

Latitudinal Variation of Freeze Tolerance in Intertidal Marine Snails of the Genus *Melampus* (Gastropoda: Ellobiidae)

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ABSTRACT

Low temperatures limit the poleward distribution of many species such that the expansion of geographic range can only be accomplished via evolutionary innovation. We have tested for physiological differences among closely related species to determine whether their poleward latitudinal ranges are limited by tolerance to cold. We measured lower temperature tolerance (LT_{50}) among a group of intertidal pulmonate snails from six congeneric species and nine locales. Differences in tolerance are placed in the context of a molecular phylogeny based on one mitochondrial (cytochrome oxidase subunit I) and two nuclear (histone 3 and a mitochondrial phosphate carrier protein) markers. Temperate species from two separate lineages had significantly lower measures of LT_{50} than related tropical species. Range differences within the temperate zone, however, were not explained by LT_{50} . These results show that multiple adaptations to cold and freezing may have enabled range expansions out of the tropics in *Melampus*. However, northern range limits within temperate species are not governed by cold tolerance alone.

Introduction

Temperature imposes geographic limits (Gaston 2003; Koehler et al. 2012) in both aquatic (Hylleberg and Siegismund 1987;

Stillman 2002; Lee et al. 2009) and terrestrial (Castaneda et al. 2004; Hellmann et al. 2008) systems. On a global scale, cooler temperatures may limit the poleward spread of tropical taxa and contribute to global patterns of species richness (Sagarin et al. 1999; Wiens and Donoghue 2004). In particular, geographical shifts into areas that freeze require a means to deal with internal ice formation and ice exposure from the environment, achieved by either freeze tolerance or freeze avoidance (Lee and Costanzo 1998). Adaptations to deal with subzero temperatures have arisen many times and can function in a variety of ways (Doucet et al. 2009; Tattersall et al. 2012), but these adaptations may be costly or evolve only rarely (Hoffmann 2009; Wiens 2011).

In marine organisms, physiological determinants of range have been well studied, particularly in the intertidal zone (Sagarin et al. 1999; Stillman and Somero 2000; Dahlhoff et al. 2002; Helmuth 2002; Rivadeneira and Fernandez 2005; Helmuth et al. 2006). Evidence from the fossil record suggests that many marine species have geographic ranges that are conserved across a phylogeny and are often centered in the tropics (Briggs 1999; Jablonski 1999; Roy et al. 2009). This suggests that the evolution of traits to tolerate cooler temperatures may underlie range expansion in these groups (Spicer and Gaston 1999). If this is true for extant species, variation in temperature tolerance is expected to correspond to differing latitudinal range limits. This can be tested in groups of related species with differing latitudinal ranges (Somero 2011; De Frenne et al. 2013).

The pulmonate snail genus *Melampus* (Ellobiidae, Say 1822) occurs in high intertidal habitats worldwide, primarily in the tropics (Martins 1996). Adults breathe air and assume a nearly terrestrial life, but they are dispersed by planktonic larvae that can travel over hundreds of kilometers, such that each generation potentially arrives in a new environment (Russell-Hunter et al. 1972; Dennis and Hellberg 2010). In the Atlantic, only two described species are found above latitude 30°N: *M. bidentatus* and *M. floridanus*.

Freeze tolerance has been well studied in the nominal species *M. bidentatus*, where production of an ice-nucleating protein enables seasonal freeze tolerance (Hilbish 1981; Hayes and Loomis 1985; Loomis 1985). This ice-nucleating protein causes freezing and confers greater freeze tolerance by influencing the location and rate of ice formation (Ansart and Vernon 2003; Sinclair et al. 2004). Three cryptic species (hereafter referred to as North, South, and Gulf) have been identified within *M. bidentatus*, two of which are geographically restricted to the temperate zone but differ in their latitudinal extent (Dennis and Hellberg 2010). Among mid-Atlantic populations, greater freeze tolerance has been found in populations located farther north (Hilbish 1981), but the genetic

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basis of this variation is not known, nor has it been tested in light of the cryptic species.

To test for physiological differences that coincide with geographic range, we have compared lower thermal tolerance (LT_{50}) among six of the nine Atlantic *Melampus*, including the three cryptic species of *M. bidentatus*. To provide context for these results, in particular, to determine whether tolerance of subzero temperature has arisen more than once, we must also determine the relationships among Atlantic *Melampus*. We have generated a molecular phylogeny that includes the species whose LT_{50} we have measured, three additional *Melampus*, and an outgroup. We also compare air temperature among locales to determine which regularly experience freezing conditions. We find that temperate species in two lineages tolerate similar subzero temperatures, while the tropical species tested is intolerant of subzero conditions. These results suggest that northern range expansions in this historically tropical group may have been enabled by the advent of multiple adaptations to freezing and that range differences among temperate species are likely determined by factors other than temperature alone.

