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NOVEL INDICES TO QUANTIFY FREEZE TOLERANCE IN AMPHIBIANS

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**NOVEL INDICES TO QUANTIFY FREEZE TOLERANCE IN
AMPHIBIANS**

by

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**B.S., BIOLOGICAL SCIENCES, UNIVERSITY OF ALASKA,
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Abstract

We propose Freeze Endurance (F_{end}) as a new measure of the ability of amphibians to tolerate internal freezing, allowing integration of time and temperature to describe each freeze event. Use of freeze endurance as a descriptor allows direct comparison between individuals, populations, and species, and may be scaled by body size, or other characteristics, to determine if abilities to survive freezing are significantly different between groups. Thermal limit examinations have long suffered from lack of comparability between publications due to differing endpoints, measurements, equipment, and organism availability, despite the value of information yielded by such studies to understanding mechanisms governing life at both cellular and organismal levels. Utilization of the freeze endurance metric will allow future studies to define, and more rigorously compare, freezing abilities of species so that potential factors contributing to variation, such as body size, glycogen storage, gene regulation, metabolic efficiency, and hydration state can be examined.

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Introduction

Thermal limit examinations have long suffered from lack of comparability between publications due to differing endpoints, measurements, equipment, and organism availability, despite the value of information yielded by such studies to understanding mechanisms governing life at both cellular and organismal levels. Examination of comparative physiological variation at multiple hierarchical levels within- and among-species, inclusion of genomic data, integration of laboratory and field experiments, protocol standardization, and global collaboration facilitate the discovery of explicit connections between biogeography and physiology and allow tests of macrophysiological processes (Gaston et al. 2009). Such interdisciplinary collaborations will ultimately improve our ability to predict potential responses to climate change (Chown and Gaston 2008), and identify target species for novel research in low temperature medicine. Studies of animals from thermal extremes are critical to advancing cryomedicine, having already identified natural proteins from animals for testing in humans and other mammals. Antifreeze Proteins I and III isolated from several species of North Atlantic fish (AFP I: winter flounder; AFP III: eelpout, ocean pout, and wolf fish) improve survivorship of Male Sprague Dawley rat hearts during nonfreezing cryopreservation (Amir et al. 2004). Transfection with a stress protein from brine shrimp (*Artemia franciscana*) combined with loading of the native cryoprotectant of that species (trehalose) improves dehydration tolerance of human embryonic kidney cells (Ma et al. 2005). Supercooling methods to preserve human lungs were developed in recognition of challenges faced by freeze tolerant ectotherms (Abe et al. 2006; Costanzo et al. 1995b), and isolated spermatozoa of the freeze tolerant Wood Frog (*Rana sylvatica*) and intolerant American Toad and

Northern Leopard Frog (respectively, *Anaxyrus americanus* and *Rana pipiens*) show differential responses to freezing storage (Beesley et al. 1998).

While numerous cryoprotectants from nature are currently used in low temperature medicine, they are insufficient to overcome the legion of challenges presented by freezing a wide array of tissues. Antifreeze protein efficacy varies by composition, purity, concentration, and target cell type (Bang et al. 2013), as a variety of physiological and molecular adaptations are necessary to survive extreme temperatures (Tattersall et al. 2012). Understanding complex challenges associated with freezing requires comparative work (Comizzoli et al. 2012) to expand our knowledge of the mechanisms underlying freeze tolerance, including evolutionary pathways, ecological drivers, and novel cryoprotectant molecules. Examining animal models of cold tolerance has potential for advances in low temperature medicine and storage. However, responses of cells to cryopreservation depend on cell type and method of preservation, and it is only with continued research into the bases of these differences can we improve survival (Baust et al. 2009).

Recent efforts in integrating ecological and physiological data (Chown et al. 2004) have already greatly improved our broad scale understanding of temperature constraints (Chown and Gaston 2016), though responses to winter conditions are still poorly represented (Williams et al. 2014). Species distribution models have also commenced inclusion of physiological data to more accurately depict potential niche space (Kearney and Porter 2009), but availability of detailed data is limited for many species and

variability of traits among life-stages is largely unknown (Chown 2012; Marais et al. 2009). Here we propose a novel measure of sub-zero thermal tolerances in amphibians, freeze endurance (F_{end}), to directly compare freeze tolerant and intolerant species from local to global scales and provide a standardized methodology to test freezing abilities of all life stages. Our intention is to simplify initial examinations of additional amphibian species and populations, provide an index integrating critical descriptive components of freeze tolerance (minimum temperature, time frozen, and survival) and improve accessibility of comparative data for future studies.

Thermal tolerance in animals is generally linked to geographic distribution and associated variation in environmental conditions, but gaps in physiological data have been identified as a major limitation in predicting responses of species to climate change (Bonino et al. 2015; Bozinovic et al. 2011; Gaston et al. 2009; Sunday et al. 2011). Temperature limits have historically been measured using a variety of methods, including onset of chill coma (CT_{min}) for low temperatures, onset of spasms (OS or CT_{max}) for high temperatures and loss of righting response (LRR) or length of time to 50% mortality (LT_{50}) for both and low and high experimental temperature descriptors (Hazell and Bale 2011; Lutterschmidt and Hutchison 1997). Each of these methods uses a clear physical response of the animal to determine the endpoint of the experiment, but is not applicable in the case of freezing survival tests. Recovery after a freeze event is a binomial event; the animal lives or dies. With death, we recover no knowledge of how far limits of freezing ability are exceeded, or if limits are due to minimum temperatures experienced, time frozen, or an interaction of the two variables. Survival alone is equally uninformative, necessitating numerous

replications under variable conditions to identify specific limitations for each facet of freeze tolerance. In addition, use of differing endpoints among studies precludes comparative approaches, and variation in acclimatization regimes, temperature ramping, and food availability may also alter experimental outcomes (Terblanche et al. 2007). While reporting variation in physiological values from studies with differing endpoints and methodology has scientific relevance (Rezende and Santos 2012), such experimental approaches limit our ability to make comparisons at broad scales necessary to link underlying mechanisms to global diversity and evolutionary patterns. Species in high latitudes introduce additional challenges, as upper thermal limits and endpoints are often less complex, and more comparable between studies than low temperature endpoints, and subzero temperatures create the potential for freezing without external indications of a change in state.

Water is essential to life and composes the majority of cell mass as the solvent for biochemical reactions; conversion of water to ice in living organisms is catastrophic. As ice forms, it damages cells by increasing solute concentrations to toxic levels, while disrupting cellular structures. For high latitude species, temperature reduction without freezing can be beneficial by reducing reaction rates and energy consumption (Chown and Terblanche 2006; Tattersall et al. 2012; Voituron et al. 2002), as cooler body temperatures allow survival on less stored energy during periods of reduced activity such as torpor or hibernation (Ruf and Geiser 2014; Storey and Storey 2010). Similar circumstances may also be desirable in medicine, as reducing temperature and metabolism can also extend time available to address traumatic injuries, perform major

surgical procedures (Kheirbek et al. 2009), store, match, and transplant organs (Abe et al. 2006; Bruinsma et al. 2015; Saad and Minor 2008), and store reproductive tissues for later use (Jakimiuk and Grzybowski 2007; Sumida 2006). These concepts are also increasingly applied in wildlife conservation efforts to help preserve genetic diversity of animal populations via storage and transportation of reproductive tissues (Comizzoli et al. 2012) and embryos (Streit et al. 2014). Despite risks associated with sub-zero body temperatures, many organisms have adapted to survive this extreme condition, and even benefit from associated reductions in resource requirements.

Animals survive subzero temperatures using two strategies, freeze tolerance or freeze avoidance. In scientific literature, a 'freeze tolerant' animal is generally considered one that survives extracellular freezing of body fluids (Bale 1996; Costanzo and Lee 2013; Nedved 2000; Renault et al. 2002; Sinclair 1999; Somme 1999; Storey and Storey 1988). Freeze avoiding, or supercooling, animals prevent internal freezing by removing potential ice nucleators and accumulating cryoprotectants and/or thermal hysteresis proteins to depress the freezing point of body fluids (Somme 1999). Freeze tolerance and avoidance are usually mutually exclusive strategies (Costanzo et al. 2013), as ice accumulation following freezing point depression rapidly overcomes adaptive mechanisms to minimize damage during freeze events (Salt 1961). However, the extent of freezing that must be endured in freeze tolerant species is debatable (Baust 1991; Storey 2006). Amphibians or reptiles take longer to freeze than smaller freeze tolerant animals such as insects and may only survive short duration freeze events in which environmental temperatures, and thus ice content stabilization, are never reached (Rexer-Huber et al. 2011; Storey 2006;

Voituron et al. 2005). If equilibrium ice is never reached, the freeze tolerance status of an animal cannot be confidently evaluated, as survival may represent extended supercooling, rather than freeze tolerance. To prevent this complication among our datasets, we limit the definition of freeze tolerance to species that survive a minimum of 24 hours frozen after freezing initiation, as the small mass of amphibians guarantees this time period is sufficiently long for all but the largest species to reach ice equilibrium. Among insects, levels of freeze tolerance have been placed on a continuum using supercooling point (SCP) and lower lethal temperature (LLT) of a species as criteria to consider insects partially freeze tolerant, moderately freeze tolerant, strongly freeze tolerant, or freeze tolerant with a low SCP (Sinclair 1999). Many authors have also defined freeze tolerance under ecological relevancy considerations (Bale 1993, 1996; Costanzo and Lee 2013; Costanzo et al. 2006; Storey 2006; Storey and Storey 1996; Terblanche and Hoffmann 2011), where definition of freeze tolerance (or intolerance) in a species is dependent on ability to survive freezing conditions likely to be encountered in its natural environment(s), and not explicit limits of temperature or time.

Among vertebrates, freeze tolerance has been most commonly documented among nine species of anuran amphibians (Storey and Storey 1992; Costanzo and Lee 2013). Isolated instances of freeze tolerance are known in other ectothermic vertebrate groups, such as the European Common Lizard, *Lacerta vivipara* (Costanzo et al. 1995a) and several turtle species. Only the Box Turtle (*Terrapene carolina*), however, is freeze tolerant as both adult and juvenile (Costanzo and Claussen 1990), other turtle species are only freeze tolerant as juveniles (Storey 2006). Two closely related urodelean amphibians, the

Siberian Newt (*Salamandrella keyserlingii*) and Schrenck's Newt (*Salamandrella schrenckii*), are also freeze tolerant (Berman and Meshcheryakova 2012, Berman et al. 1984, 2010). Freeze tolerance in anurans encompasses approximately 150 million years of evolution (Pyron 2014; Pyron and Wiens 2011) in two of the most speciose amphibian families, *Ranidae* and *Hylidae*. Within *Ranidae*, a single genus contains all three freeze tolerant frogs, the Moor Frog (*Rana arvalis*, tolerance first described in Voituron et al. 2009), the Common Frog (*Rana temporaria*, tolerance first described in Pasanen and Karhapää 1997), and the Wood Frog (*Rana sylvatica*, tolerance first described in Schmid 1982). The Moor and Common Frog are both distributed throughout Europe, though the Moor Frog extends farther east into Russia and is less cosmopolitan within European countries. The Wood Frog range extends northwest across much of North America from the eastern coast of the United States into Canada and Alaska, but is found in only isolated pockets in central and western continental states.

Freeze tolerant hylids are known within two genera, *Hyla* and *Pseudacris*. All five freeze tolerant hylids are North American, and *Hyla* is the only genus containing species outside the New World (Hua et al. 2009). Within the genus *Hyla*, only the cryptic diploid-tetraploid complex of Cope's Gray Treefrog, *Hyla chrysoscelis* (diploid) and Eastern Gray Treefrog, *Hyla versicolor* (tetraploid), are known to be freeze tolerant. Cope's Gray Treefrog is found throughout the eastern half of the United States, extending into southeastern Canada. Similarly, the Eastern Gray Treefrog is also found throughout most of the central and northeastern United States and extends into southeastern Canada, but is not found in many southeastern United States. Cope's and Eastern Gray treefrogs occur

in sympatry across much of their distributions, hybridize in regions of co-occurrence (Espinoza and Noor 2002), have similar life histories (Ptacek 1996), and their tetraploid lineages are thought to have arisen multiple times from diploids (Ptacek et al. 1994). Within the genus *Pseudacris*, freeze tolerance is known to occur among three species with disparate distributions across the United States and Canada: the Spring Peeper, *Pseudacris crucifer* (Schmid 1982); the Boreal Chorus Frog, *P. maculata* (Storey and Storey 1986); and the Pacific Treefrog, *P. regilla* (Croes and Thomas 2000). Spring Peepers are distributed in a pattern similar to Gray Treefrogs, throughout the eastern half of the United States and into southeastern Canada, whereas Pacific Treefrogs are found only along the western coast of North America, from Baja California to British Columbia and southeast Alaska. The Boreal Chorus frog extends farthest north of any North American hylid species, into Canada (Appendix 1). Though the Boreal Chorus Frog does not occur as far north into as the Wood Frog, it is one of the most widely distributed anurans in North America and is found in much of the central United States and Canada.

Though variation in freeze tolerance within and among known tolerant anuran species and a few closely related species has been examined (See Tables 1, 2, and 3), freeze tolerance status of most anuran amphibians is unknown, and all are assumed to be intolerant. A few additional species that share similar geographic distributions and environmental factors with freeze tolerant species have been examined (Bazin et al. 2007; Berman et al. 1984; Rexer-Huber et al. 2011; Steiner et al. 2000; Storey and Storey 1986; Swanson and Graves 1995). However, in some cases utility of these studies is reduced by extremity of freeze tolerance tested (Berman et al. 1984; Swanson and Graves 1995).

Extremity of freeze tolerance testing refers to factors known to alter freezing survivorship, including (but not limited to) duration (Layne and Kefauver 1997; Layne et al. 1998), minimum temperature (Layne and Kefauver, 1997; Layne and Stapleton 2009; Zimmerman et al. 2007), temperature reduction rates (Costanzo et al. 1991), and acclimation regimes prior to a freeze event (Layne and Stapleton 2009; Zimmerman et al. 2007). Juveniles of the Plains Spadefoot Toad (*Spea bombifrons*, previously *Scaphiopus bombifrons*, see Appendix 1) from South Dakota were tested for freeze tolerance due to the harsh winter conditions in the region, relatively high dehydration tolerance of other species within the clade (Jørgensen 1997, Shoemaker et al. 1992; Thorson and Svihla 1943), and probability of juveniles as the only life stage exposed to freezing temperatures (Swanson and Graves 1995). Freezing tests of juvenile spadefoots were conducted with standard acclimation regimes, records of necessary morphological characteristics, temperature reduction rates (1°/hour), body temperature and exotherm tracking, recovery periods and testing. However, freezing was not initiated at high subzero temperatures, and animals were allowed to reach their supercooling points during freezing experiments (Swanson and Graves 1995). Supercooling prior to freezing increases rate of ice formation (Claussen and Costanzo 1990) and decreases survival probability during freeze events (Claussen et al. 1990). The results of freezing experiments with juvenile spadefoot toads indicate this species is intolerant of freezing under the conditions tested, but does not exclude freeze tolerance under other conditions such as shorter duration, higher subzero temperatures, and initiation of ice formation at higher subzero temperatures (Swanson and Graves 1995).

Here we propose a novel measure of freezing survival, freeze endurance (F_{end}), which integrates minimum temperature, time, and survivorship during freeze events for better direct comparisons between freeze tolerant and intolerant species. We apply this measure to amphibians in a meta-analysis of species previously tested for freeze tolerance to depict how freeze endurance compares to freeze tolerance. We also compare fit of different physiological models integrating variables of freeze tolerance testing to identify physiological parameters best predicting freezing ability in amphibians. We use these data to suggest a protocol for future tests aimed to facilitate direct comparison between studies of vertebrate freeze tolerance, and expand applicability of such experiments to our understanding of broad patterns of diversity in amphibians.

Methods

Literature Search and Data Extraction

Initial literature searches were performed using Thomson Reuters' Web of Science Web Portal on August 7, 2015 by searching papers for terms related to freeze tolerance in amphibians and collecting electronic copies of publications containing freeze tolerance testing. Searches were performed with all possible combinations of an organismal identification (amphibia*, urodel*, caudat*, anura*, frog*, or salamander*) and a subzero temperature indicator (freez*, froz*, or subzero temperature*). Additional papers not obtained from electronic database searches were identified from literature cited sections of publications. Publications reporting original tests of amphibian freeze tolerance were retained and data were extracted for inclusion in our database. Two potential strategies may be employed to survive subzero temperatures in ectotherms, supercooling (also

called freeze avoidance) and freeze tolerance. These strategies differ due to ice crystal formation within the body of freeze tolerant organisms, but not in supercooling, with the two strategies requiring different physiological adaptations. Thus, inclusion of data from a published study required two criteria be met:

- 1) amphibians were tested at subzero temperatures; and
- 2) freezing was confirmed by direct examination, or observation of body temperature recordings during freezing.

Physiological Data

Experimental data extracted from publications were collated into a single database, with each experiment of differing conditions recorded as a separate data point, resulting in multiple data entries from most publications. Variables extracted and recorded included taxon, collection locality, life stage, mass, sex, number of individuals tested, survival, and testing conditions. When nomenclature reported in publications differed from currently recognized taxonomy, both the current species and original published name were recorded. Collection locality, year, and season were extracted, with locality recorded as latitude and longitude if provided, or approximated using the GEOLocate web application (Rios and Bart 2010). Across studies, amphibians were obtained and tested at various life stages, including embryos in eggs, tadpoles, metamorphs, juveniles, and adults. Both stage of collection and life stage at which freeze tolerance testing occurred were recorded, including Gosner stages if reported. Within the database, survival was reported across three data columns: successes (number of individuals surviving treatment), failures (number of mortalities), and survivorship (successes over total individuals tested).

