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Seasonal freezing adaptations of the mid-intertidal gastropod *Nucella lima* from southeast Alaska

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ABSTRACT

Nucella lima from the mid-intertidal zone of Bridget and Sunshine Cove, Alaska is exposed to multiple freezing emersion events during the winter. The average duration of low tide when the air temperature fell below 0 °C increased from 2.91 to 6.78 h between the lower limit and upper limit of the intertidal range of *N. lima*. Air temperatures below freezing were observed between October 20, 2007 and April 20, 2008. Snails cease feeding and move into crevices, under boulders or into the sediment at the base of rocks in the winter which potentially minimizes their exposure to freezing events. Egg capsules were also observed in the snail habitat between September 27, 2007 and March 12, 2008. Snails supercool below the freezing point of seawater which delays freezing during tidal cycle related emersion. The supercooling point of snail tissues does not vary seasonally. Air temperatures below the maximum supercooling temperature of snails (-4.94 °C) occurred multiple times in December 2007 and January and February 2008. The freeze tolerance of *N. lima* varies seasonally and is always below the supercooling point indicating that *N. lima* physiologically tolerates freezing. It is likely that the seasonal synthesis of cellular compatible osmolytes is responsible for the seasonal variation in freeze tolerance: Quantitatively important compatible osmolytes which are found in higher concentration in the winter versus the summer in foot tissue of snails are total free amino acids, taurine (119 mol.Kg wet^{-1}), and glycine (43 mol.Kg wet^{-1}).

1. Introduction

The rocky intertidal zone is one of the most physically complex and variable environments on earth. The interaction of climate and the timing of low tides in the rocky intertidal zone of the west coast of North America create a complex mosaic of thermal environments during emersion that is more thermally stressful at northern locations than at southern sites (Helmuth et al., 2002). Seasonal changes in seawater temperature at the surface are relatively constrained, ranging from 12.1 to 15.4 °C (1983 to 1993) at Monterrey, CA (Barry et al., 1995), from 6 to 13 °C in the San Juan Islands, WA (Stickle, 1970) and from 1 to 15 °C at Sunshine (Ravioli) Cove along the Lynn Canal north of Juneau, AK (Stickle, 1970; Stickle and Denoux, 1976). Although seasonal variation in seawater temperature on the west coast of North America is small, tidal amplitude is large and exposure to air temperatures which deviate from seawater temperature may be significant in both the summer and winter (Helmuth et al., 2006). Air temperatures during tidal emersion are ameliorated on the outer coast of the continental United States because maximum low tides occur at night during the summer and in the day during the winter. In contrast, maximum low tides occur during the day in the summer and during the night in the winter in the inside waters north of Seattle. Air temperatures reach summer highs of 32 °C in the San Juan Islands, WA and 27 °C at Sunshine Cove, AK. Air temperatures fall as low as -10 °C in the winter at Sunshine Cove (personal observations). Consequently, more extreme air temperatures during the emersion of intertidal organisms at the northern intertidal sites render them more vulnerable to emersion temperature stress (Helmuth et al., 2002, 2006).

The severity and duration of temperature stress of intertidal organisms are dependent on the air–water temperature differential and the intertidal height of the species. Intertidal invertebrates living in temperate regions of the world are generally freeze tolerant. Osmotic and mechanical damage to their tissues must be limited as a result of freezing of the extracellular fluids that draws water out of the intracellular fluid compartment (Murphy, 1983; Loomis, 1995). The intertidal height distribution of the species and pattern of tidal cycles determine the duration of exposure to emersion events (Murphy, 1983). Adaptations of intertidal molluscs to freezing include supercooling, the synthesis of proteinaceous or the use of bacterial ice nucleators, the synthesis of cryoprotectants and behavioral avoidance through migration to a lower position on the shore, into crevices, into

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sediment at the base of rocks, or under rocks (Loomis, 1995; Sinclair et al., 2004).

Gastropods are important members of rocky intertidal communities. *Nucella lima* is a mid-intertidal predator which exhibits a latitudinal range from northern Vancouver Island to northern Japan where it preys on barnacles and mussels (Collins et al., 1996). Cold hardiness of rocky intertidal molluscs has been thoroughly reviewed by Ansart and Vernon (2003). All species studied are freeze tolerant including *Thais* (*Nucella*) *lapillus* (Murphy, 1979b). An understanding of freeze tolerance of the upper intertidal salt marsh pulmonate snail, *Melampus bidentatus* has been gained (Hilbish, 1981; Loomis, 1985; Hayes and Loomis, 1985; Madison et al., 1991). *M. bidentatus* utilizes a supercooling point for freeze avoidance which lowers the freezing point of the snail below that of the osmolality of the extracellular fluid compartment during the initial aerial exposure (Loomis, 1985; Hayes and Loomis, 1985; Madison et al., 1991). Eventually, adaptations of the ICF to freezing of the ECF must occur. Because the duration of aerial emersion increases with intertidal height, the duration of freeze exposure should be considerably shorter during emersion for the mid-tidal *N. lima* than the upper tidal *M. bidentatus* (Loomis, 1995). The time elapsed as a result of the snail not freezing because of their supercooling point should occupy a higher percentage of the emersion time of *N. lima*.