Material and Methods

DNA Sampling

To infer relationships among Atlantic *Melampus*, we sampled eight of the nine western Atlantic species, two Pacific species, and the ellobid *Tralia panamensis* as an out-group (fig. 1; table 1). This included all described *Melampus* in the Western Atlantic except for *M. paranus*, a species reportedly found in Surinam and Brazil (Martins 1996), for which samples could not be obtained.

DNA was extracted from ethanol-preserved samples using the QIAamp DNA Mini Kit (Qiagen; catalog no. 51304) with overnight tissue digestion at 56°C. Portions of three genes were sequenced to infer phylogenetic relationships across *Melampus*. A 448-bp region of the mitochondrial cytochrome oxidase subunit I (*COI*) gene was amplified using HCOI (Folmer et al. 1994) and a modified version of their LCOI (MCOI: GGTCAA-CAAATCATAAGGATATGG). Samples that were unsuccessful with these primers were amplified by polymerase chain reaction (PCR) using degenerate primers designed to overlap the same region (COIF-degen: ACAAATCAYAARGAYATYGG; COIR-degen: TTCAAGRTGNCCRAARAAYCA). This product was isolated by TOPO TA cloning (Invitrogen; catalog no. K4575) and sequenced using the M13 primers provided by Invitrogen. A 316-bp region of the histone 3 (*H3*) gene was amplified using H3F and H3R (Colgan et al. 2000). A 159-bp nuclear region that is tentatively identified as a portion of a mitochondrial phosphate carrier protein was amplified using MCPL/MCPR (Dennis and Hellberg 2010). PCR was used to amplify *COI* and *H3* reactions with an annealing temperature of 50°C and *MCP* with an annealing temperature of 52°C. All sequence data were edited and aligned in Geneious (ver. 5.6; Biomatters; <http://www.geneious.com>); there were no indels.

Phylogeny

The closest available approximations of the models of molecular evolution identified by jModeltest (Guindon and Gascuel 2003; Posada 2008) were implemented in MrBayes (ver. 3.2.1; Ronquist et al. 2012). The following departures from default settings in MrBayes were implemented. All markers used the general time-reversible (GTR) model with gamma-distributed rate variation across sites. *COI* was designated haploid, with a designated prior for each of the six substitution rates for the GTR model (revmatpr) set at (1.0000, 24.2101, 2.5881, 2.5881, 39.9434, 1.0000) and the estimated prior state frequencies (statefreqprior) set at (0.2640, 0.1756, 0.2256, 0.3349). *H3* also used an estimated revmatprior (1.0000, 1.7787, 1.0000, 1.0000, 9.7527, 1.0000) and had a fixed statefreqprior. *MCP* used an estimated revmatprior (1.0000, 16.3274, 7.1978, 7.1978, 16.3274, 1.0000) and a fixed statefreqprior. This partitioned analysis was run for 100 million generations with a sampling frequency of 1,000 and was summarized with a 30% burn-in.

Cold-Tolerance Trials

In January and February of 2008 and 2009, six Atlantic *Melampus* species were collected for immediate cold-tolerance trials (table 2). In 2008, trials included *M. bidentatus* North and South collected from four sites and were conducted at Connecticut College. In 2009, this was expanded to include *M. bidentatus* Gulf, *M. floridanus*, *M. coffeus*, and *M. bullaoides* (table 2; fig. 2), and trials were conducted at Louisiana State University. In both years, Fenwick Island, Delaware, was included because *M. bidentatus* North and *M. bidentatus* South occur there in sympatry. Trials from Barn Island, Connecticut (table 2, 2009b), were conducted several weeks late due to thick ice cover and were accompanied by a repeated test of *M. bidentatus* South from Fourchon, Louisiana (table 2).

Before testing, live snails were maintained for a minimum of 5 d in humid incubators at a constant temperature (5°–7°C) with a 12L : 12D light-dark cycle and no food. With the exception of *M. bullaoides*, mortality in the incubators was very low (less than 5%). Collections of *M. bullaoides* maintained at 5°C experienced a die-off within several days. The remaining healthy animals were transferred to a maintenance temperature of 25°C and experienced little subsequent mortality; only those moved to 25°C were used in the trials presented here.