Experimental characteristics extracted included holding conditions prior to acclimation, acclimation conditions immediately prior to freeze tolerance tests, cooling regime, ice inoculation temperature/supercooling point, thawing regime, and time held at each temperature. Data on holding and acclimation regime extracted for analysis included temperature, time, and hours of light exposure per day. Experimental cooling regime was recorded as reduction in degrees Celsius per hour. Inoculation temperature, the subzero temperature at which ice crystallization began, was frequently initiated through application of ice or aerosol coolant spray to animals or containers holding animals. For the purpose of these analyses, supercooling point and artificial inoculation temperature were regarded as physiologically equivalent and recorded as inoculation point in Celsius degrees. In cases where a supercooling point was obtained separately from the freeze tolerance experiment, the temperature at which freezing was initiated in the experiment was recorded as the inoculation point and the supercooling point was recorded separately, as was method of ice crystal inoculation (or lack thereof). Thawing regime was recorded within two separate columns as temperature at which animals were held while thawing and maximum time observed post-freeze. Minimum experimental freezing temperature reached was recorded as minimum temperature in °C and time was recorded in hours as both time held at the minimum temperature and total time frozen, including hours spent ramping between temperatures. Reporting of time in hours was preferred to minutes due to the prohibitively large upper limits of time tested. When values were reported as ranges, data were split into multiple columns to report minimum, maximum, and average reported values. Where multiple experiments were performed on the same individual,

total time frozen including previous experiments was also included in an additional column. Data on body temperature recording (True/False/Unknown, frequency per hour), tetracycline bath prior to experiment (True/False/Unknown), and if bladders were emptied prior to experimentation (True/False/Unknown) were also recorded.

Freeze Survival Modeling

Relationships between survival and freezing conditions were examined using generalized linear models (GLMs) as implemented in R (R Core Team 2015). Because freeze tolerance testing results in either survival or death, normal linear regression analyses are inappropriate for binomially distributed data. We used GLMs with a link function transformation (logit link function with a dispersion parameter of 1) on the binomial response of success (number of individuals survived), or 2) failure (number of individuals died) under each tested condition (Fox 2008). GLMs can utilize maximum likelihood to estimate regression coefficients and residual deviance of non-normally distributed data. This fixed dispersion parameter makes differences between residual deviances in nested models the same as the likelihood ratio test statistic (Fox 2008).

The likelihood ratio test statistic was used to test for significant differences in model fit among GLM nested models and we compared measures of model mean squared error, adjusted mean squared error, Analysis of Variance (ANOVA), residual sum of squares values (RSS), and Akaike Information Criterion (AIC) to determine best fit among non-nested models. Cross-validation was performed to assess model quality by breaking the dataset into five non-overlapping sections, fitting the model to four sections, using the

fitted model to predict responses in the remaining section, and comparing predictions to actual responses. This process was repeated until all sections had been held out and compared to predicted values once for each of the GLMs. Eight GLMs (Figure 1) were compared to determine predictive power of variables representing each stage of freeze tolerance experimentation and population level variation in amphibians. Choice of character inclusion for each model was determined based on primary literature on ice formation and survival in vertebrates (Claussen and Costanzo 1990, Costanzo et al. 1991a), freeze tolerance strategies and energetics (Jenkins and Swanson 2005; Layne. et al. 1998; Voituron et al. 2002, 2005), potential effects of acclimation time and temperature (Irwin and Lee 2003; Lotshaw 1977), and post-freeze treatment and observation (Berman et al. 1984, Costanzo et al. 1992; Layne and First 1991).

Model Name ~ Predictor Variables

1. Population Variation ~ Mass, Geographic Locality
2. Acclimation ~ Acclimation Temperature, Duration
3. Initiation ~ Cooling Regime, Inoculation Temperature
4. Treatment ~ Minimum Temperature, Duration
5. Recovery ~ Thawing Temperature, Duration
6. Full Physiological ~ All predictors included in all models above
7. General Physiological ~ Full Physiological without Species/Population Variation
8. Minimal Physiological ~ Mass, Minimum Temperature, Duration of Freeze
9. Informed ~ All predictors included in all models above, and known freeze tolerance or intolerance species status

Figure 1. Predictor models of freeze tolerance data.

Because not all publications reported all predictor variables, a reduced dataset including only experiments reporting all predictors is used to test model fit, otherwise differences in degrees of freedom would prevent direct comparison between models. Localized adaptation and individual variation were encompassed by the Population Variation model, with predictor variables in grams of mass, and geographic locality as latitudinal and longitudinal coordinates of the source population. The potential for preparatory loading of glucose, or other freeze inhibiting molecules and variation of energetic stores are represented by the Acclimation model, including temperature, and time held at said temperature immediately prior to testing. The Initiation model is composed of the conditions used to initiate freezing and includes the cooling regime in degrees of temperature reduction per hour and temperature at which ice crystal growth begins within the body. Importance of ice crystal growth patterns and distribution of freeze tolerance

related compounds throughout the circulatory system are represented by the Initiation model. The Treatment model is composed of minimum temperature and time held at the minimum temperature during freezing. Treatment relates to the inherent variation in freezing abilities within and between species, and glycogen storage variation between groups and experimental conditions. Time observed after thawing initiation and temperature of thawing post-freeze in the Recovery model accounts for variation between experiments in time animals were observed post-freeze. The Full Physiological model included all predictors from each of the previously described models to examine relative importance of each variable in predicting survival of freeze events. The General Physiological model is identical to the Full model, however, population-level variation predictor variables have been removed. The Minimal Physiological model includes only mass, time held at minimum temperature and minimum temperature. An additional Informed model integrated prior knowledge of species freeze tolerance or intolerance status with the variables of the Full Physiological models to observe how prior knowledge of freezing ability altered significance of physiological variables (Figure 1).

Freeze Endurance

Freeze endurance, our proposed novel descriptor of freezing ability, was calculated for each database entry using a simple equation:

$$\text{Freeze Endurance (F}_{\text{end}}) = \text{Days Frozen} * \text{Minimum Temperature} * \text{Survivorship}$$

This calculation method is a condensed summation model of freezing experiment survival for each individual and is equivalent to averaging survival among individuals. For example, in the case of one individual of five in an experimental group surviving a treatment of -2° for one day, freeze endurance of a surviving individual is -2°Days , and a mortality has freeze endurance of 0°Days , the entire experimental group has an F_{end} of -0.4°Days . Lower freeze endurance values indicate higher survival at low subzero temperatures for a longer period of time. Each individual tested in an experiment will have an individual freeze endurance value $F_{\text{end}}(\text{Ind})$, each population tested will have freeze endurance value $F_{\text{end}}(\text{Pop})$, and each species tested will have a freeze endurance value $F_{\text{end}}(\text{Sp})$ for direct comparison of freezing ability, using parameters which are clearly defined in all freeze tolerance literature. We use days, instead of hours or weeks, due to the generally accepted value of one day frozen as a standard for freeze tolerance, and it is also the working definition we use in this paper. Freeze tolerant amphibian species vary widely in mass, a characteristic known to alter ice accumulation and freeze tolerance related metabolite production, such as cryoprotectants (Claussen and Costanzo 1990; Costanzo et al. 2013). We can calculate freeze endurance per gram, and in the case of our example population, if the average mass of the amphibians tested is 4.48g, the $F_{\text{end/g}}$ of the population is $0.0893^{\circ}\text{Days/g}$. This allometric scaling of freeze endurance corrects for variation in freezing ability due to mass, per gram comparison of freezing abilities among amphibians, and comparisons to abilities of other animal groups, including insects.

Results

Literature Search and Database Construction

Physiological data from tests of freeze tolerance were extracted for 29 amphibian species from 56 papers published between 1941 and 2014, representing a total of 1,610 individuals tested at various life stages including eggs, tadpoles, juveniles, and adults. Data were from 10 countries (Brazil, Canada, Denmark, Finland, France, New Zealand, Russia, Scotland, Turkey, and United States of America), though 44 of 56 publications originated in North America. Of the 29 species, seven were Order Caudata from five families, and the remaining 22 amphibians were from four anuran families. The most studied species (Table 1), *Rana sylvatica*, was tested in nearly half of the studies (27 of 56 publications), subjected to 87 separate experiments, and represented approximately one-third of the individuals ($n = 557/1610$). By number of publications, *Hyla versicolor* was second most studied, with 27 separate experiments among six publications ($n = 120$), though *Rana temporaria* was represented by more individuals ($n = 191$) among three publications and 32 experiments. For most species (16 of 29), a single publication represented all available data, while the remaining 10 species were examined in two to four papers. Testing across life stages (Table 1) was performed primarily on adult or juvenile individuals ($n = 1455$), with tadpole data only from *Rana temporaria* and egg data from *Rana sylvatica* and *Pseudacris regilla*.

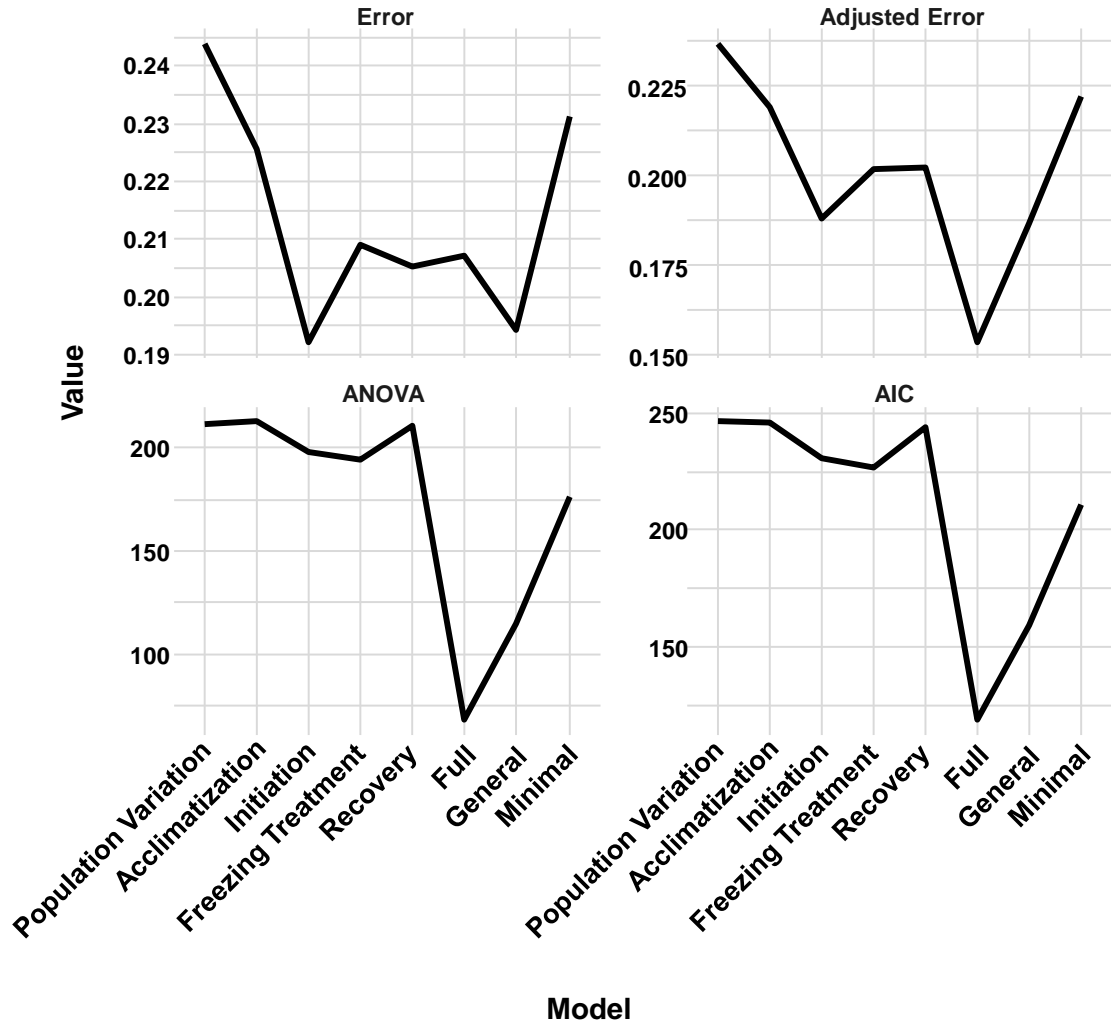


Figure 2. Comparisons of fit of physiological models tested by raw error, adjusted error, analysis of variance, and Akaike information criterion. Smaller error values indicate better fit of the models to the observed data.

| Order | Family | Species | Number of Publications | Number of Experiments | | | | | | Total |
|----------------|-------------------|--|------------------------|-----------------------|----------|-----------|-----------|-----------|----------|-----------|
| | | | | Adult/ | Juvenile | Juvenile | Tadpoles | Eggs | Unknown | |
| Anura | Bufo | <i>Anaxyrus americanus</i> | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Anura | Bufo | <i>Rhinella schneideri</i> | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| Anura | Hylidae | <i>Acris blandchardi</i> | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| Anura | Hylidae | <i>Hyla chrysoscelis</i> | 2 | 8 | 0 | 0 | 0 | 0 | 0 | 8 |
| Anura | Hylidae | <i>Hyla versicolor</i> | 6 | 22 | 3 | 1 | 0 | 0 | 0 | 26 |
| Anura | Hylidae | <i>Litoria ewingii</i> | 2 | 1 | 6 | 0 | 0 | 0 | 0 | 7 |
| Anura | Hylidae | <i>Pseudacris crucifer</i> | 4 | 7 | 4 | 2 | 0 | 0 | 0 | 13 |
| Anura | Hylidae | <i>Pseudacris maculata</i> | 4 | 7 | 0 | 0 | 0 | 0 | 0 | 7 |
| Anura | Hylidae | <i>Pseudacris regilla</i> | 3 | 6 | 0 | 0 | 0 | 1 | 1 | 8 |
| Anura | Ranidae | <i>Pelophylax esculentus</i> | 1 | 10 | 0 | 0 | 0 | 0 | 0 | 10 |
| Anura | Ranidae | <i>Pelophylax lessonae</i> | 1 | 9 | 0 | 0 | 0 | 0 | 0 | 9 |
| Anura | Ranidae | <i>Pelophylax ridibundus</i> | 2 | 18 | 0 | 0 | 0 | 0 | 0 | 18 |
| Anura | Ranidae | <i>Rana arvalis</i> | 1 | 0 | 0 | 8 | 0 | 0 | 0 | 8 |
| Anura | Ranidae | <i>Rana aurora</i> | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Anura | Ranidae | <i>Rana catesbeiana</i> | 2 | 1 | 0 | 3 | 0 | 0 | 0 | 4 |
| Anura | Ranidae | <i>Rana dalmatina</i> | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 5 |
| Anura | Ranidae | <i>Rana pipiens</i> | 3 | 8 | 1 | 0 | 0 | 0 | 0 | 9 |
| Anura | Ranidae | <i>Rana septentrionalis</i> | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Anura | Ranidae | <i>Rana sylvatica</i> | 27 | 56 | 7 | 14 | 0 | 10 | 0 | 87 |
| Anura | Ranidae | <i>Rana temporaria</i> | 3 | 11 | 0 | 11 | 10 | 0 | 0 | 32 |
| Anura | Scaphiopodidae | <i>Spea bombifrons</i> | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Caudata | Ambystomatidae | <i>Ambystoma laterale</i> | 2 | 0 | 1 | 0 | 0 | 0 | 4 | 5 |
| Caudata | Ambystomatidae | <i>Ambystoma maculatum</i> | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Caudata | Hynobiidae | <i>Salamandrella keyserlingii</i> | 2 | 4 | 6 | 0 | 0 | 0 | 0 | 10 |
| Caudata | Hynobiidae | <i>Salamandrella schrenckii</i> | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 5 |
| Caudata | Plethodontidae | <i>Eurycea bislineata</i> | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Caudata | Plethodontidae | <i>Plethodon cinereus</i> | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Caudata | Salamandridae | <i>Notophthalmus viridescens</i> | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Total | | | 56 | 182 | 31 | 40 | 10 | 11 | 9 | |

Table 1. Summary of data extracted from primary literature on amphibian freeze tolerance used in this paper. Data in bold text and highlighted in gray are freeze tolerant amphibians, as defined by minimum criteria of at least one individual surviving 24 hours frozen.

Physiological Model Testing

The reduced subset of data used to test physiological model fit contained 36 experiments from 11 publications, utilizing 254 individuals. Due to missing data, only a single freeze intolerant species (*Litoria ewingii*, 31 individuals from seven experiments) was included in the subset, and no Caudata, or other freeze intolerant anurans were in the physiological model fit evaluation. The remainder of the data subset was composed of three hylid species (*Hyla chrysoscelis*, *Pseudacris crucifer*, and *P. maculata*) and two ranids (*Rana arvalis*, *R. sylvatica*), with most of the data points coming from *R. sylvatica* (16 of 29 experiments, and 116 of 223 individuals). Though model performance differed slightly between evaluation methods, most models showing minimized errors also had equivalently low AIC and ANOVA residual sum of squares (RSS) values (Figure 2). Raw error of models tested varied between 0.19 and 0.24, and adjusted error rates between 0.15 and 0.24, while ANOVA RSS were between 68.03 and 212.75, and AIC values between 118.95 and 246.37. Adjusted error, RSS, and AIC values were lowest in the Full Physiological model (respectively 0.15, 68.03, and 118.95), with inclusion of all available characters providing best fit, and prediction of freezing survival. Raw error was lowest in the Initiation model (0.19), but when error values were adjusted for cross-validation method error, this model was a worse fit than both the Full and General Physiological models. Error and adjusted error rates (both 0.24), and AIC were highest in the Population Variation model, indicating poor fit of the model using only mass and geographic locality coordinates as predictor variables. The poor fit of the Population Variation model was consistent in ANOVA evaluations, though the Acclimation model performed slightly worse with RSS = 212.75 versus 211.44. Models including only a

single stage of freeze cycles or population level variation only, generally fit survival data worse than physiological models integrating data across stages and including population variation. Best model fit was described by utilizing all available data in the Full Physiological model and fit decreased as fewer variables were included. Among the stage and individual variation models, Freezing Treatment (length of time and minimum temperature) fit the reduced dataset best, though the time predictor did not significantly contribute to the model.

