, K. B. Storey, Adaptations of metabolism for freeze tolerance in the gray tree frog, Hyla versicolor. *Can. J. Zool.* **63**, 49–54 (1985).

59. K. B. Storey, Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am. J. Physiol.* **258**, R559–R568 (1990).

60. D. Hedgecock, Population subdivision and genetic divergence in the Red-belliew Newt, Taricha rivularis. *Evolution (N. Y).* **32**, 271–286 (1978).

61. G. D. Sutherland, thesis, University of British Columbia (2000).

62. R. L. Essner Jr. *et al.*, A Technique for Field Maintenance and Transport of Cold-water Amphibians. *Herpetol. Rev.* **43**, 247–249 (2012).

63. R. L. Essner Jr., M. E. Jorgensen, B. W. Ringer, S. J. Wright, S. M. Reilly, An Improved Husbandry Setup for Cold-water Amphibians. *Bull. Chicago Herpetol. Soc.* **49**, 24–27 (2014).

64. J. Layne *et al.*, Inoculation triggers freezing at high subzero temperatures in a freeze-

tolerant frog (Rana sylvatica) and insect (Eurosta solidaginis). *Can. J. Zool.* **68**, 506–510 (1990).

65. J. P. Costanzo, R. E. Lee, Biophysical and physiological responses promoting freeze tolerance in vertebrates. *News Physiol. Sci.* **9**, 252 (1994).

66. K. a. Berven, Density Dependence in the Terrestrial Stage of Wood Frogs: Evidence from a 21-Year Population Study. *Copeia*. **2009**, 328–338 (2009).

67. W. Schmid, Survival of frogs in low temperature. *Science (80-.).* **215**, 697–698 (1982).

68. R. E. Lee, J. P. Costanzo, Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu. Rev. Physiol.* **60**, 55–72 (1998).

69. D. L. Swanson, B. M. Graves, Supercooling and Freeze Intolerance in Overwintering Juvenile Spadefoot Toads (Scaphiopus bombifrons). *J. Herpetol.* **29**, 280–285 (1995).

70. S. B. Adams, C. A. Frissell, Thermal Habitat Use and Evidence of Seasonal Migration by Rocky Mountain Tailed Frogs, Ascaphus montanus, in Montana. *Can. Field-Naturalist*. **115**, 251–256 (2001).

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

72. J. P. Costanzo *et al.*, Physiological responses of freeze-tolerant and -intolerant frogs : clues to evolution of anuran freeze tolerance. *Am. J. Physiol.* **265**, 721–725 (1993).

73. J. P. Costanzo, M. C. F. do Amaral, A. J. Rosendale, R. E. Lee, Seasonality of freeze tolerance in a subarctic population of the wood frog, <i>Rana sylvatica<i/>. *Int. J. Zool.* **2014**, 1–13 (2014).

74. J. L. Jenkins, D. L. Swanson, Liver glycogen, glucose mobilization and freezing survival in chorus frogs, Pseudacris triseriata. *J. Therm. Biol.* **30**, 485–494 (2005).

75. B. J. Sinclair, Field ecology of freeze tolerance: interannual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach Celatoblatta quinquemaculata. *Oikos*. **93**, 286–293 (2001).

76. D. L. Swanson, S. L. Burdick, Overwintering Physiology and Hibernacula Microclimates of Blanchard's Cricket Frogs at Their Northwestern Range Boundary. *Copeia*. **2010**, 247–253 (2010).

77. B. J. Sinclair *et al.*, Real-time measurement of metabolic rate during freezing and thawing of the wood frog, Rana sylvatica: implications for overwinter energy use. *J. Exp. Biol.* **216**, 292–302 (2013).

78. R. D. Brown, D. A. Robinson, Northern Hemisphere spring snow cover variability and change over 1922–2010 including an assessment of uncertainty. *Cryosph.* **5**, 219–229 (2011).