To calculate lower lethal temperature (LT_{50}), dose mortality responses were measured at intervals of 1°C across temperatures between –4° and –15°C. The temperature range of these trials was chosen to include temperatures at which species experienced both zero and 100% mortality and to include LT_{50} 's previously observed across the year in *Melampus* collected in Connecticut (–5° to –13°C; Loomis 1985). Once zero and 100% mortality were observed, the population was excluded from more extreme trials. As we recorded results, half-degree steps (–7.5°, –8.5°, and –9.5°C) were also included to increase precision. *Melampus coffeus* and *M. bullaoides* were tested at warmer temperatures than the other species. These were +3°

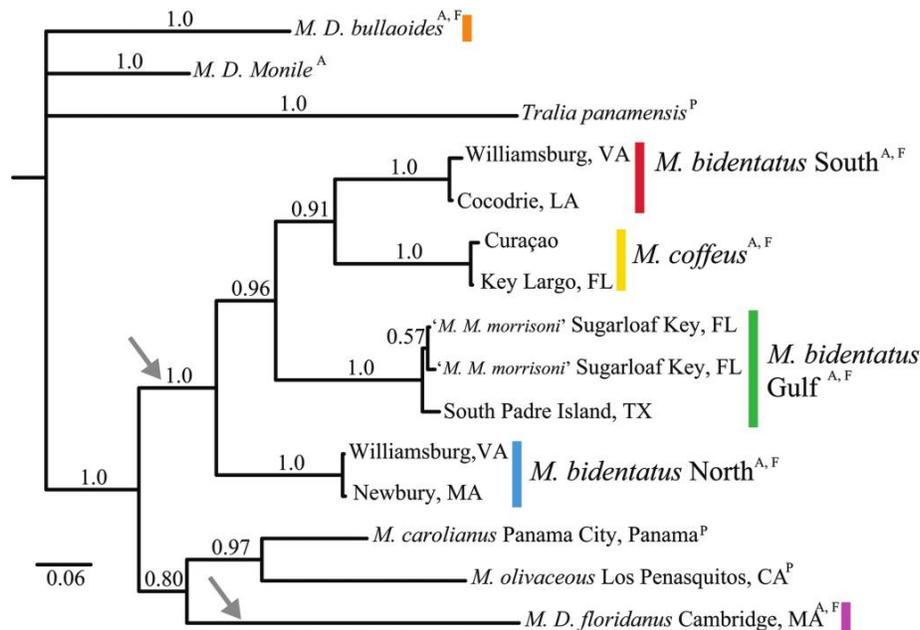


Figure 1. Bayesian consensus tree based on three genes (*COI*, *H3*, and *MCP*). Gray arrows indicate lineages that tolerated freezing conditions. Species included in tests of lower lethal limit are marked by a superscript F, Pacific species are marked by a superscript P, and Atlantic species are marked by a superscript A. Posterior probabilities are shown above each branch. Colored bars correspond to the range map (fig. 2).

and -3°C for *M. coffeus* and $+5^{\circ}$, $+3^{\circ}$, $+1^{\circ}$, and -3°C for *M. bullaoides*. The full list of trials and their results is available in table A1 (tables A1–A4 are available online). In most exposures (table A1 details exceptions), 10 individuals from each site were placed in 50-mL tubes with a clean paper towel dampened with 25-ppt sterile sea water. For the Fenwick Island population, two replicates of 10 individuals were tested, and the species they contained were later identified using *COI* sequence as described below. Tubes were sealed and submerged in a $+5^{\circ}\text{C}$ ethylene glycol bath (Forma Scientific in 2008 and Thermo Digital One in 2009). After a 5-min acclimation, temperature was reduced at 1°C per minute and held constant for 24 h before being returned to $+5^{\circ}\text{C}$.

To assess mortality, snails were transferred to fresh containers and placed aperture down for 24 h at $+5^{\circ}$ to $+8^{\circ}\text{C}$. They were then flipped onto their backs and given 24 h at 25°C to right themselves. Mortality was assessed by whether the snail had righted itself or whether the foot responded to a poke from a metal probe. LT_{50} was calculated in GraphPad Prism (ver. 5.0.2; <http://www.graphpad.com>), in which the mortality was fit as a function of the logarithm of temperature. Survival curves were best fit by the sigmoidal curve (i.e., three-parameter logistic equation), for which r^2 averaged 0.78 (0.44–1.0). Wide confidence intervals around LT_{50} in several 2009 trials may have been due to acclimation by the snails during the 1-mo trial period, over which time survival increased slightly. To test for a relationship between the latitudinal distribution of a species and its lower lethal limit, a Spearman's rank correlation was

calculated between the calculated LT_{50} and the northernmost range limit of each species (table A3).

Molecular Identification of Sympatric Cryptic Species

The co-occurring cryptic species (*M. bidentatus* North and South) collected from Fenwick Island, Delaware, cannot be morphologically distinguished. Thus, the 20 individuals tested in each trial were differentiated by subsequent restriction digest. DNA was extracted using Chelex beads (Sigma; catalog no. C7901) and was amplified by PCR for *COI* as above. Product was then digested using two restriction enzymes, *RsaI* (NEB; catalog no. R0167S) and *ScrFI* (NEB; catalog no. R0110S), and verified on 1% agarose gel. Inconclusive results were sequenced using the HCOI primer and compared with previously collected sequences for these species (Dennis and Hellberg 2010).