All predictor variables in the Full Physiological model were significant at $p = 0.05$. Among other predictors, three of the 11 variables lost significance when a more stringent Bonferroni corrected p -value of $7.14e-03$ was applied: cooling regime, inoculation temperature, and freeze treatment duration. Cooling regime and inoculation temperature together compose the Initiation model and both were significant predictors using both corrected and uncorrected p -values. However, fit of the Initiation model to the dataset was significantly worse than the Full model when comparing adjusted error, RSS, and AIC values, suggesting these variables are only important in isolation, and less critical to survival of freezing. Freeze treatment duration was used as a predictor variable in the Treatment, Full, General, and Minimal Physiological models, and was not a significant predictor of survival in the Treatment or Minimal Physiological models. In both the Full and General Physiological models, duration of freezing treatment was only significant using an uncorrected p -value of 0.05, suggesting duration of freeze is also not as important to survival as other predictor variables. The best predictor of freeze tolerance survival among all variables in the Full model was acclimation duration (-0.25 , $p = 3.98e-$

07), followed closely by absolute Latitude (-2.69, $p = 7.83e-06$) and minimum temperature (1.18, $p = 7.36e-06$). For the remaining predictor variables — mass, acclimation temperature, days observed post-freeze, longitude, and thaw temperature — p-values were between $4.71e-06$ and $7.05e-03$ and all significant contributors to fit of the Full Physiological model. The data could not be further split to compare freeze tolerant and intolerant species responses due to an inadequate number of freeze intolerant species, however, results were similar among only freeze tolerant species of the Full subset.

Full model testing could only be performed on a subset of experiments complete for all test variables, as missing data result in differing degrees of freedom in model fit tests and prevent direct comparisons. The Informed data subset included 45 experiments of freeze tolerant and intolerant hylids and ranids, including 309 individuals, 49 of which were freeze intolerant. Of the 49 intolerant frogs tested, 11 were adults, 18 were juveniles, and 20 were unspecified adults or juveniles. Freeze tolerant individuals were mostly specified as adults ($n = 124$) or juveniles ($n = 105$), though 31 were unspecified adults or juveniles as well. The Informed model fit of combined freeze tolerant and intolerant data found best predictors of survival minimum temperature (0.68, $p = 5.77e-08$) and maximum acclimation time (-0.03, $p = 2.57e-05$), though ice inoculation temperature (-0.97, $p = 1.39e-03$) and cooling regime (-0.05, $p = 6.06e-03$) were also significant contributors. Again, inadequate data for number of freeze intolerant species prevented comparison between tolerant and intolerant models. Freeze tolerant data were fit separately with the Informed model, with similar results to the all-inclusive Informed data set, except only ice inoculation temperature was not a significant part of the model. When the Minimal

Physiological model was fit on the Informed subset, only mass and minimum temperature were good predictors of survival, as was the model fit on only freeze tolerant species data. However, when the Minimal Physiological model was fit to freeze intolerant data, minimum temperature was the only informative predictor of survival. When the dataset was restricted to experiments reporting mass, freeze duration, and minimum temperature of freezing, the combined model fit of freeze tolerant and intolerant species indicated none of the predictor variables was statistically significant in the Minimal Physiological model. Division into freeze tolerant and freeze intolerant species datasets showed mass was the only significant variable in freeze tolerant species modeling, while all three variables were important in freeze intolerant species, with freeze duration as most significant.

While most freeze tolerance experiments on vertebrates are performed at subzero temperatures above -7° and for a week or less, some of the experiments in our dataset are from tests of species with extreme freeze tolerance, surviving at lower temperatures and for longer times than most other freeze tolerant species. The Minimal Physiological dataset was further reduced by removing experiments longer than 7 days and/or at temperatures below -7° . In the less than 7 days subset, minimum temperature was the most informative variable across freeze tolerant and intolerant species, and in a dataset of freeze tolerant species only, mass was the only significant predictor. In freeze intolerant species all predictors were significant, with duration of freeze being the best predictor in the less than 7 days frozen subset. In the higher than -7° dataset, minimum temperature was also the most important variable for the combined tolerant and intolerant dataset,

followed by freeze duration, while mass was not a significant predictor of survival. When split into tolerant and intolerant model tests, all variables were significant predictors, but survival of freeze tolerant anurans was best predicted by minimum temperature, while freeze duration was the best predictor among intolerant species. In the dataset restricted by both 7 days in duration and -7° as the minimum temperature, all variables included (minimum temperature, time frozen, and mass) were significant predictors of survivorship in freeze intolerant species, but only mass and minimum temperature were significant in freeze tolerant species. In intolerant species the best predictor was duration of freeze, while in freeze tolerant species minimum temperature best fit survivorship data.

Freeze Endurance

Freeze tolerance tests reached minimum temperatures as low as -52° and as high as -0.5° and varied in duration between 0.04 days (1 hour) and 87.33 days (2096 hours).

Geographic distribution of freezing tests was generally limited within and between species, covering only a small portion of local and global amphibian distributions (Figure 3). Appendix 2 includes individual maps of each experiment by location and species. No individuals of any species survived temperatures lower than -35° , and only one species was found to recover after testing lower than -16° , the Siberian Newt (*Salamandrella keyserlingii*, Table 2). Of seven individuals tested at -35° for 0.21 days, six individuals initially moved after thawing, but only three salamanders survived more than four days post-freeze, and the remaining three succumbed to internal hemorrhaging, ultimately resulting in an F_{end} of -3.15° days for the population (Berman et al. 1984).

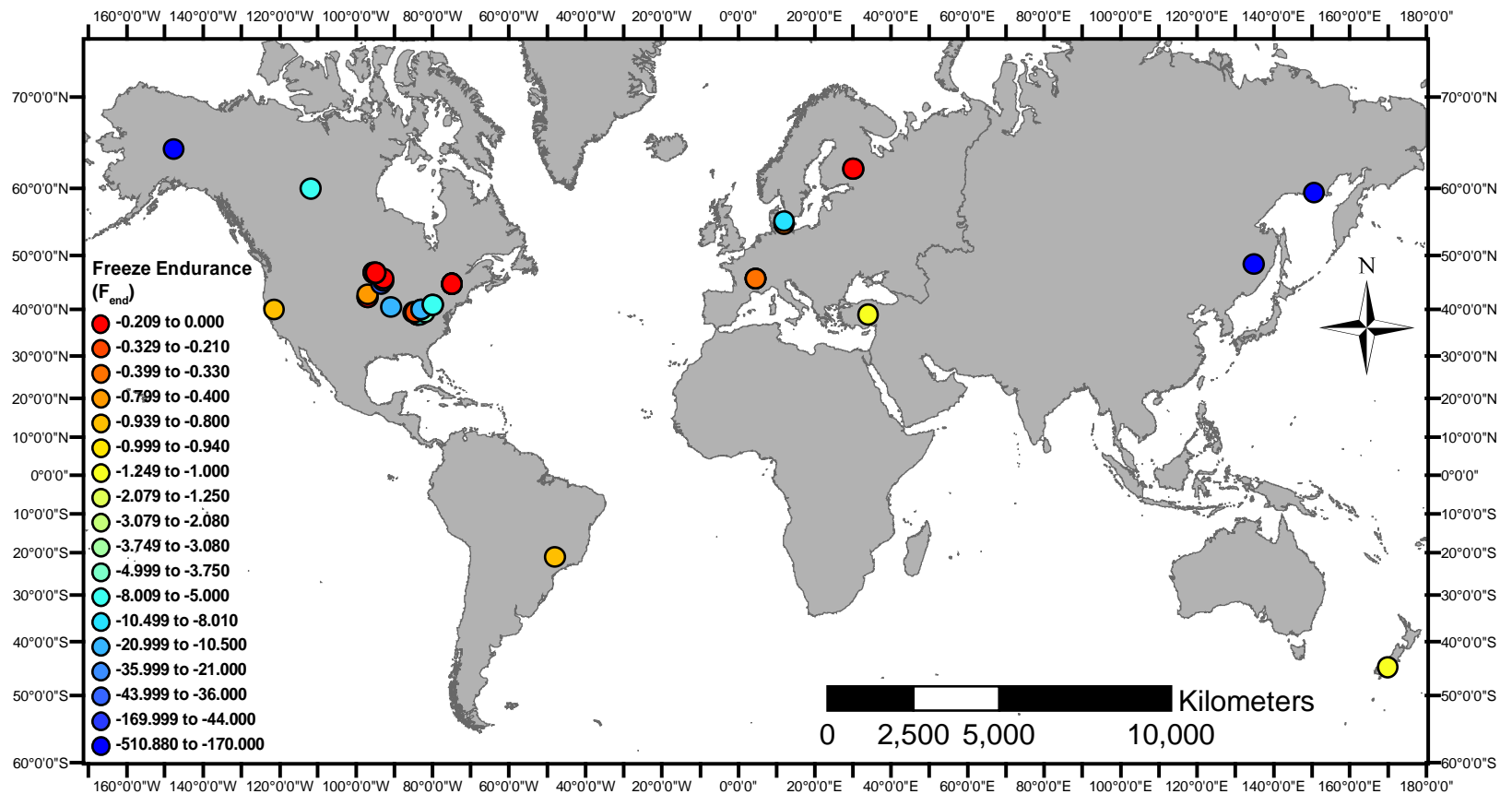


Figure 3. Global distribution of freeze endurance showing localities of the lowest freeze endurance tested, and thus most extreme freezing ability, of each amphibian species.

Table 2. Summary of predictor variable p-values in three physiological models tested for fit to freeze tolerance survival data. Numbers shown in bold type are p-values < 0.05 for significant predictors of surviving a freeze event. Cells showing NA represent values that could not be calculated.

| | | | <u>Full Physiological</u> | | | <u>General Physiological</u> | | | <u>Minimal Physiological</u> | | |
|---|--------------------------------|----------|---------------------------|---------------|------------|------------------------------|---------------|------------|------------------------------|---------------|------------|
| | | | Both | Tolerant | Intolerant | Both | Tolerant | Intolerant | Both | Tolerant | Intolerant |
| N Experiments | | | 36 | 29 | 7 | 36 | 29 | 7 | 36 | 29 | 7 |
| Individual/ Population Variation | Mass | Estimate | -4.32 | -4.47 | 524.10 | - | - | - | 0.12 | 0.11 | 524.10 |
| | | p-value | 0.0000 | 0.0000 | 1.0000 | - | - | - | 0.0001 | 0.0003 | 1.0000 |
| | Absolute Latitude | Estimate | 1.18 | 1.30 | 30.30 | - | - | - | - | - | - |
| | | p-value | 0.0000 | 0.0000 | 1.0000 | - | - | - | - | - | - |
| | Absolute Longitude | Estimate | -0.37 | -0.38 | -292.50 | - | - | - | - | - | - |
| | | p-value | 0.0011 | 0.0013 | 1.0000 | - | - | - | - | - | - |
| Acclimation | Acclimation Temperature | Estimate | -2.69 | -12.65 | NA | 1.05 | 0.95 | 30.30 | - | - | - |
| | | p-value | 0.0000 | 0.9900 | NA | 0.0000 | 0.0000 | 1.0000 | - | - | - |
| | Acclimation Duration | Estimate | -0.35 | 2.01 | NA | -0.18 | -0.18 | -292.47 | - | - | - |
| | | p-value | 0.0002 | 0.9910 | NA | 0.0337 | 0.0326 | 1.0000 | - | - | - |
| Initiation | Cooling Regime | Estimate | -4.76 | -4.74 | NA | -1.78 | -3.27 | -63.92 | - | - | - |
| | | p-value | 0.0026 | 0.0028 | NA | 0.0000 | 0.0001 | 1.0000 | - | - | - |
| | Inoculation Temperature | Estimate | 10.04 | -6.66 | NA | -1.83 | -3.30 | NA | - | - | - |
| | | p-value | 0.0021 | 0.9970 | NA | 0.0065 | 0.0023 | NA | - | - | - |
| Freeze Treatment | Minimum Temperature | Estimate | 4.35 | -4.20 | NA | 0.53 | 0.62 | NA | 0.73 | 0.86 | 30.30 |
| | | p-value | 0.0000 | 0.9963 | NA | 0.0094 | 0.0065 | NA | 0.0000 | 0.0000 | 1.0000 |
| | Freeze Duration | Estimate | -0.25 | 0.04 | NA | -0.03 | -0.04 | NA | 0.05 | 0.03 | -292.50 |
| | | p-value | 0.0000 | 0.9989 | NA | 0.0002 | 0.0025 | NA | 0.4360 | 0.6234 | 1.0000 |
| Recovery | Recovery Temperature | Estimate | 2.07 | -0.67 | NA | -0.40 | -0.71 | NA | - | - | - |
| | | p-value | 0.0071 | 0.9982 | NA | 0.3160 | 0.1415 | NA | - | - | - |
| | Duration of Recovery | Estimate | -4.80 | -2.43 | NA | -0.02 | 0.03 | NA | - | - | - |
| | | p-value | 0.0000 | 0.9925 | NA | 0.7577 | 0.7256 | NA | - | - | - |
| Overall | AIC | 118.65 | 94.18 | 10.13 | 159.53 | 125.00 | 10.13 | 211.10 | 167.22 | 10.13 | |

Table 3. Summary of most extreme freezing regimes, freeze endurance, and freeze endurance per gram of Caudata in this study. Data in bold text and highlighted in gray are freeze tolerant amphibians, as defined by minimum criteria of at least one individual surviving 24 hours frozen.

| Caudata | | | | | | |
|--|---------------------------|--------------|----------------------------|--------------|-------------------------------|---------------------------------|
| <i>Species</i> | Lowest Temperature | | Longest Time Frozen | | Lowest F_{end} | Lowest F_{end/g} |
| | ° | Days | ° | Days | °Days | °days/g |
| <i>Ambystoma laterale</i> | -2.50 | 0.17 | -2.50 | 0.17 | -0.14 | - |
| <i>Ambystoma maculatum</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Eurycea bislineata</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Notophthalmus viridescens</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Plethodon cinereus</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Salamandrella keyserlingii</i> | -35.00 | 0.21 | -21.00 | 56.00 | -1176.00 | -43.59 |
| <i>Salamandrella schrenckii</i> | -35.00 | 30.00 | -35.00 | 30.00 | -510.88 | - |

Table 4. Summary of most extreme freezing regimes, freeze endurance, and freeze endurance per gram of Anura in this study. Data in bold text and highlighted in gray are freeze tolerant amphibians, as defined by minimum criteria of at least one individual surviving 24 hours frozen.

| Anura | | | | | | |
|-----------------------------------|--------------------|--------------|---------------------|--------------|-------------------------|---------------------------|
| Species | Lowest Temperature | | Longest Time Frozen | | Lowest F _{end} | Lowest F _{end/g} |
| | ° | Days | ° | Days | °Days | °days/g |
| <i>Acris blandchardi</i> | -2.00 | 0.25 | -1.20 | 2.00 | -0.80 | - |
| <i>Anaxyrus americanus</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Hyla chrysoscelis</i> | -6.50 | 5.90 | -6.50 | 5.90 | -21.36 | -5.34 |
| <i>Hyla versicolor</i> | -6.50 | 5.90 | -2.50 | 14.00 | -36.00 | -23.97 |
| <i>Litoria ewingii</i> | -2.00 | 0.50 | -2.00 | 0.50 | -1.00 | -0.84 |
| <i>Pelophylax esculentus</i> | -2.50 | 0.54 | -2.50 | 0.54 | -1.35 | -0.06 |
| <i>Pelophylax lessonae</i> | -2.50 | 0.38 | -2.50 | 0.38 | -0.94 | -0.09 |
| <i>Pelophylax ridibundus</i> | -3.00 | 0.29 | -2.50 | 0.83 | -2.08 | -0.06 |
| <i>Pseudacris crucifer</i> | -6.00 | 6.00 | -1.50 | 7.00 | -36.00 | -5.02 |
| <i>Pseudacris maculata</i> | -3.00 | 3.00 | -2.50 | 3.00 | -9.00 | -10.00 |
| <i>Pseudacris regilla</i> | -2.00 | 1.00 | -2.00 | 1.00 | -0.80 | -0.18 |
| <i>Rana arvalis</i> | -4.00 | 3.38 | -4.00 | 3.38 | -8.01 | -2.53 |
| <i>Rana aurora</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Rana catesbeiana</i> | -2.00 | 0.42 | -2.00 | 0.42 | -0.83 | -0.02 |
| <i>Rana dalmatina</i> | -2.00 | 0.33 | -2.00 | 0.33 | -0.33 | -0.06 |
| <i>Rana pipiens</i> | -2.00 | 0.33 | -2.00 | 0.33 | -0.50 | -0.02 |
| <i>Rana septentrionalis</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| <i>Rana sylvatica</i> | -16.00 | 13.58 | -4.00 | 59.33 | -217.33 | -30.19 |
| <i>Rana temporaria</i> | -2.00 | 2.00 | -2.00 | 2.00 | -3.08 | -0.10 |
| <i>Rhinella schneideri</i> | -2.00 | 0.42 | -2.00 | 0.42 | -0.83 | 0.00 |
| <i>Spea bombifrons</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

Individuals dying during and immediately after testing ($n = 4$), such as the salamanders that awoke, but subsequently perished, have F_{end} of 0.0°days , while surviving individuals have F_{end} of -7.35°days . Some individuals survived -37.5° for 0.21 days, but how many or for how long post-freeze was not reported (Berman et al. 1984), and F_{end} could not be reported with confidence. Mass was not reported in Berman et al. (1984), and age was reported as a range from yearlings to adults, so $F_{\text{end/g}}$ could not be calculated. Lowest F_{end} may also be from the same publication (Berman et al. 1984), but the data reported are unclear as translated from Russian into English. Lack of clarity is due to a statement that 17 individuals of various ages from yearlings to mature adults were tested in selected sites in the external environment. Those sites were estimated to endure at most two to three days at -21° each winter, and all individuals placed in those sites were reported to have survived, then died upon exposure to air, “Все животные, бывшие в «мягкой» и «нормальной» зимовках, весной пробудились, но находившиеся на воздухе погибли” (Berman et al. 1984, page 324, paragraph 2, sentence 4). Endurance of subzero temperatures for an entire winter, with a minimum temperature of -21° would place F_{end} somewhere between -1176 and -63°days , depending upon survivorship, and actual time at minimum temperatures. Schrenck’s newt (*Salamandrella schrenckii*), a close relative to the Siberian Newt estimated to have diverged 7 million years ago based on allozyme and cytochrome b data (Matsui et al. 2008), was found to have freezing tolerance limits similar to the Siberian Newt. One of three adults survived freezing at -35° for 30 days (Berman et al. 2010), resulting in $F_{\text{end}} = -357.00^{\circ}\text{days}$, the minimum temperature and longest time Schrenck’s newt was frozen. Again, mass was unreported, so $F_{\text{end/g}}$ was not calculated for these experiments. Although $F_{\text{end/g}}$ could not be calculated among the

experiments listed above, similar experiments include mass measurements for Siberian Newts tested at -10° for 17 days (Berman and Meshcheryakova 2012). No mortalities among the five individuals and an average weight of 3.9 g resulted in $F_{\text{end}} = -170^{\circ}\text{days}$, and $F_{\text{end/g}} = -43.59^{\circ}\text{days/g}$ for this population, which is located near Magadan, Russia; this is the lowest reported value for amphibians. With only one minor exception, Schrenck's Newt and the Siberian Newt were the only freeze tolerant salamander species, with all others dying upon exposure to subzero temperatures. In tests of tetraploid *Ambystoma laterale*, one-third were found to tolerate freezing for up to 4 hours at -2.5° ($F_{\text{end}} = -0.14^{\circ}\text{days}$), though no diploid or triploid individuals recovered under any conditions (Storey and Storey 1992). Freeze tolerance data for Caudata are summarized in Table 4.

Anuran species (Table 4) were tested under conditions between -30° and -0.5° , though the lowest temperature survived by an anuran was -16° for 13.58 days by *Rana sylvatica* from Alaska, with no mortalities among four adults (Costanzo et al. 2013). Freeze endurance of this population is $-217.33^{\circ}\text{days}$, and $F_{\text{end/g}} = -30.19^{\circ}\text{days/g}$, both the lowest recorded values among anurans. Freeze endurance of freeze tolerant and intolerant frogs overlapped at the high range of values, with tolerant species values varying from $-217.33^{\circ}\text{days}$ to 0°days , and intolerant from $F_{\text{end}} = -2.08^{\circ}\text{days}$ (*Pelophylax ridibundus*, Voituron et al. 2005) to 0°days . Similarly, freeze tolerant anuran $F_{\text{end/g}}$ varied from $-30.19^{\circ}\text{days/g}$ to 0°days/g , while intolerant species were between $-0.84^{\circ}\text{day/g}$ (*Litoria ewingii*, Rexer-Huber et al. 2011) to 0°day/g .

Discussion

Freeze endurance is a new measure of the ability of amphibians to tolerate internal freezing that integrates freeze duration, minimum temperature of exposure, and time held at subzero temperatures. Use of freeze endurance as a descriptor allows direct comparison among individuals, populations, and species, and may be scaled by body size, or other characteristics, to determine if abilities to survive freezing are significantly different. Use of only two categories, tolerance or intolerance, inhibits assessment of differing levels of freezing ability among species and identification of individual evolutionary trajectories. The Siberian Newt can survive freezing temperatures down to -35° , as can Schrenk's salamander a species recently diverged from the same evolutionary lineage (Matsui et al. 2008; Berman et al. 2010). These two salamanders are the only species known to survive extremely low temperatures and appear to endure them for long periods of time with little or no loss of viability. The Wood Frog is known to survive over 13 days frozen when exposed -16° , and exhibits the most extreme freezing ability among anurans. Whether physiological mechanisms responsible for freezing survival in salamanders are identical to those in frogs is unknown, though the basic premise of cryoprotectant accumulation is consistent (Storey 1984; Berman et al. 1984). Both Siberian Newts and Wood Frogs are able to survive freezing for 24 hours; however, when examining freeze endurance of these species, -1176°day for Siberian Newts and $-217.33^{\circ}\text{days}$ among Wood Frogs, a large difference in freezing ability exists. This new freeze endurance metric will allow future studies to more rigorously compare freezing abilities of species so that potential factors contributing to variation, such as body size,

glycogen storage, gene regulation, metabolic efficiency, and hydration state can be examined.

The method of calculating freeze endurance contains an inherent flaw that potentially produces the same values, despite being derived from different conditions and survival rates. For example, animals held at -2.5° for 1 day with 100% survival will have the same freeze endurance value as animals held at -5° for 1 day with 50% survival ($F_{\text{end}} = -2.5^{\circ}\text{day}$). Future studies should carefully examine how this measure may differ across freeze tolerance abilities, size, species, or other characteristics related to freeze survival. Extension of this metric to non-freezing temperature constraints may also be viable, though examination of equivalencies and differences would also require exploration individually for minimum and maximum temperature experiments, and explicit statements of supercooling or freezing for subzero experiments. Thermal tolerance landscapes have been suggested as a model to better depict thermal limits of species (Rezende et al. 2014), and are likely superior to our method in describing non-freezing conditions. However, the calculation of thermal tolerance landscapes relies on the observation of a trait change in the animal being tested, such as 50% or 100% mortality, or onset of spasms. As previously stated, there is no observable state change when a frozen animal reaches the limit of freezing ability. Measures utilizing percent mortality could be useful, but would require multiple experiments, and percent survival would vary with time and temperature. Freeze endurance is a comparative descriptor for a single experiment, a population, a species, or a single individual. Although multiple experiments are always preferable, they are often unfeasible due limitations of resources and animals. We do not promote the use of freeze endurance to the exclusion of other

metrics, such as T_{\min} and T_{\max} ; instead we suggest freeze endurance as an extension of other descriptive indices of extreme temperature tolerances, with a distinct advantage for inclusion of multiple important factors into a single descriptor that may be allometrically scaled, or assigned to populations of individuals for comparative studies.

Of over 7,000 species of amphibians, only 28 (< 0.01% of global diversity) have been formally tested for freeze tolerance; however, the majority of amphibians are assumed to be unable to survive freezing. Of only seven species of Caudata tested, two are freeze tolerant, Schrenck's newt and the Siberian Newt. Among tested frogs, 47.6% meet freeze tolerance criteria of surviving 24 hours frozen, suggesting either biased sampling of species or a wider evolutionary dispersal of freeze tolerance among amphibians than previously recognized. Amphibians tolerate dehydration more readily than other vertebrates, likely due to skin permeability necessitating adaptations for transition to terrestrial ecosystems (Hall 1922; Jørgensen 1997; Thorson 1955; Thorson and Svihla 1943). Connections between dehydration and freeze tolerance evolution are well established among vertebrates and invertebrates, with cryoprotective dehydration playing a critical role in increasing osmolarity of circulating body solutes and depressing intracellular freezing points (Churchill and Storey 1993; Clark and Worland 2008; Sinclair et al. 2003). Despite knowledge of connections between freeze and dehydration tolerance, few studies carefully isolate responses to these stressors, with only a single known dehydration tolerant species, the Plains Spadefoot Toad, also subsequently tested for freeze tolerance.

Freezing tolerance tests of the extremely dehydration tolerant Plains Spadefoot toad were performed on six juveniles from South Dakota, USA at -4.4° for a full day and none survived (Swanson and Graves 1995). The conditions of the study were extreme for an initial investigation of freeze tolerance, as even known freeze tolerant species have expired under milder conditions. For example, no individuals survived an experiment on three adult or juvenile Wood Frogs from Minnesota at -2.9° for just 0.42 days (Lotshaw 1977), the model species for amphibian freeze tolerance. Tests of freeze tolerant Boreal Chorus Frogs adults from South Dakota (like the Plains Spadefoot Toad juveniles), at -2.5° for a single day also resulted in none of the eight tested individuals surviving (Jenkins and Swanson 2005). Though the Plains Spadefoot Toad may be freeze intolerant (Swanson and Graves 1995), because two individuals supercooled without freezing and survived the same experimental conditions, the experiment does not entirely exclude a less extreme freezing ability, as juveniles are the most likely to experience freezing conditions due to inefficient overwintering site selection as compared to adults (Swanson and Graves 1995). Testing at less extreme freezing conditions may allow separation of freeze endurance abilities due to dehydration tolerance alone from additional adaptations required to achieve freeze tolerance.

Varying levels of seasonal aridity have been present throughout the majority of amphibian evolution (end-Carboniferous, early Permian, Mesozoic, and Paleocene), resulting in many lineages retaining some level of dehydration tolerance (Hillman 1980; Jørgensen 1997; Thorson 1955). An ancestral state of dehydration tolerance may give rise to low levels of freeze endurance, and likely will be reflected by measurements of -

$1^{\circ}\text{Days} < F_{\text{end}} < 0^{\circ}\text{Days}$, such as those seen in the Pool Frog, Bullfrog, Agile Frog, Northern Leopard Frog, and Cururu Toad (Table 4). Two species, Blanchard's Cricket Frog and Pacific Treefrog (Table 4), meet the criteria for freeze tolerance and also have F_{end} values within the range for low freeze endurance resulting from ancestral dehydration tolerance, thus highlighting a need for more extensive physiological data on dehydration tolerance and freeze endurance, and limitations of a binary tolerant/intolerant descriptor. Although those species meet the strict requirement of freeze tolerance with at least one individual surviving ice-water equilibrium freezing (Bale 1996; Costanzo and Lee 2013; Nedved 2000; Renault et al. 2002; Sinclair 1999; Somme 1999; Storey and Storey 1988), low levels of survival among tested specimens indicate variability of the trait within each species.

Testing freeze endurance is an inherently complex process, with experimentation requiring seven phases: 1) acclimation, 2) temperature reduction to freezing, 3) internal ice crystal initiation, 4) observation of an exotherm and continued temperature reduction until equilibration with surrounding environment, 5) time held frozen, 6) thawing, and 7) post-freeze observation and evaluation of survival. Each stage can and usually does vary in time, temperature, and methodology, making direct comparisons between trials difficult. We propose a standardized methodology for testing freeze endurance to maximize utility of experiments for comparative physiology, allow direct comparisons to legacy studies, and increase accessibility of data (Figure 4). In this protocol, acclimation should occur entirely in darkness and without food, immediately prior to testing. Acclimation times should not be extended beyond 14 days, due to potential effects of

long term storage above freezing without replenishment of depleting energy stores. Insect freeze tolerance studies often include long term storage prior to freeze tolerance testing, which may result in dehydration and starvation of individuals and underestimate thermal tolerance (Terblanche and Hoffmann 2011). In our examination of physiological models of freezing survival, we also found a significant, negative correlation between acclimation duration and survival (Table 4), indicating reduced survival in experiments with longer acclimation times. Presence of food in the gut is also known to alter ice formation temperature within freeze tolerant frogs (Costanzo et al. 2003), making acclimation without feeding a critical variable to control. Studies of freeze tolerance in amphibians vary in acclimation time from 0 (Bazin et al. 2007; Layne and First 1991) to 90 days (Jenkins and Swanson 2005). Responses to low temperature exposure often occur quickly, with changes in gene expression evident within hours (Lee and Denlinger 2010; Ronges et al. 2012; Storey 2004). However, alterations of lipid membrane composition and anticipatory accumulation of cryoprotectants may take much longer and are factors known to alter freezing ability in numerous taxa (Tattersall et al. 2012), and acclimation time and feeding differences between anuran studies have been identified as confounding factors in direct comparison of freezing ability between populations of *Hyla versicolor* (Irwin and Lee 2003; Layne 1999; Layne and Lee 1989).

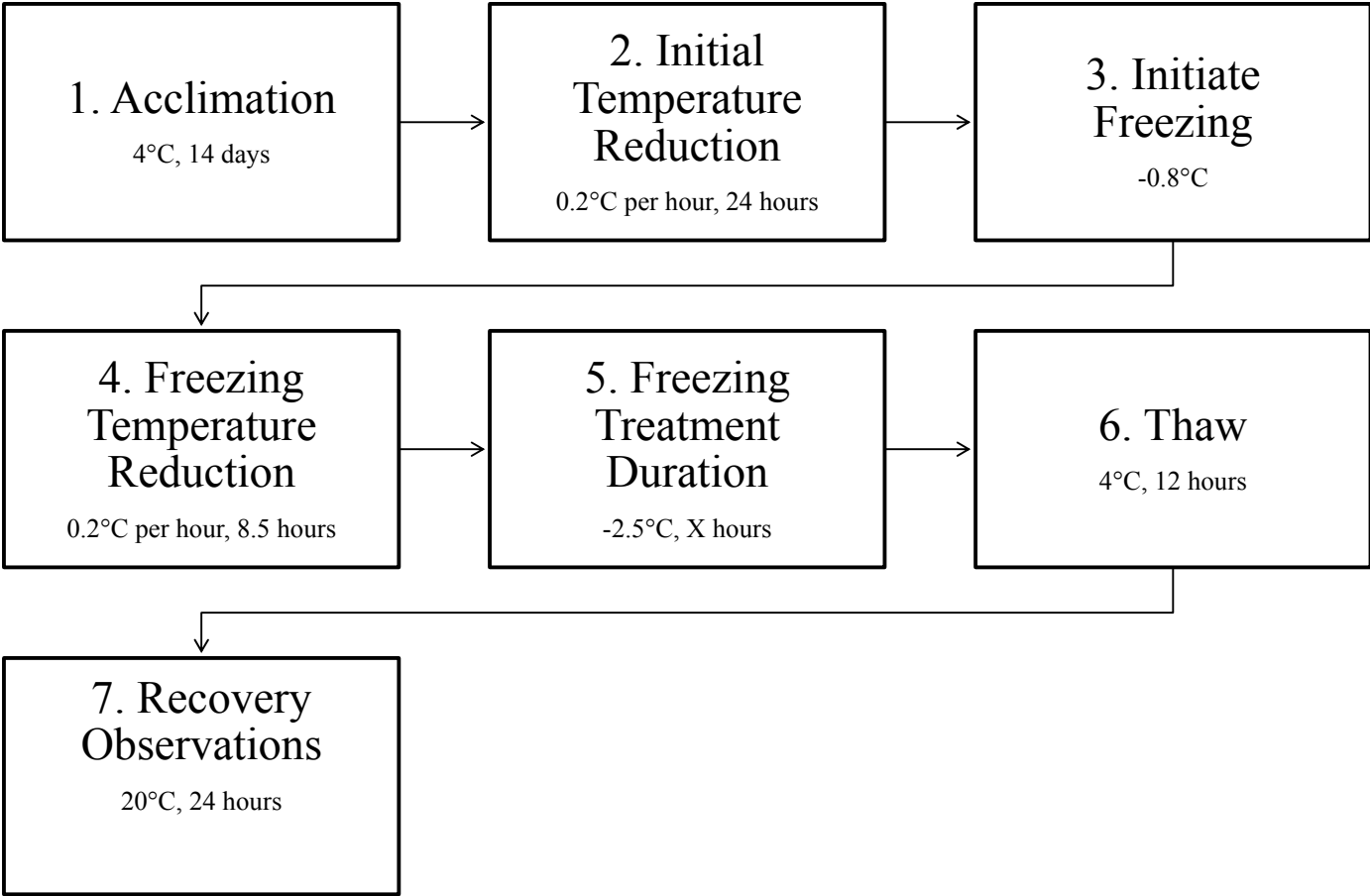


Figure 4. Recommended freeze endurance testing protocol for standardization among studies and laboratories.

The recommended acclimation regime of 14 days in darkness without feeding will be sufficient to clear the gut of food and allow initiation of molecular changes to prepare for freeze events. This time is similar to the median (14 days) and mean (19 days) minimum acclimation times of all previous experiments (*data not shown*). During acclimation animals must be provided with free access to water in the form of a moist paper towel or water bowl to prevent dehydration, unless dehydration is being intentionally included in experimental protocols. Alterations in hydration status affect osmolarity of body fluids, potentially impacting freezing ability. In all studies of freeze tolerance, body temperature should be recorded at least once an hour for the duration of the experiment to prevent confusion between supercooling and freeze tolerance survival strategies at low temperatures. Ideally, studies will use a data logger attached to sensors placed within the cloaca, or in direct contact with the body of the frog prior to initial temperature reduction to record body temperatures continuously. Extremities experience different rates of temperature change, and should not be used to record freeze tolerance temperature data, unless a main body temperature is also being recorded. Initial temperature reduction following acclimation in our protocol requires 24 hours to reduce from 4° to -0.8°, at a rate of 0.2° per hour. We found faster rates of temperature reduction are inversely related to survival in amphibians in our physiological models (Table 4), consistent with another study on rates of temperature reduction and survival (Costanzo et al. 1991). The rate of 0.2° degrees per hour is close to the median rate in our dataset (0.19° per hour), and will allow comparison with prior studies, without negative impacts on survival. Internal ice crystal formation at subzero temperatures is critical to control locations and speed of ice crystal growth within the body to minimize damage (Claussen and Costanzo 1990; Cai

and Storey 1997). We suggest internal ice crystal formation initiation at -0.8° , through application of an aerosol to the outside of the tube holding the animal, or direct exposure to ice crystals on the body. These methods are regularly used in studies of freeze tolerance, ensure freezing occurs without supercooling, are similar to most common value within our dataset of previous studies (-1°), and make the timing between cooling initiation and freezing initiation 24 hours, a methodologically convenient period. A temporary increase in body temperature (exotherm) should be observed following initiation of ice formation due to the release of heat as water molecules enter a crystalline formation. If an increase does not occur, the specimen should be manually checked for signs of internal freezing, such as opaque eye lenses and a rigid body, as freezing may not have occurred. Unexpectedly, lower freeze initiation temperatures are associated with increased survival in the General Physiological model, though the Full model found the expected pattern of increasing survival with high temperatures (Table 4). Inoculation temperature relationships were not calculated for freeze intolerant species in the physiological models due to the small sample size of freeze intolerant species. Only in the combined tolerant and intolerant species dataset did we find the expected pattern of increasing survival with higher subzero temperature freeze initiation, thus, initiation temperature may be more important among freeze intolerant than tolerant species.

Following freeze initiation, we recommend continued reduction in temperature at the same rate, 0.2° per hour for 8.5 hours, ending at -2.5° , with the specimen held at this temperature for the desired duration of the experiment. For species from thermally stable, warm regions (such as the tropics), we suggest initial experiments should be short,

continuing only until a temperature equilibrium is reached with the surrounding environment after the exotherm. Lower freezing temperatures and longer durations are associated with reductions in survival in both tolerant and intolerant species (Table 3). Thawing should proceed at 4° for 12 hours while checking for movement and recovery of righting response every 3 hours. If righting response is not recovered in the first 12 hours, specimens should still be moved to the final recovery temperature of 20° and monitoring should proceed for 24 hours with checks and recording of post-freeze survival responses at 3, 6, 12, and 24 hours. We found a significant positive relationship between recovery temperature and survival, and a decrease in survival with increasing duration, in the Full physiological model only (Table 3). These relationships were not present in other models, and likely reflect an increased probability of recording death at longer post-freeze observations due to a brief initial recovery, before animals succumb to freeze induced injuries. More rapid thawing would also reduce time for accumulation of damage associated toxins in body cavities, increasing periods of ephemeral initial recovery.