Supercooling Point

The temperature at which an organism freezes, or its supercooling point, is a useful measure to determine its cold-tolerance strategy. If *Melampus* is freeze tolerant, internal ice formation will occur at a temperature that is warmer than its LT_{50} ; if it is freeze avoidant, ice formation is lethal, and the supercooling point will be colder than, or near, its LT_{50} . Loomis (1985) found that winter collection *M. bidentatus* originating from Connecticut were freeze tolerant. To confirm this and test *M. bidentatus* South, the supercooling point was measured

Table 1: Summary of collections of *Melampus*

Species	Geographic range	Collection locale(s)
<i>M. Melampus bidentatus</i> (Say) North	Southern Canada to northern Florida	Newbury, MA ^a Beebe Cove, CT; Barn Island, CT Williamsburg, VA ^a Indian River, DE; Fenwick Island, DE
<i>M. M. bidentatus</i> (Say) South	Eastern Texas to Florida and North Carolina to Delaware	Williamsburg, VA ^a Fenwick Island, DE Cambridge, MD Cocodrie, LA;^a Fourchon, LA
<i>M. M. bidentatus</i> (Say) Gulf	Southern Texas and southern Florida; likely occupies the northern Caribbean	Florida Keys, FL South Padre Island, TX ^a
<i>M. M. coffeus</i> (Linnaeus 1758)	Florida, southern Gulf of Mexico, West Indies, and South American coast to Brazil (Martins 1996)	Naples, FL Florida Keys, FL ^a Curaçao ^a
<i>M. Detracia morrisoni</i> (Martins 1996)	Southern Florida, Bahama Islands, Cuba, and the Yucatán, Mexico (Martins 1996)	Sugarloaf Key, FL ^a
<i>M. D. bullaoides</i>	Southern Florida, West Indies to Suriname (Martins 1996)	Florida Keys, FL^a
<i>M. D. floridanus</i>	New Jersey to Florida and Gulf of Mexico to Vera Cruz; absent from Florida Keys (Martins 1996)	Cambridge, MD^a
<i>M. D. monile</i> (Bruguière 1836)	Florida, West Indies, Central America to Brazil (Martins 1996)	Florida Keys, FL ^a
<i>M. olivaceous</i> (Carpenter 1856)	Southern California to Mazatlán, Mexico, including the Gulf of California (Keen 1971)	Los Penasquitos lagoon, CA ^{a,b}
<i>M. carolianus</i> (Lesson 1842)	Costa Rica to Ecuador and the Galapagos Islands (Keen 1971)	Panama City, Panama ^{a,b}
<i>Tralia panamensis</i>	Costa Rica, Panama City, and Perlas Islands (Keen 1971)	Punta Culebro, Panama ^{a,b}

Note. Boldfacing indicates locales used in tests of lower lethal limit.

^aLocales included in the phylogenetic analysis.

^bSamples from the Pacific.

alongside the 2008 cold-tolerance trials. Supercooling point was measured in snails collected from two sites containing *M. bidentatus* North (Beebe Cove, CT, and Barn Island, CT) and one site containing *M. bidentatus* South (Cocodrie, LA). For this, individual snails were suspended in a 50-mL tube with a thermocouple (OMEGA H309 data logger) attached to the shell surface with a rubber band to measure surface temperature. Data were recorded to a computer every 1 s using SE309 software, v2.5.0.0. Sealed tubes were submerged in the ethylene glycol bath in the same manner as described above. Freezing point was identified by a positive spike in temperature, caused by the release of the latent heat of crystallization. This value was averaged across all trials for each site ($n = 5-9$; table A2), and the standard deviation was calculated in Excel.

Environmental Data

Temperature at each locale was approximated using hourly air temperature data (National Oceanic and Atmospheric Administration National Climatic Data Center) collected at nearby

(1–29 km) recording stations. These were used to calculate the number of days below 0°C, the number of months with freezing temperatures, and the minimum absolute temperature at each collection site (table 3).