Our division of freeze tolerance testing regimes into sets of variables allowed separate determination of how population variation, acclimation, initiation, treatment, and recovery related variables each contributed to survival of freeze events in freeze tolerant versus freeze intolerant amphibians. While including all variables provided the best fit for the data, removal of data related to local and individual variation from models fit the data almost as well (Figure 2, Full versus General Model), indicating freezing protocols may contribute as much to animals surviving freeze events as local adaptations and evolutionary history. The freezing Treatment model utilizes the same variables as

calculations of freeze endurance, and is the best model among the four treatment single stage models (Acclimation, Initiation, Treatment, or Recovery predictors only), indicating our choice of these characteristics for inclusion in the metric freeze endurance, and use in future comparisons among studies is physiologically robust. Our formal definition of freeze tolerance is defined upon duration of freezing survived, a minimum of 24 hours post ice inoculation, and may be driving the relationship of significant decrease in survival with freeze duration.

Mass was consistently a significant predictor of survival among freeze tolerant species in all models, survival decreasing with mass in the Full model, but increasing in the Minimal. Freezing survival increasing with mass is consistent with previous knowledge of increased storage of liver glycogen, and higher probabilities of survival in larger individuals (Jenkins and Swanson 2005). Mass did not predict survival in freeze intolerant species, supporting the concept that mass importance in survival in freeze tolerant species is related to cryoprotectant accumulation, not decreased cooling rates due to increased size or basic thermodynamic principles of heat loss being slower in larger mass objects with a decreased surface to area ratio. The finding that mass is negatively associated with survival in the full model is inconsistent with prior studies, but could allow for higher liver mass to body size ratios, allowing for a relatively larger accumulation, and thus cryoprotectant production, in smaller individuals.

Absolute latitude is also positively associated with freeze survival in freeze tolerant, but not intolerant, amphibians. Freeze tolerant amphibians are generally distributed at higher

latitudes (Figure 3), possibly due to biased sampling of species in regions of high human population density and reduced annual temperatures. Distinction between sampling bias, and increasing probability of freeze tolerance evolving in high latitude lineages cannot be made using these data, though cold tolerance is a conserved trait among northern distributed amphibians (Chejanovski and Wiens 2014; Olalla-Tárraga et al. 2011), suggesting an evolutionary basis to our findings. We found Absolute Longitude to be negatively associated with freezing survival, but this is most likely an artifact due to sampling bias of species with the most extreme freezing abilities being found only in North America and Eastern Russia, *Rana sylvatica* and *Salamandrella keyserlingii* (Figure 3).

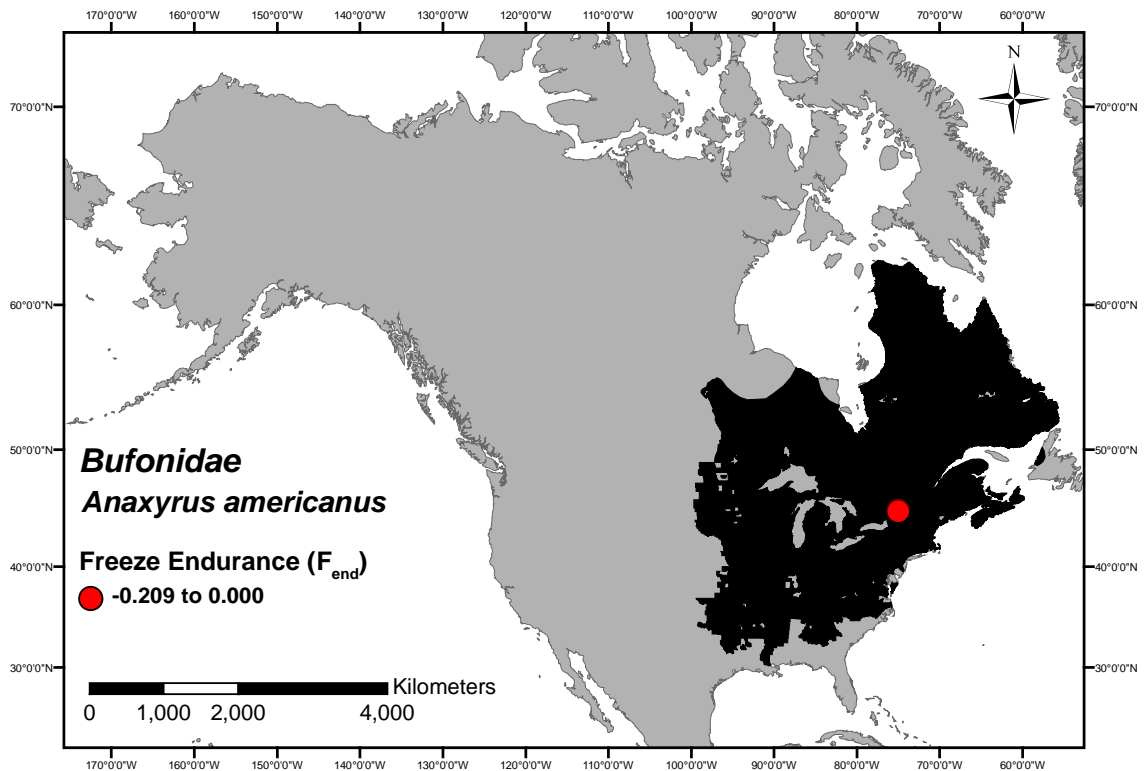
Freeze endurance, our new proposed measuring of freezing ability, varies widely among species, where lower values indicate higher survival rates at lower temperatures for longer time periods. This measure includes the highly significant predictors (duration and minimum temperature) of freeze survival from our models, provides direct comparison of freezing ability between freeze tolerant and intolerant amphibian species, and can be allometrically scaled to account for differences in body mass contributions to survival (Tables 2, 3, and 4). The lack of comparability between studies of freeze tolerance is widely recognized (Irwin and Lee 2003; Layne 1999; Rezende et al. 2014) and prevents hierarchical comparisons of individual, population, regional, and global variations in an already complex physiological trait. Temperature tolerance is thought to be a direct driver of global diversity patterns, making comparative studies particularly important as we expand macrophysiology to include predictive outcomes based on global climate change

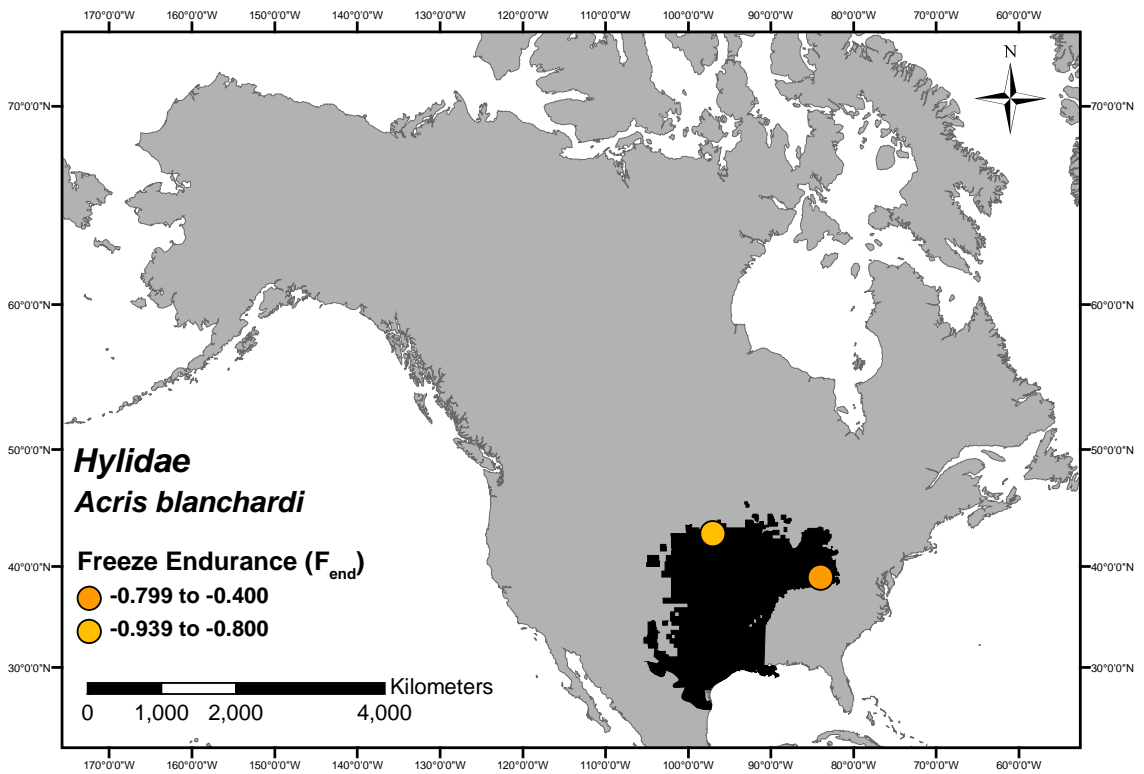
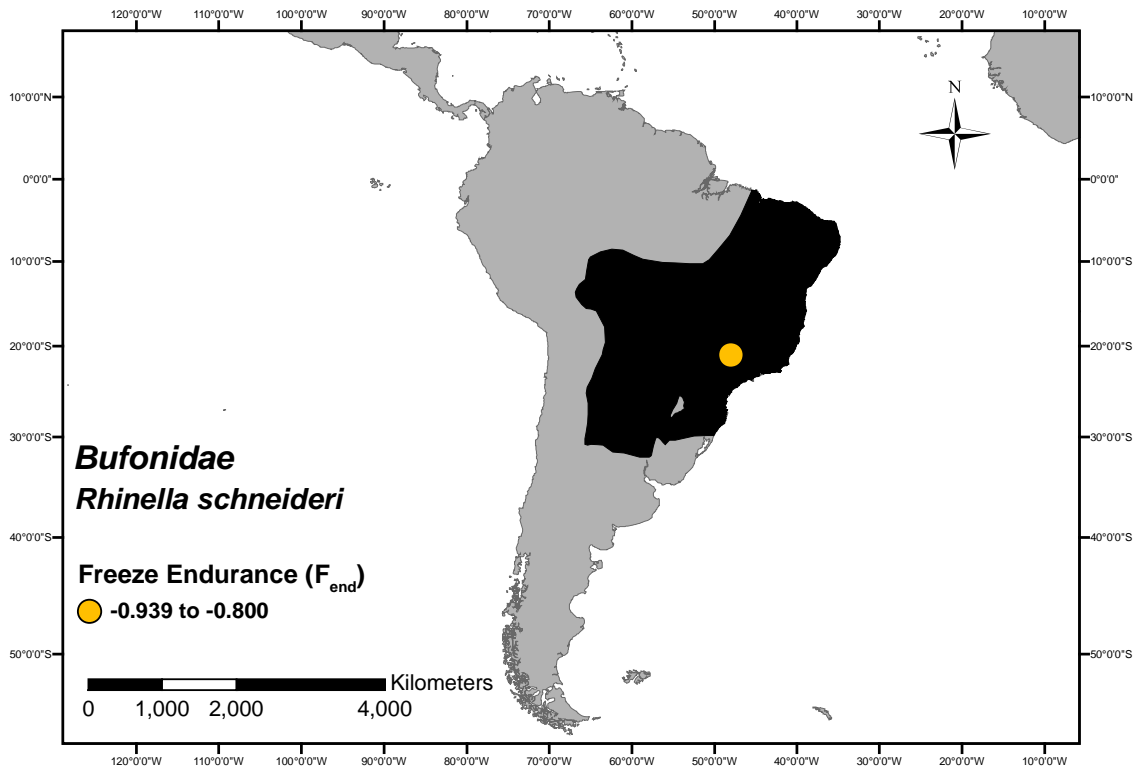
scenarios. Freeze endurance is a simple measure to calculate and can be applied to past studies to extend temporal comparisons. To date, freeze tolerance literature has generally lacked phylogenetic (Tables 2 and 3) and geographic (Figures 2) depth and breadth necessary to adequately separate adaptations related to dehydration and others stressors from those specific to freeze tolerance in amphibians. This standardized freezing protocol and freeze endurance metric will provide a framework for additional studies on freezing abilities in amphibians to increase data that are relevant to broader applications to medicine, macrophysiology, and species conservation.

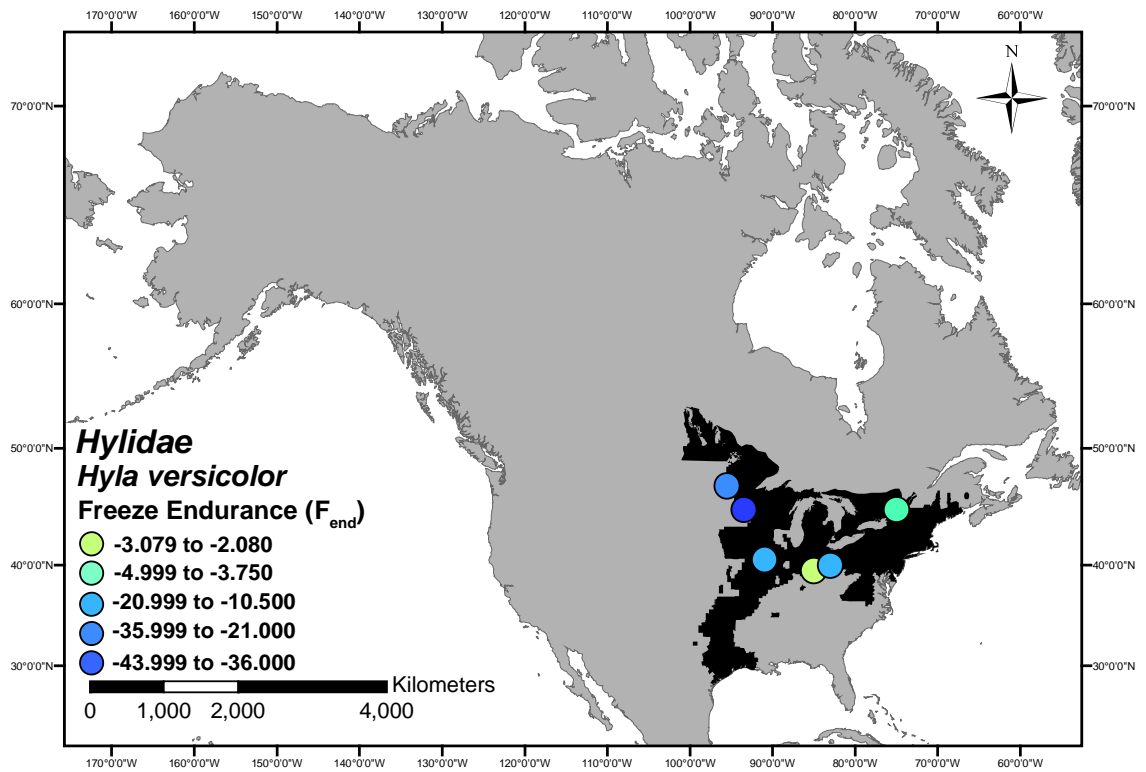
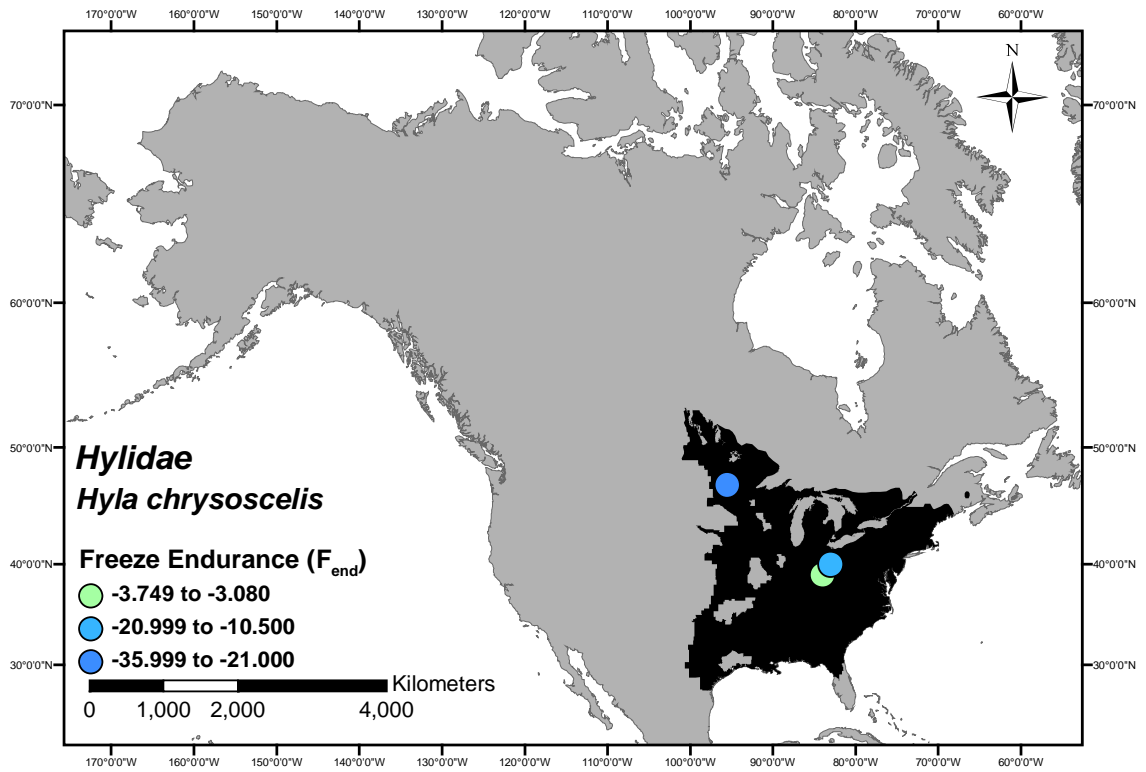
Appendix 1. Summary of species tested for freeze tolerances that have subsequently changed taxonomic species names, and the current taxonomy of each, as used in this publication.