Results

Relationships among Atlantic *Melampus*

Atlantic *Melampus* are not a monophyletic group with respect to the Pacific species, and the two tropical species (*M. bullaoides* and *M. monile*) are only distantly related to the other species sampled (fig. 2). Relationships among the cryptic species of *M. bidentatus* and *M. coffeus* (subgenus *M. Melampus*) were well supported (posterior probability >0.9). However, this data set places *M. coffeus* not as sister to the *M. bidentatus* group but as nested within it. Despite the morphological placement of *M. morrisoni* within the subgenus *Detracia* (Martins 1996), individuals morphologically identified as *M. morrisoni* grouped within *M. bidentatus* Gulf; *M. bidentatus* Gulf sampled from

Table 2: Species and collections used in tests of the lower lethal limit (LT₅₀) of *Melampus*

Collection locale	Latitude	Longitude	Date	Species	LT ₅₀ (°C; 95% CI)
2008:					
Beebe Cove, CT	41.3333	-71.9932	1/18/2008	<i>M. bidentatus</i> North	-12.6 (-12.8, -12.3)
Barn Island, CT	41.3302	-71.8665	1/12/2008	<i>M. bidentatus</i> North	-8.9 (-9.0, -8.7)
Fenwick Island, DE	38.4603	-75.0777	1/11/2008	<i>M. bidentatus</i> North	-7.6 (-8.8, -6.5)
				<i>M. bidentatus</i> South	-7.6 (-8.4, -6.8)
Cocodrie, LA	29.2543	-90.6628	1/7/2008	<i>M. bidentatus</i> South	-8.4 (-9.3, -7.5)
2009a:					
Fenwick Island, DE	38.4603	-75.0777	1/5/2009	<i>M. bidentatus</i> North	-11.9 (-14.6, -9.2)
				<i>M. bidentatus</i> South	-11.7 (-13.1, -10.2)
Indian River, DE	38.6045	-75.0685	1/6/2009	<i>M. bidentatus</i> North	-12.1 (-12.6, -11.6)
Cambridge, MD	38.5713	-76.0648	1/6/2009	<i>M. bidentatus</i> South	-10.6 (-11.7, -9.5)
				<i>M. floridanus</i>	-9.3 (-10.7, -7.9)
Fourchon, LA	29.1035	-90.1865	1/13/2009	<i>M. bidentatus</i> South	-11.8 (-12.6, -11.0)
Naples, FL	26.1357	-81.7932	1/10/2009	<i>M. coffeus</i>	-4.3 (-5.6, -3.00)
Florida Keys, FL	25.024	-80.4952	1/10/2009	<i>M. bidentatus</i> Gulf	-8.0 (-9.8, -6.3)
				<i>M. bullaoides</i>	.18 (.12, .23)
2009b:					
Barn Island, CT	41.3302	-71.8665	2/19/2009	<i>M. bidentatus</i> North	-11.6 (-12.2, -11.1)
Fourchon, LA	29.1052	-90.1865	2/21/2009	<i>M. bidentatus</i> South	-9.5 (-11.0, -8.0)

other locales in both Florida and Texas are morphologically more similar to other *M. bidentatus* than to *M. morrisoni*. *Melampus floridanus* grouped most closely with the two Pacific species (fig. 1); these Pacific species are not found farther north than 34° of latitude (Hall 2002). Complete gene sequences (*COI* and *H3* only) used to generate these relationships are available in GenBank (accession nos. KJ609102–KJ609129).

Environmental Temperatures

On a broad geographical scale, the more northward a site is, the more days below freezing it experiences and the lower its minimum temperature (table 3). The two northernmost sites, which contained *M. bidentatus* North, experienced nearly 100 annual days below freezing and minimum air temperatures of -19.4° and -22.2°C. In contrast, the next most northerly species, *M. bidentatus* South and *M. floridanus*, experienced roughly half as many freezing days and a minimum air temperature of -13.3°C, even though they were collected near their northern range limits. The remaining species for which we tested cold tolerance (*M. bidentatus* Gulf, *M. coffeus*, and *M. bullaoides*) all occur farther south and experienced minimum temperatures very near or just above freezing. The southernmost collection site, containing *M. bullaoides* and *M. bidentatus* Gulf, had a minimum temperature of +5°C, suggesting that freezing events are either very rare or nonexistent there.

Lower Lethal Temperature

Across all data points, the Spearman's rank correlation test was significant ($\rho = -0.57$, $P = 0.02$; raw data in table A3). This is primarily driven by *M. bullaoides*, which was the sole species

that could not tolerate temperatures below freezing (LT₅₀ = +0.2°C). *Melampus bullaoides* has the southernmost northern range limit among the six species tested (Martins 1996), and the molecular data suggest that it is not closely related to other species sampled here.

Among the three cryptic species of *M. bidentatus*, LT₅₀ varied from -7.6° to -12.6°C (table 2) and did not differ significantly among species. Despite having a more southerly range, the lower lethal temperature for *M. bidentatus* Gulf fell within the range measured in the other two more northerly cryptic species (-8.1°C). The mean lethal temperature of *M. coffeus*, which the molecular data placed within the *M. bidentatus* group, was significantly warmer (LT₅₀ = -4.3°C).