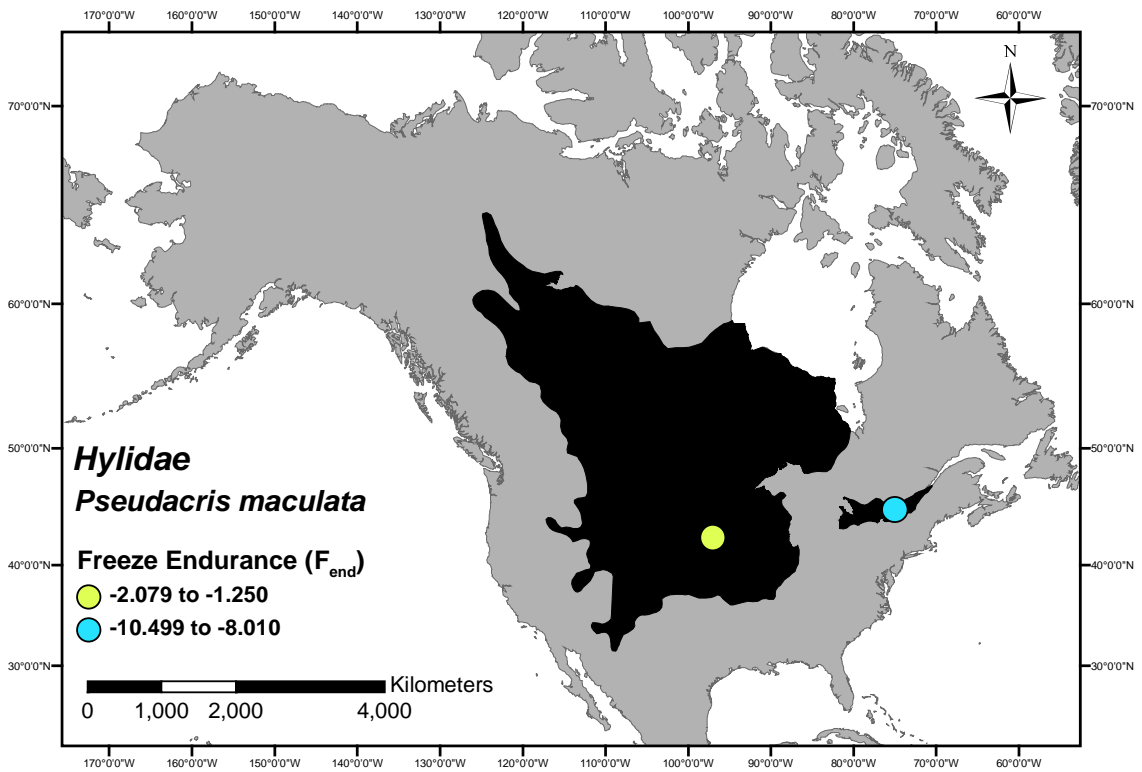
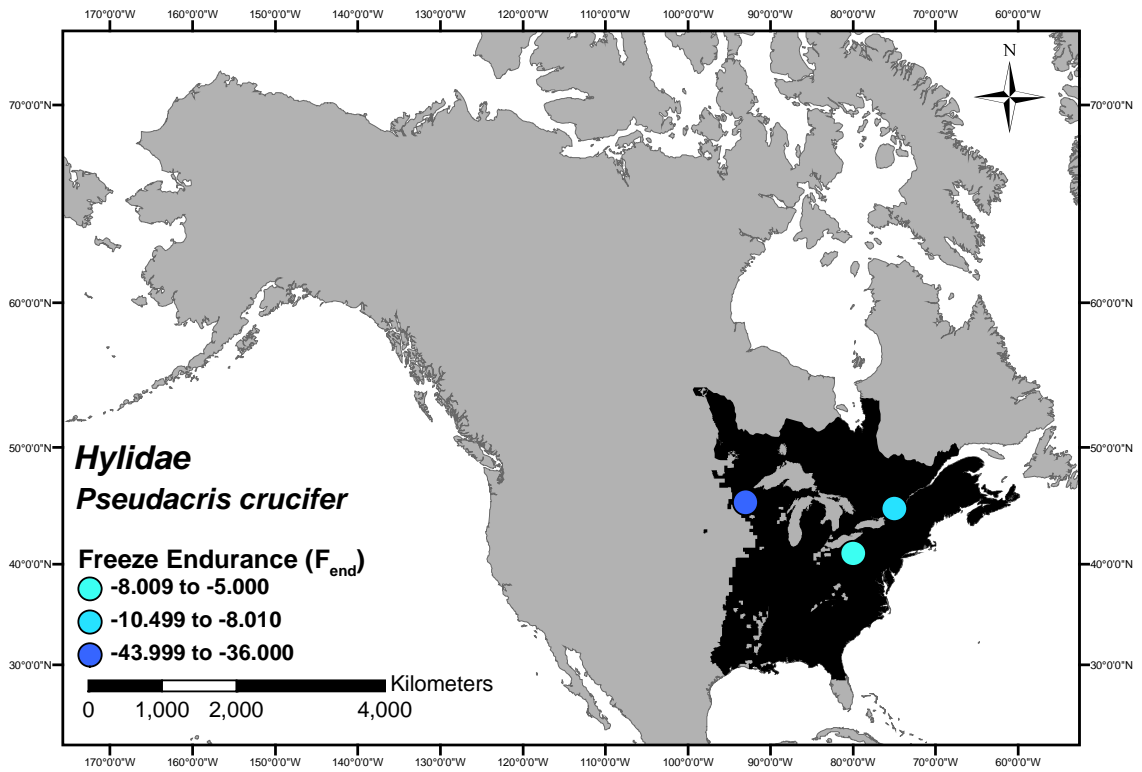
| Current Species Name | Species Name When Tested | Reason for Change in Species Name | Publication(s) |
|------------------------------|---------------------------------|--|--|
| <i>Acris blanchardi</i> | <i>Acris crepitans</i> | Sample locality, Taxonomic revision | (Irwin et al. 1999; Swanson and Burdick 2010) |
| <i>Anaxyrus americanus</i> | <i>Bufo americanus</i> | Taxonomic revision | (Storey and Storey 1986) |
| <i>Pelophylax esculentus</i> | <i>Rana esculenta</i> | Taxonomic revision | (Voituron et al. 2005) |
| <i>Pelophylax lessonae</i> | <i>Rana lessonae</i> | Taxonomic revision | (Voituron et al. 2005) |
| <i>Pelophylax ridibundus</i> | <i>Rana ridibunda</i> | Taxonomic revision | (Voituron et al. 2003, 2005) |
| <i>Pseudacris maculata</i> | <i>Pseudacris triseriata</i> | Sample locality, Taxonomic revision | (Storey and Storey 1986; Swanson et al. 1996) |
| <i>Rhinella schneideri</i> | <i>Bufo paracnemis</i> | Taxonomic revision | (Steiner et al. 2000) |

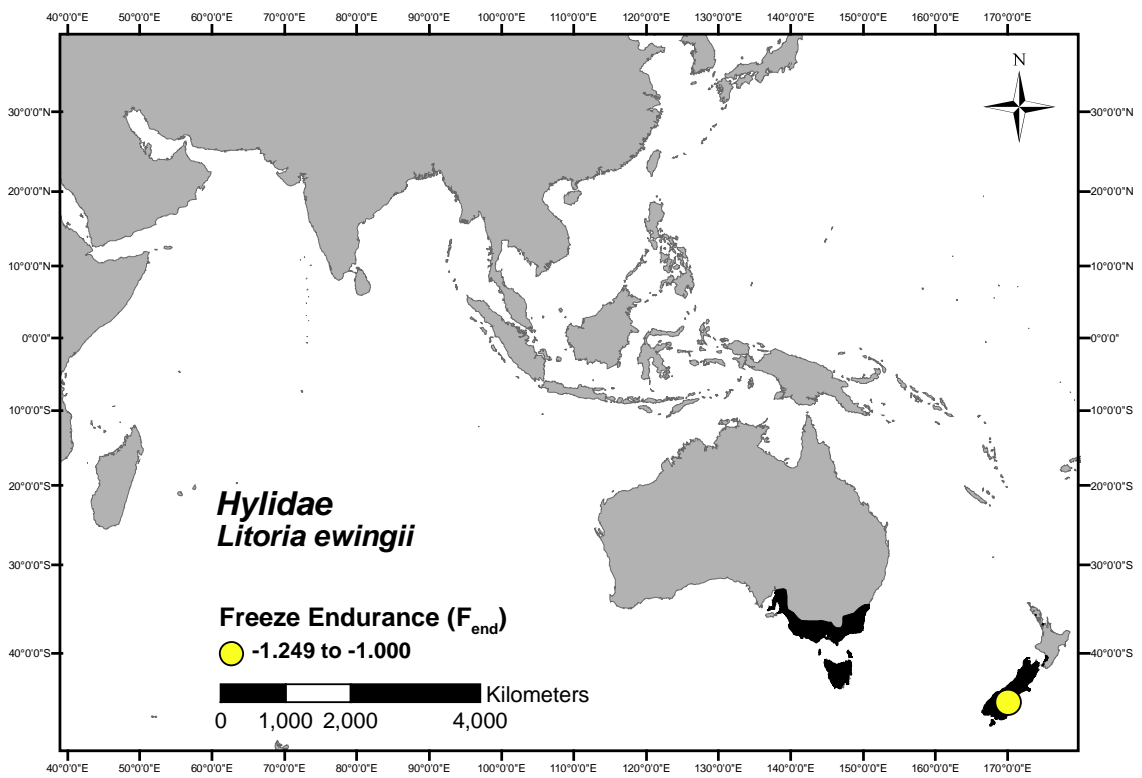
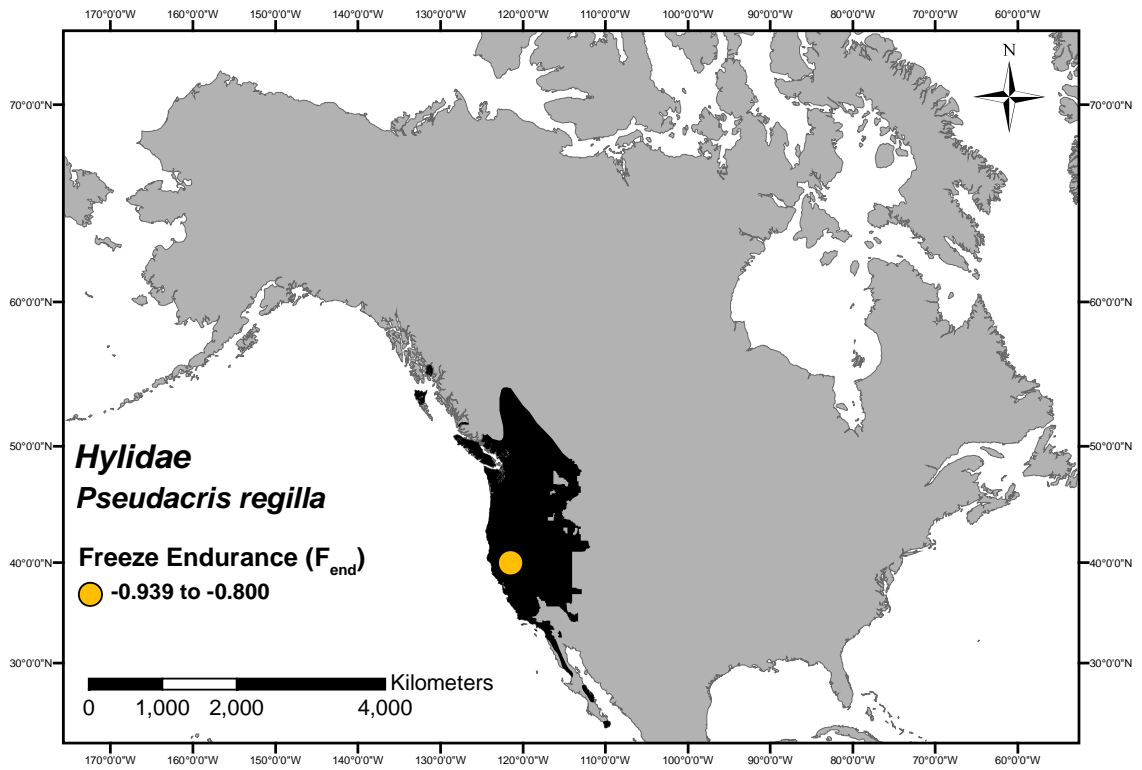
Appendix 2. Freeze endurance values for each species by locality. The red to blue color scale represents extremity of freeze endurance and is overlaid on the distribution of the species (black). Dark blue dots are localities where individuals showed extreme freeze endurance; bright red dots are localities where individuals showed little or no freeze endurance. Each species is shown alphabetically by family, genus, and species.

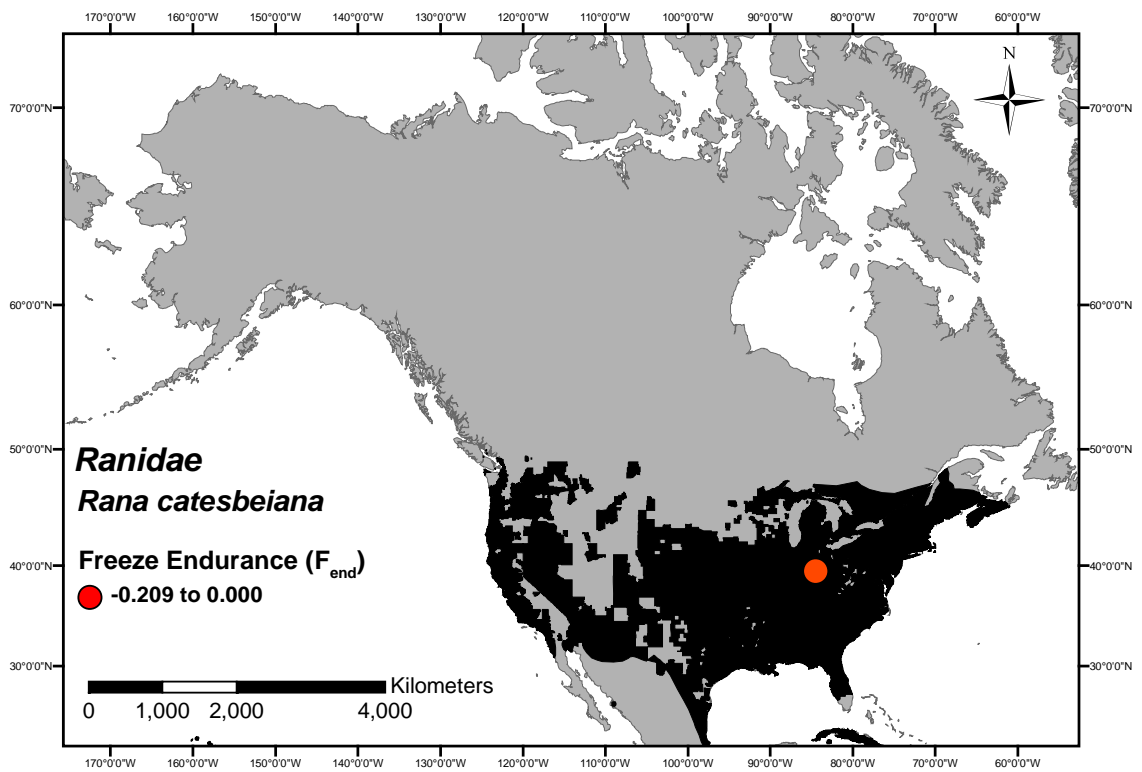
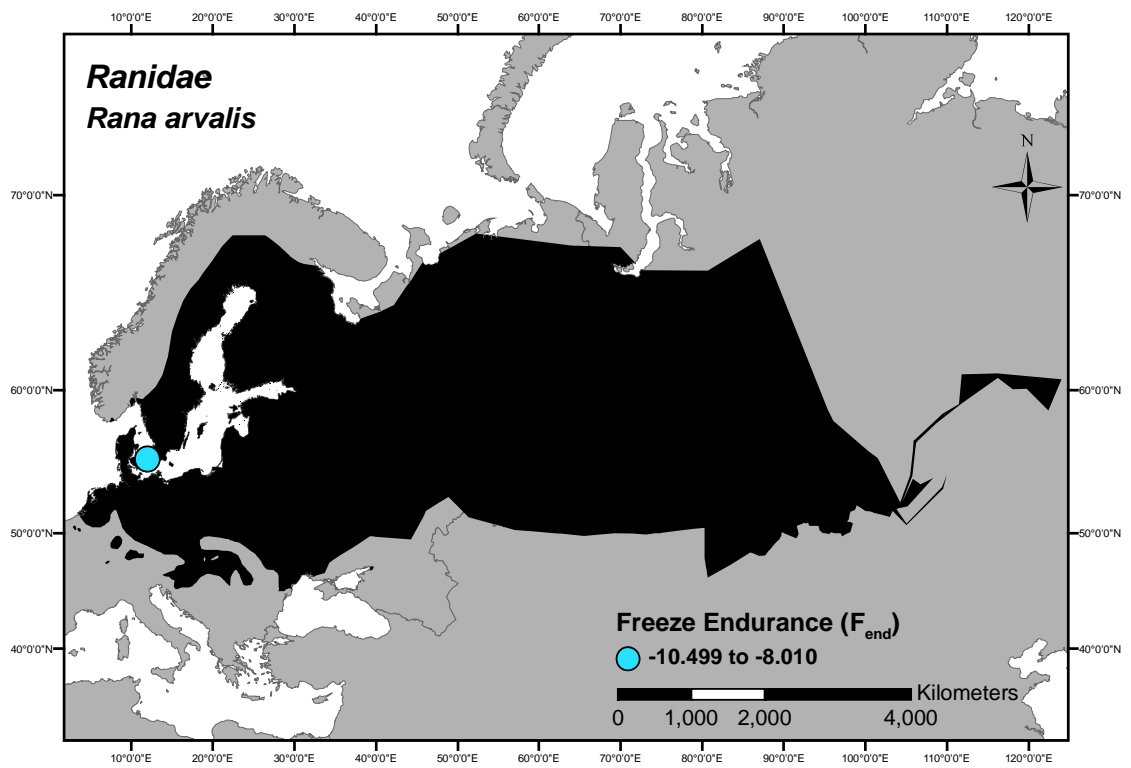