Among the three cryptic species of *M. bidentatus*, the coldest values of LT₅₀ were found in the species with the northernmost distribution (*M. bidentatus* North), but there was significant variation among years and among sites. For example, the two sets of trials in 2009 that included Fourchon, Louisiana, had significantly different LT₅₀'s (-11.8° and -9.5°C). Furthermore, LT₅₀ did not differ between *M. bidentatus* North and *M. bidentatus* South when they were collected in sympatry (Fenwick Island, DE; table 2; fig. 2). Within *M. bidentatus* North and South, there were no clear latitudinal patterns of LT₅₀. Measures of *M. bidentatus* North at Beebe Cove, Connecticut (-12.6°C), were very close to previous measures made at the same site (Loomis 1985; January LT₅₀ = -12.9°C).

Tolerance of subzero temperature was also seen in the lineage containing *M. floridanus*, which was more closely related to the two Pacific samples rather than the other temperate Atlantic species. *Melampus floridanus* had an LT₅₀ that did not differ from *M. bidentatus* collected from the same site (-9.3° and -10.6°C, respectively; fig. 2; table 2).

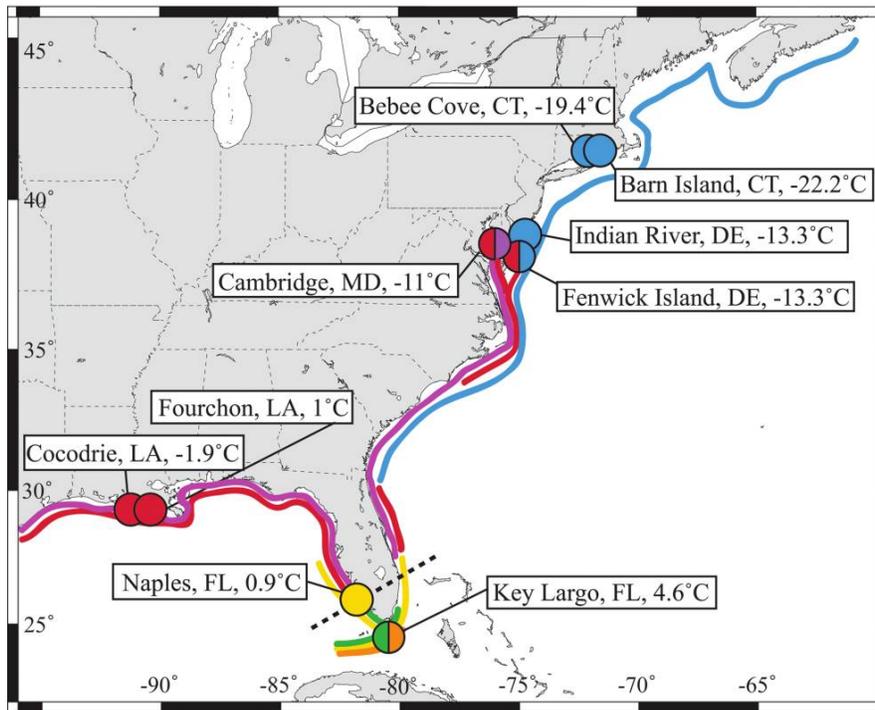


Figure 2. Approximate range of species included in physiological tests: *Melampus bidentatus* North (blue), South (red), and Gulf (green); *Melampus floridanus* (purple); *Melampus coffeus* (yellow); and *Melampus bullaoides* (orange). Circles indicate sampled populations, and labels include the coldest recorded temperature in 2008–2009.

Supercooling Point

Supercooling points ranged between -4.0° and -7.2°C and did not differ significantly between *M. bidentatus* North (Beebe Cove, -5.4°C ; Barn Island: 5.8°C) and *M. bidentatus* South (average, -5.4°C ; fig. 3; table A2). For *M. bidentatus* South, supercooling point was significantly warmer than any measures of LT_{50} in that species (-7.6° to -11.8°C ; table 2; fig. 3). For *M. bidentatus* North, the supercooling point was significantly warmer than LT_{50} measured at the same sites (-8.9° to -12.6°C), but the confidence intervals overlap slightly with those around the LT_{50} calculated at Fenwick Island, Delaware, in 2008 (fig. 3; tables A2, A3).

Discussion

Geographical range shifts into cooler climates likely require adaptive shifts in temperature tolerance (Hoffmann 2009; Wiens 2011). Survival of freezing conditions is especially important because of the damage caused by ice formation. We have tested for differences in lower lethal limit (LT_{50}) among snail species spanning different latitudes. Temperate lineages survived subzero temperatures, while the tropical lineage was unable to survive temperatures below 0°C . Measures of LT_{50} are useful to compare thermal performance. Many species live close to their thermal limits (Zippay and Hofmann 2010), and

temperature is often important in setting range limits (Gaston 2003; Koehler et al. 2012). Measuring cold-associated mortality in a group that is inferred to have expanded out of the tropics allows us to directly test for niche diversification that could have enabled historical range shifts (Martins 1996; Spicer and Gaston 1999). We found LT_{50} 's well below freezing in two temperate lineages. Warmer supercooling points in one of these lineages (two species measured from three locales; fig. 3) support previous findings (Loomis 1985) that *Melampus* is freeze tolerant and suggest that it has acquired the ability to tolerate freezing to enable its existence at high latitudes.