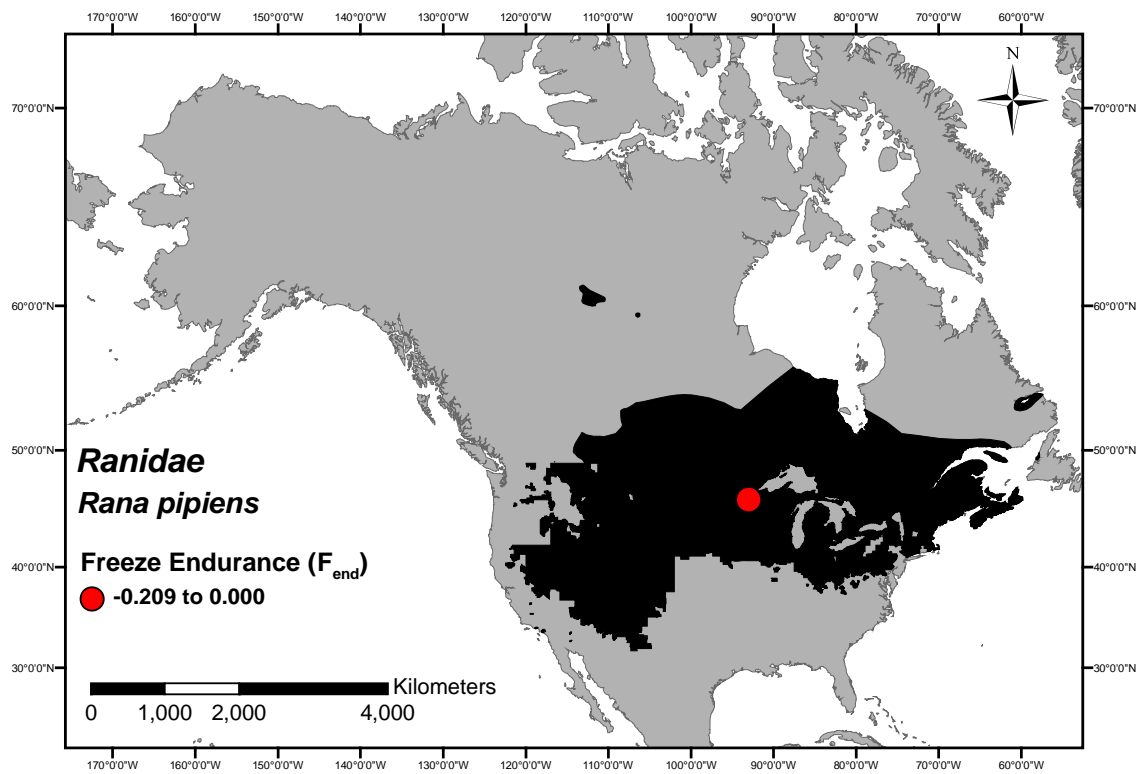
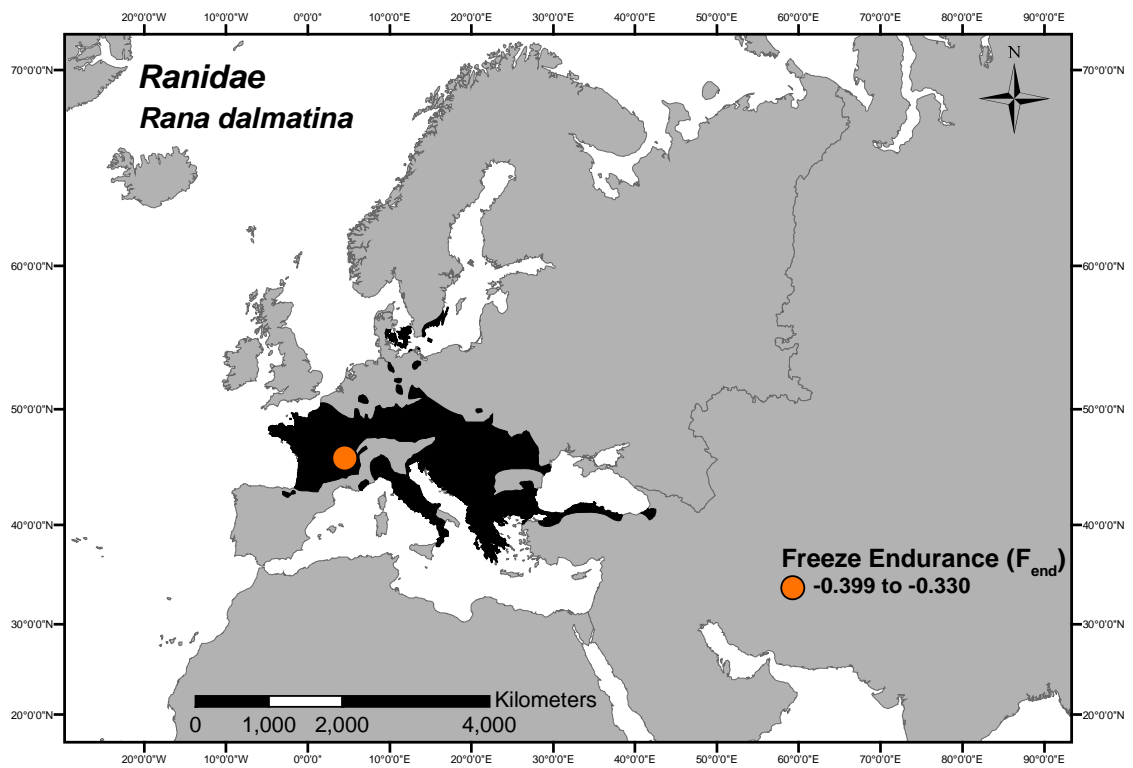


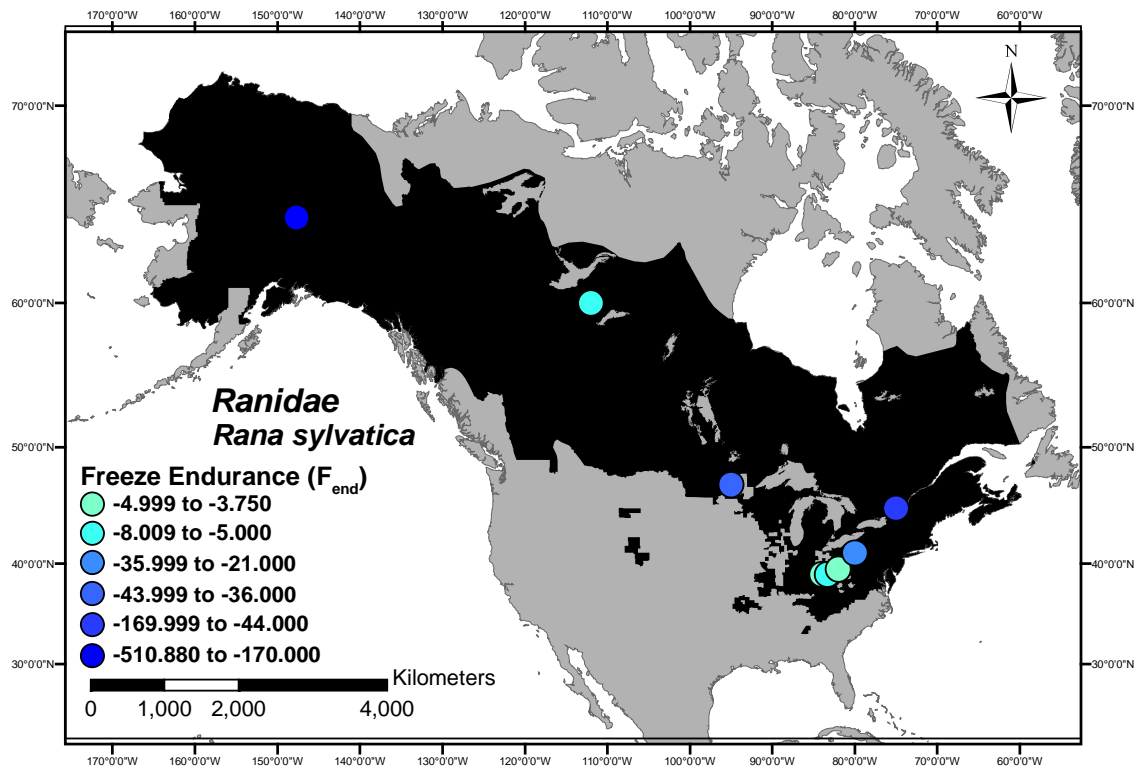
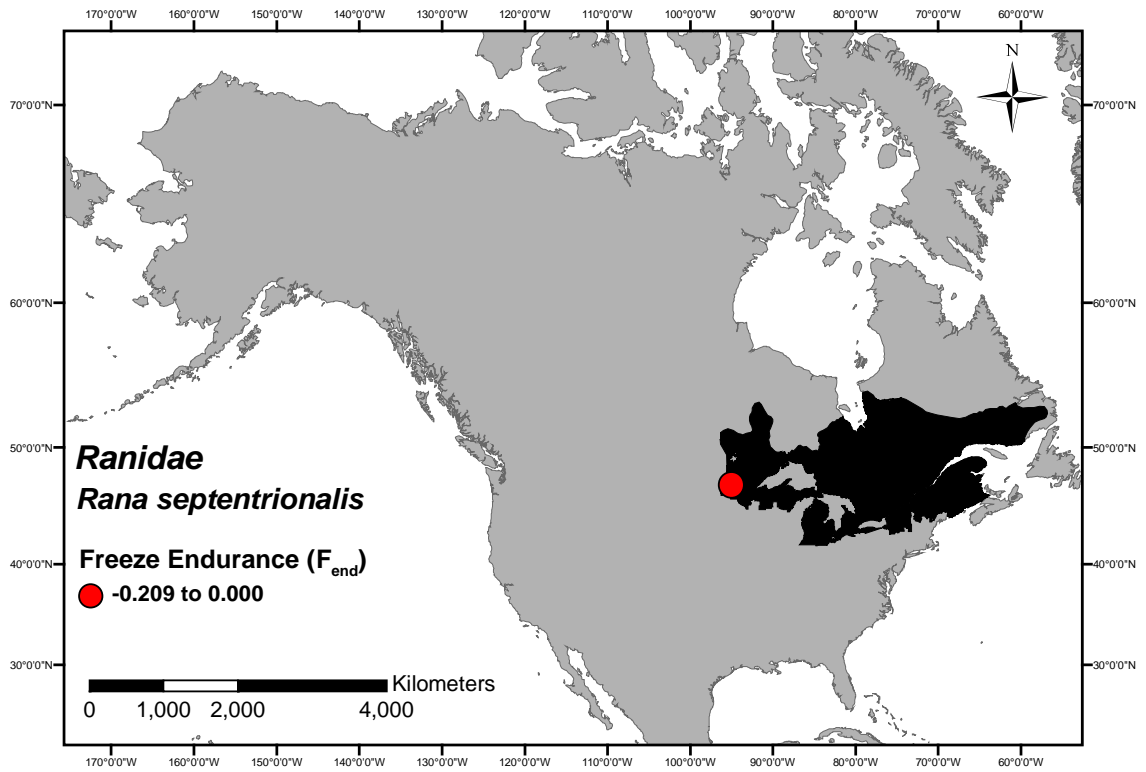


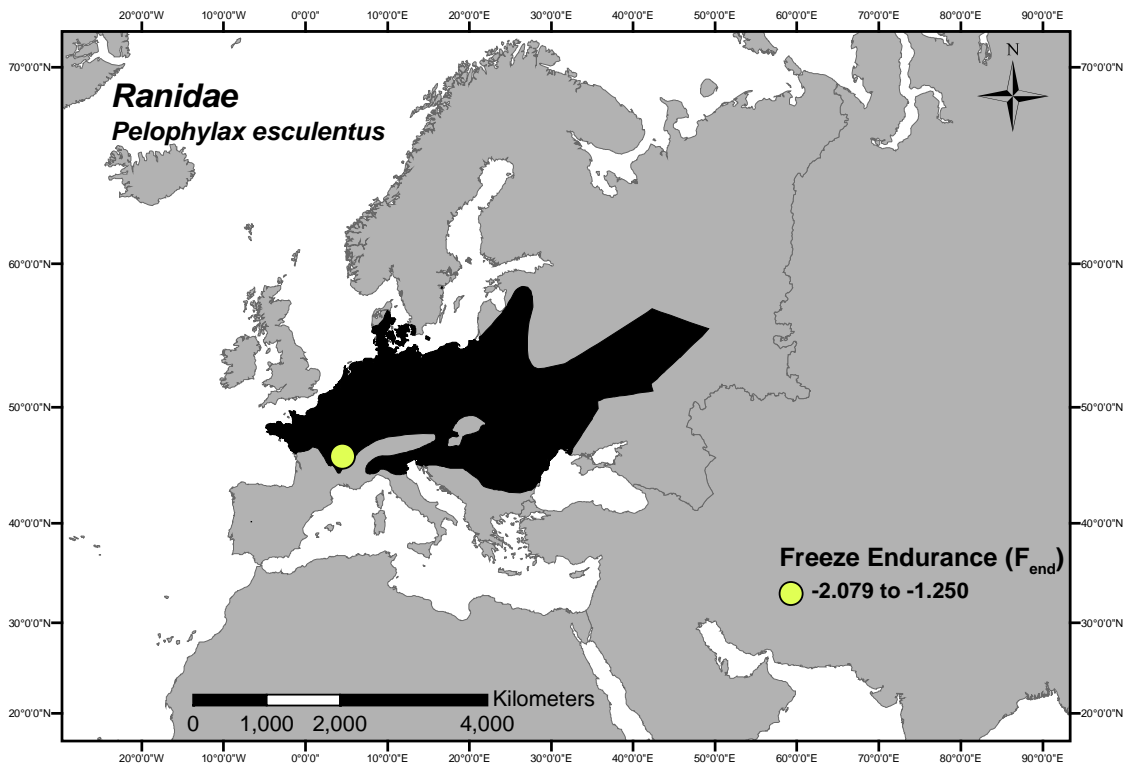
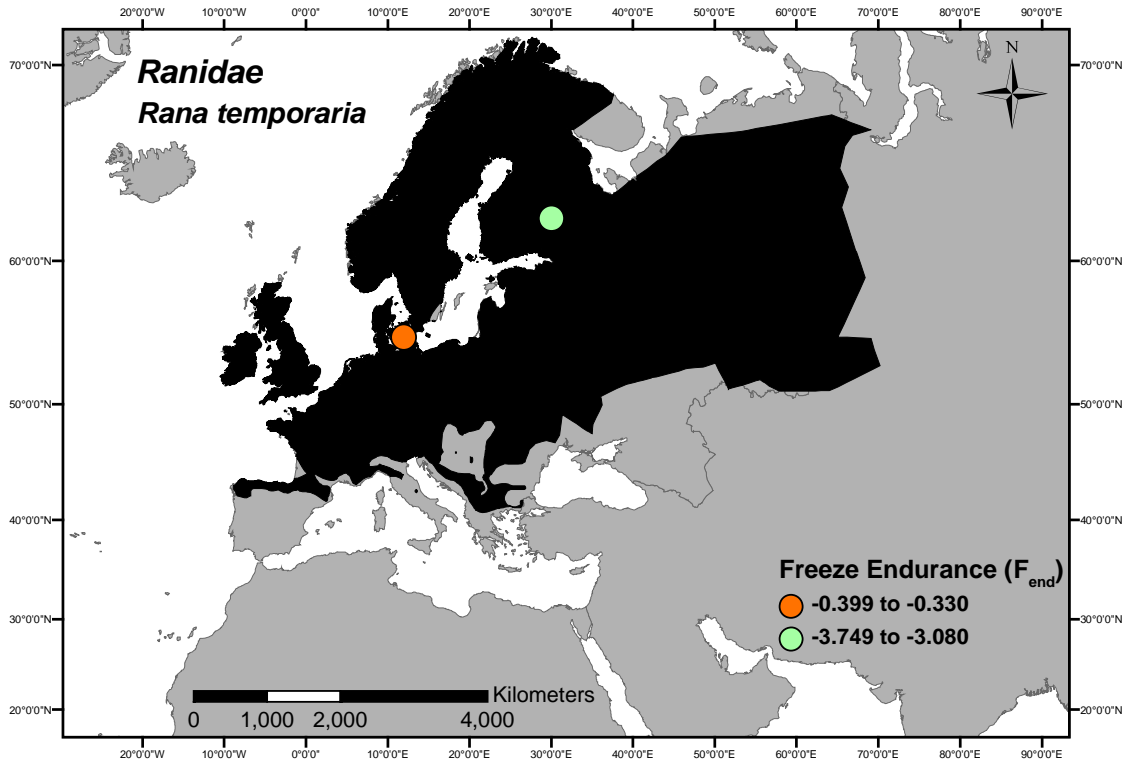