All members of the monophyletic subgenus *M. Melampus* (*M. coffeus* and the *M. bidentatus* species complex; fig. 1) survived subzero temperatures to some extent. We infer that these species survive these temperatures by being freeze tolerant, as in *M. bidentatus* North and South (Hilbish 1981; Loomis 1985). In *M. bidentatus* Gulf, LT_{50} (-8.1°C) exceeds the lowest temperatures encountered in its range; its northernmost known locale dropped below 0°C only once in 2008–2009 (South Padre Island; table 3). The apparent freeze tolerance in *M. bidentatus* Gulf suggests that the *M. Melampus* group acquired the ability to tolerate freezing before these species diverged. Freeze tolerance also may not be particularly costly, as its expression would presumably provide a selective disadvantage in *M. bidentatus* Gulf. The molecular analysis placed *M. coffeus* within

Table 3: Summary of air temperature (2008–2009) near collections used in tests of lower lethal limit (table 1) of *Melampus*

Data recording station	Collection site	Distance from data station, km	Species	Days	Days	Coldest temperature 2008–2009	Months with days below freezing 2008–2009
				below 0°C 2008	below 0°C 2009		
Groton, CT	Beebe Cove, CT	5	<i>M. bidentatus</i> North	98	94	−19.4	9
Westerly, RI	Barn Island, CT	5	<i>M. bidentatus</i> North	98	96	−22.2	6
Cambridge, MD	Cambridge, MD	5	<i>M. bidentatus</i> South	55	42	−11	5
			<i>M. floridanus</i>				
Port Isabel, TX	South Padre Island ^a	8	<i>M. bidentatus</i> Gulf	0	1	−.1	1
Ocean City, MD	Indian River	29	<i>M. bidentatus</i> North	47	56	−13.3	5
	Fenwick Island	14	<i>M. bidentatus</i> North				
LUMCON, LA	Cocodrie, LA	<1	<i>M. bidentatus</i> South	2	0	−1.9	1
Fourchon, LA	Forchon, LA	<2	<i>M. bidentatus</i> South	0	0	1	0
Naples, FL	Naples, FL	2	<i>M. coffeus</i>	0	0	.9	0
Molasses Key, FL	Key Largo, FL	11 offshore	<i>M. bidentatus</i> Gulf	0	0	4.6	0
			<i>M. bullaoides</i>				

Note. LUMCON = Louisiana Universities Marine Consortium.

^aSouth Padre Island was not included in physiological trials, but it is included here as the northernmost collection point for *M. bidentatus* Gulf.

the *M. bidentatus* group. Thus, it is expected that *M. coffeus* would possess the same LT_{50} as the other species in this clade. However, *M. coffeus* had a significantly warmer LT_{50} (−4.3°C) despite having northern range limits similar to *M. bidentatus* Gulf (fig. 2). This relatively warmer LT_{50} suggests that *M. coffeus* may have lost some ability to tolerate cold.

The least cold-tolerant species was *M. bullaoides*, which could neither tolerate temperatures below freezing ($LT_{50} = +0.2^{\circ}\text{C}$) nor survive a maintenance temperature of +5°C (see “Material and Methods”). *Melampus bullaoides* shares much of its northern range with *M. coffeus*, but *M. coffeus* was tolerant of temperatures below freezing (table 2; fig. 3). The northern range limits of these two species straddle a region in southern Florida where climate shifts occur from temperate to tropical conditions for both marine (Spalding et al. 2007) and terrestrial (Olson et al. 2001) systems. This suggests that *M. bullaoides* only occupies locales lacking freezing events, whereas *M. coffeus* can survive in the cooler, occasionally freezing conditions in the warm temperate zone.

Among temperate species, range differences appear to be set by something other than temperature alone. The greatest cold tolerance was found in the northernmost population of the northernmost distributed species (Beebe Cove, CT; table 2), just as Loomis (1985) found 25 years ago. However, significantly warmer LT_{50} 's were found in the same species only 10 km east at Barn Island, Connecticut (−8.9°C). Furthermore, similar tolerance was measured in *M. bidentatus* South collected more than 10 latitudinal degrees to the south in Cocodrie, Louisiana (−8.4°C; table 2; fig. 2). Most importantly, sympatric populations of *M. bidentatus* North and South (Fenwick Island, DE) had LT_{50} 's that varied in parallel among years (table 2), showing that when these two species develop in the same environment, they have the same temperature tolerance.