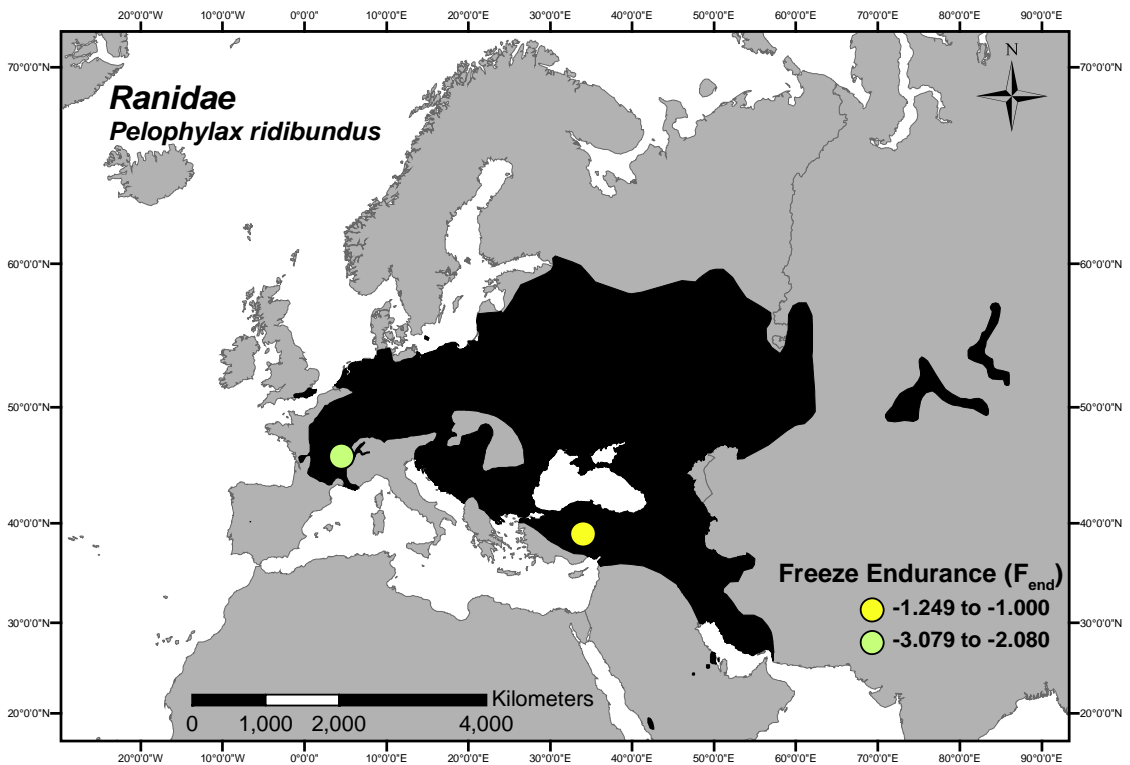
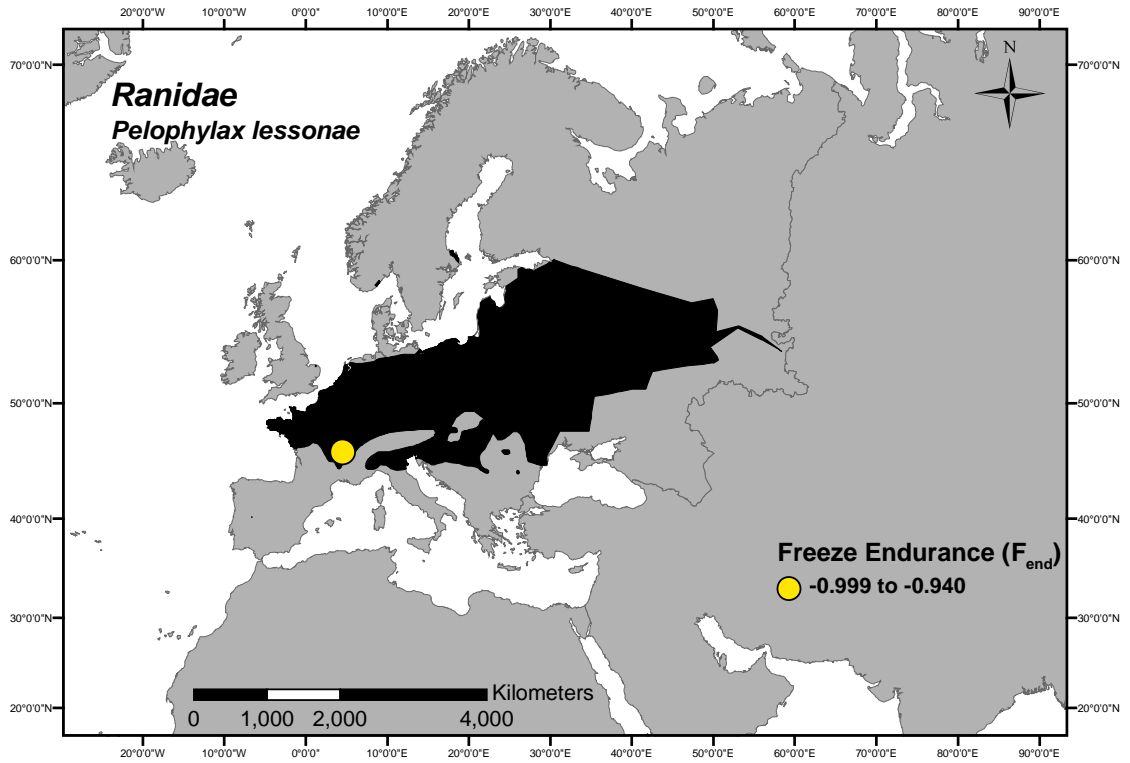


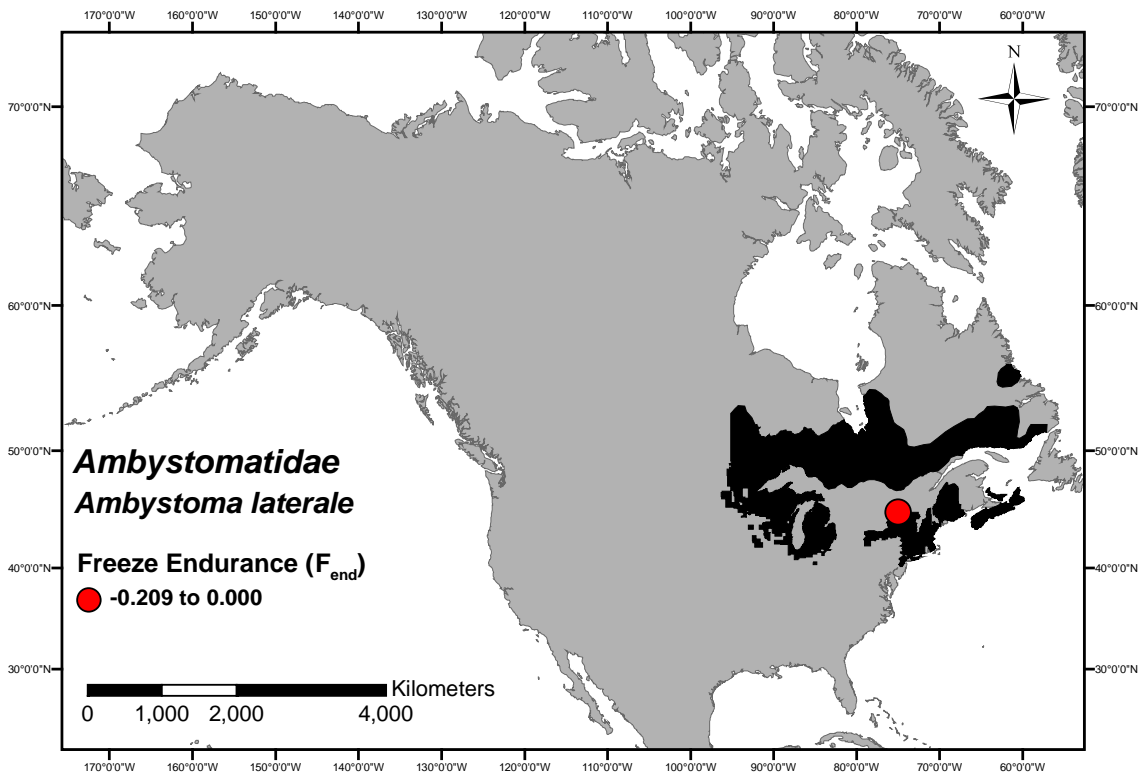
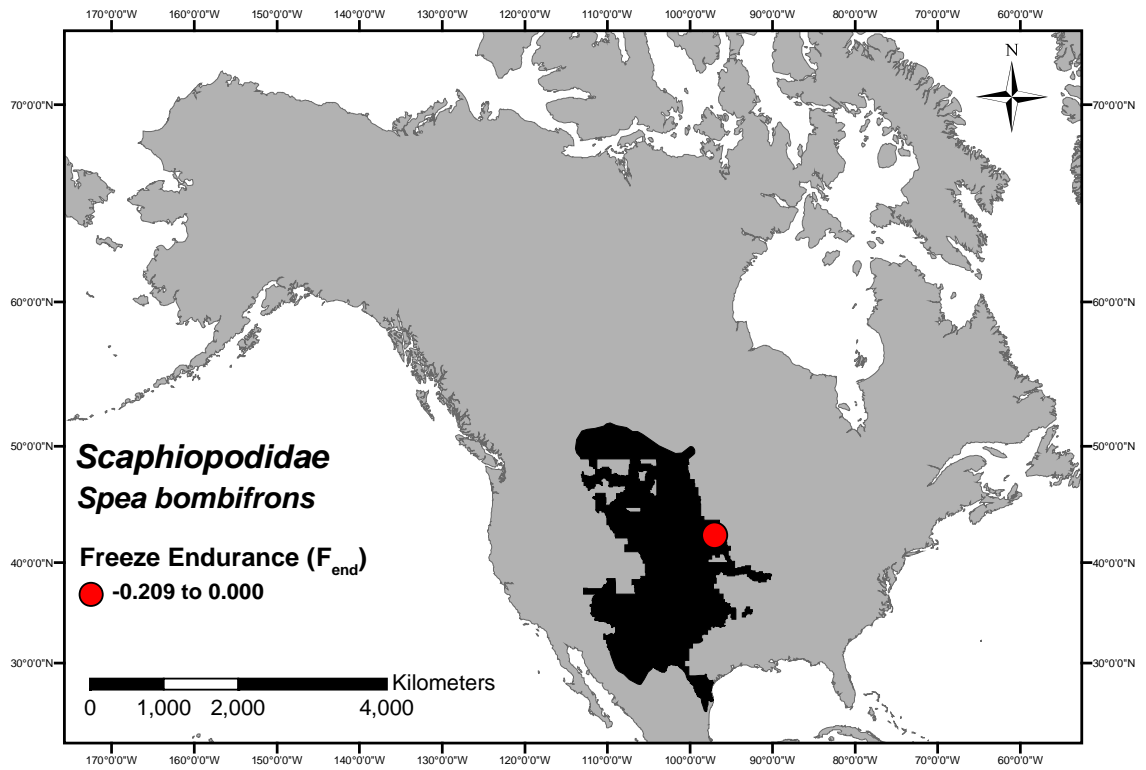


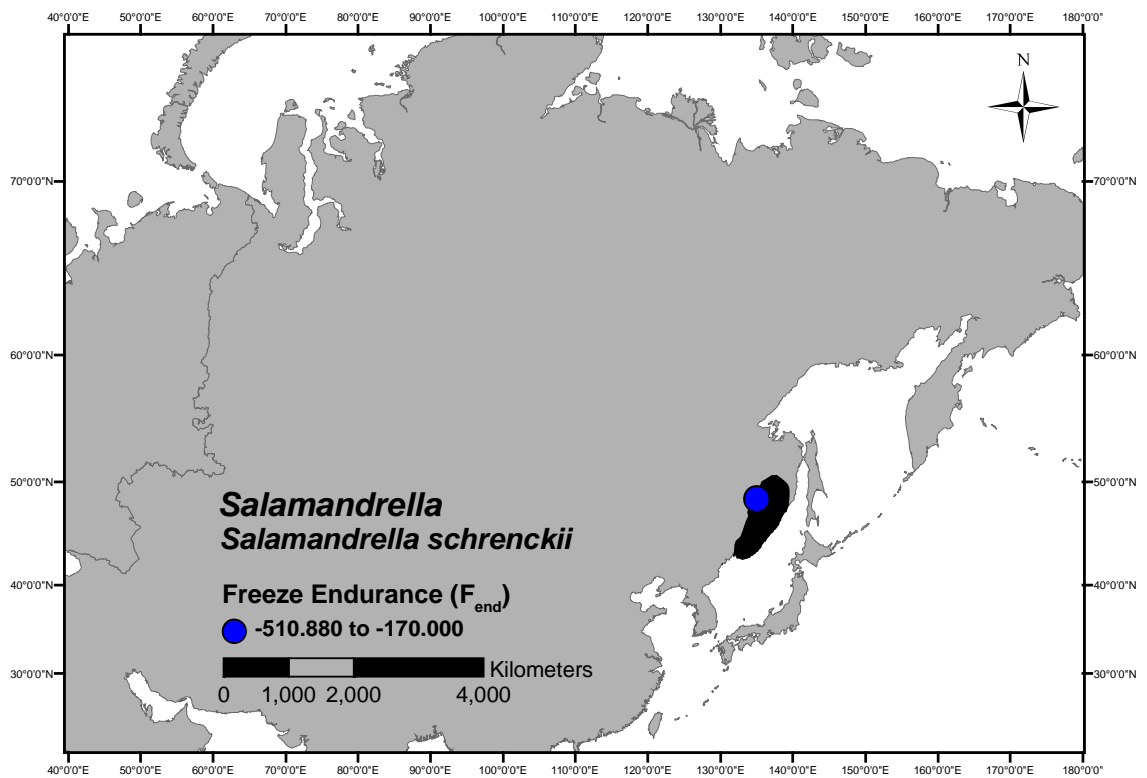
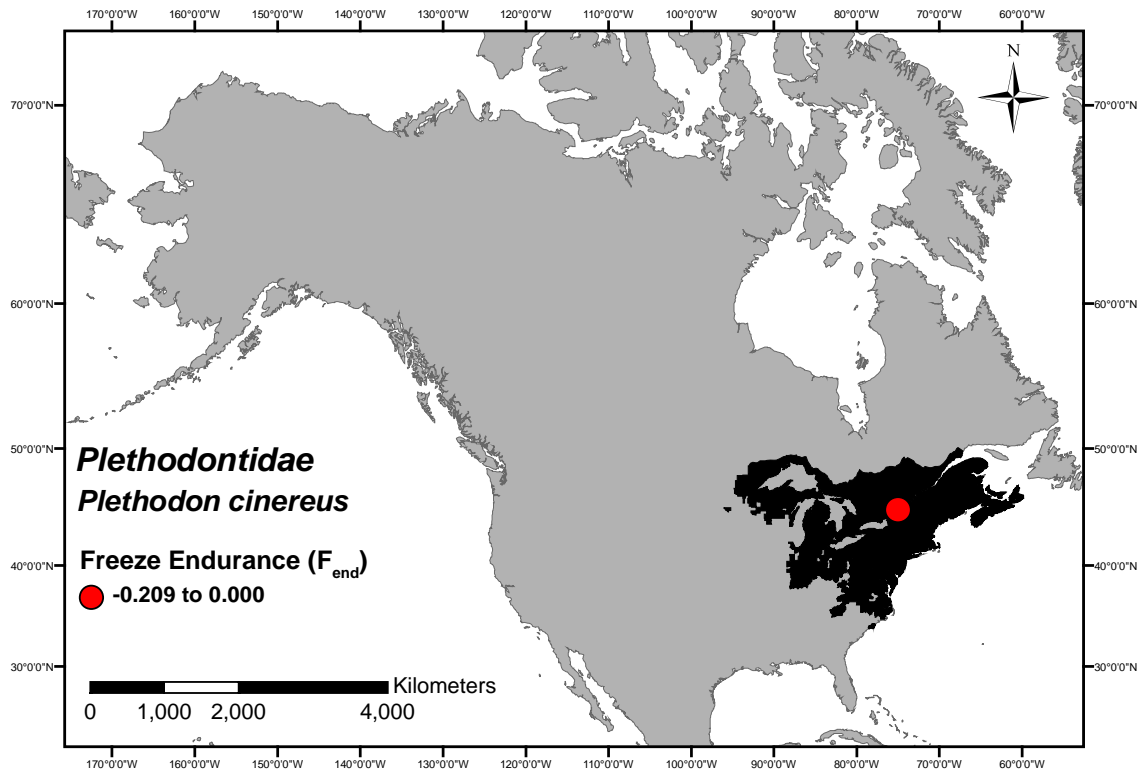


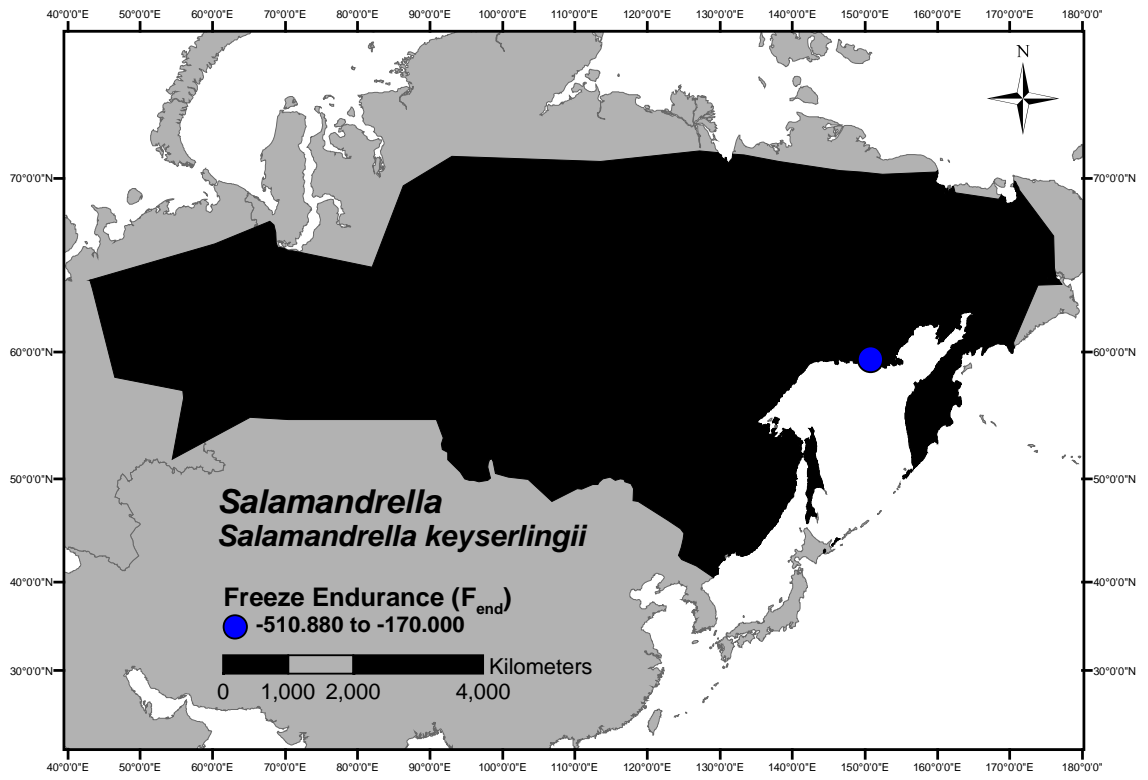












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