Microhabitat can influence physiological tolerance. In the intertidal zone, thermal adaptation can vary over just meters

of vertical distribution (Stillman 2002), and expression of genes associated with thermal stress varies on small spatial scales (Halpin et al. 2002). Species with overlapping geographical ranges may differ in subtle microhabitat preferences (Beavis et al. 2011). In molluscs, salinity and desiccation have been shown to alter freeze tolerance (Murphy and Pierce 1975; Hylleberg and Siegismund 1987), and niche modeling suggests that both salinity and desiccation stress may differ over the cryptic species ranges of *M. bidentatus* (Dennis and Hellberg 2010). Thus, small disparities in the microhabitat preferred by *M. bidentatus* North and South could influence their exposures to salinity and desiccation stress, alter basal gene expression, and cause differences in freeze tolerance that lead to differences in geographical range. These results highlight the need to better understand how microhabitat differences, climatic variation, and transcriptional response of organisms affect survival.

Conclusion

Our results suggest that an inability to withstand freezing temperatures likely restricts tropical *Melampus* from expanding their ranges into temperate regions. This is highlighted by the complete inability of the most tropical species sampled (*M. bullaoides*) to tolerate subzero temperatures. Tolerance of freezing conditions appears to enable species to inhabit the temperate zone. However, once in temperate climes, differences in lower lethal limit do not explain geographical range differences among species. Instead, geographic range differences may be influenced by abiotic factors, such as microhabitat.

Warming temperatures have been causally linked to northward range expansion in many taxa, including intertidal gastropods (Sagarin et al. 1999; Mieszkowska et al. 2007; Hawkins et al. 2009). Our findings suggest that factors besides thermal tolerance may underlie poleward range limits in *Melampus* and that it, and perhaps other temperate species, will not necessarily

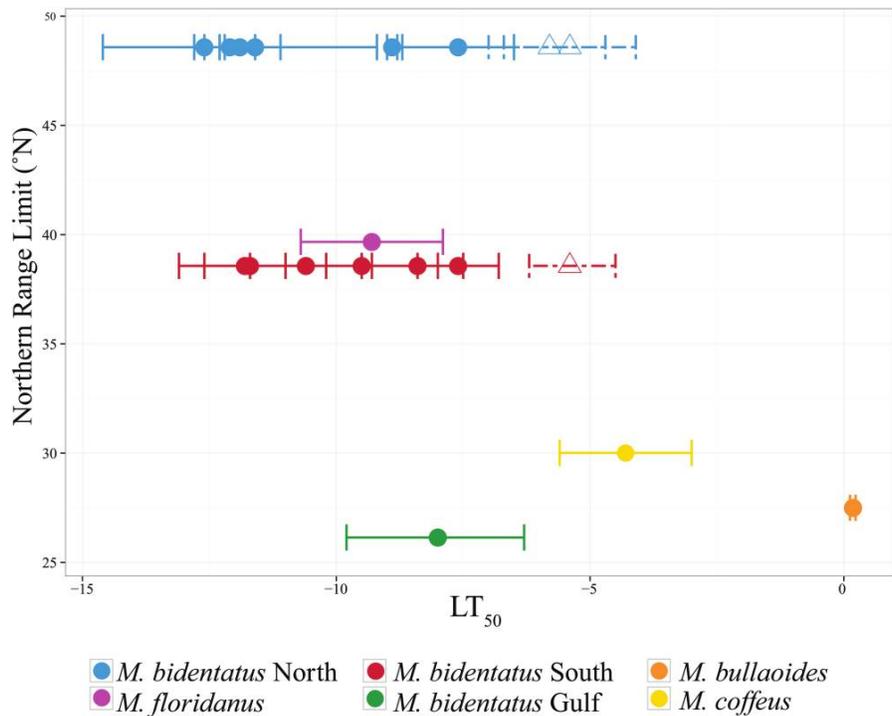


Figure 3. Lower lethal temperature (LT_{50} ; circles) as reported in table 2 and supercooling point (triangles) plotted as a function of each species' northern range limit. Colors correspond to those used in figures 1 and 2.

expand northward as climate warms. Niche modeling has suggested that microhabitat differences such as salinity and desiccation may be important (Dennis and Hellberg 2010), but the range of *Melampus* could also be determined by variation in competition (Lee and Silliman 2006) or resource availability. Further study of tolerance to high temperatures, salinity, desiccation exposure, metabolic rate under these stresses, and the interaction among all of these (McAllen and Block 1997; Sinclair et al. 2013) would help better understand what limits geographical range. Recent advances in sequencing technology would also enable differences in stress response among these species to be examined at the molecular level.

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