Freeze avoidance of gastropods is advantageous during the initial aerial emersion exposure to freezing air temperatures. Gastropods must be tolerant of longer term extracellular fluid compartment ice formation where freeze tolerance to temperatures below the tissue supercooling point is enhanced by the synthesis of cryoprotectant molecules (Loomis, 1985, 1995; Ansart and Vernon, 2003; and Yancey, 2005). Organic compatible osmolytes fall into four chemical categories: (1) small carbohydrates including sugars, polyols, and derivatives, (2) amino acids and derivatives, (3) methlyammonium and methylsulfonium compounds, and (4) urea. Compatible osmolytes in shallow-water invertebrates, such as the polychaete worm *Glycera* sp., snail *Mitrella carinata* and the clam *Saxidomis giganteus*, are typically dominated by taurine, betaine, and α-amino acids such as glycine (Yancey, 2005). The dominant intracellular free amino acid in the intertidal gastropod *Nucella lapillus* from Plymouth England was taurine which comprised 74.5% of the free amino acids at 30 and 35 psu (Stickle et al., 1985).

In order to assess the effects of freezing air temperatures on the supercooling point and freeze tolerance of *N. lima* from the mid-intertidal zone we monitored seasonal change in ambient temperature at the upper and lower limits of *N. lima* distribution with ProV2 Hobo temperature loggers as well as the temperature of partially buried probes at the lower end of their distribution. We determined seasonal variation in the activity patterns of *N. lima*. We also determined 5 h supercooling and freeze tolerance experiments on *N. lima* quarterly. Finally, we determined seasonal variability in the degree of hydration and free amino acid concentrations of *N. lima*. We designed our experiments to test the null hypothesis that there is no seasonal thermal regime effect on the activity patterns, supercooling point, freeze tolerance, degree of hydration, and free amino acid composition in the foot of *N. lima*.

2. Materials and methods

Two vertical transects of ProV2 Hobo temperature probes covered with protective sleeves were established at Bridget Cove which is 1.1 km north of Sunshine Cove (Latitude 58°30.8′ N; Longitude 134°55.8′ W) along Lynn Canal, AK. Both of these sites exhibit significant vertical salinity stratification of the water column during the summer as a result of freshwater outflow of the Herbert and Eagle glaciers via the Eagle River (Stickle and Denoux, 1976).

Probes recorded temperature every 5 min between May 26, 2007 and September 6, 2008. No data were collected between September 6 and October 6, 2007. For this study, data are reported from probes that were deployed at the upper and lower edges of the mid-intertidal range of *Nucella lima*. Two additional probes were partially buried at the lower edge of the snail intertidal range. One of the buried probes was inserted into loose particulate material and one was placed in tightly packed particulate material.

Four aspects of the temperature records were extracted from the data set with respect to this study. The number of days where each probe recorded air temperatures <0 °C, for those days when the air temperature fell below 0 °C, the hours per day when the air temperature was <0 °C, the number of days when probe air temperatures were <-4.94 °C which is the maximum average snail supercooling point throughout the experiments, and the degree hours when the ambient air temperature was below -4.94 °C. This value was determined by multiplying the difference between the negative temperature value below -4.94 °C every time it occurred times 5 min divided by 60 min. The result of these calculations represents the °h of exposure.

The distribution, activity and density of visible *N. lima* within 1 m on either side of both transects (T1 and T2) were observed seasonally within the snail's vertical distribution at Bridget Cove. The substrate was not disturbed during the surveys. This assessment was made at Bridget Cove rather than at Sunshine Cove so snail density was not altered by snail collection during the study. This procedure is similar to that used by Sorte and Hofmann (2004) except that we surveyed the entire transect in the *N. lima* zone rather than using timed counts. The activity level of all snails observed was recorded during each count.

N. lima were collected quarterly at low tide from Sunshine Cove and returned to the flow through seawater system at the NMFS laboratory. Collection dates were January 30, 2007 (seawater surface temperature = 2.2 °C), March 30, 2007 (4 °C), July 19, 2007 (14.5 °C), October 8, 2007 (9.0 °C), January 7, 2008 (3.5 °C), March 12, 2008 (2.8 °C), and June 2, 2008 (9.8 °C).

N. lima tolerance was determined by placing 15 snails into a 500 ml screw cap centrifuge bottle in the upright position with air in it in a refrigerated water bath along with an Onset Hobo Water Temp Pro v2 data logger which recorded air temperature every 3 min at each of five experimental temperatures below 0 °C for 5 h. The condition of snails was determined 24 h after each freeze experiment began at the ambient seawater temperature in the seawater holding tank. Snails were placed in a pyrex dish filled with seawater at ambient lab seawater temperature 23 h after the start of the freezing experiment and individual snail status was recorded 1 h later according to a behavioral index where a value of 0 = snail dead and non-irritable, 0.5 = foot partially extended and irritable, and 1.0 = foot attached to the substrate (glass dish). The average index for each experimental temperature was calculated for every experimental date. The actual average air temperature over the 5 h emersion exposure was used in the determination of the LT_{50} temperature. The LT_{50} temperature for each sampling date was calculated by the Spearman Karber method. Statistical comparisons among LT_{50} freeze tolerance data after 5 h of exposure to freezing conditions were made on the basis of non-overlap of 95% confidence limits.

Snail supercooling points were determined for the July 19, and October 8, 2007 and January 7, March 12, and June 2, 2008 collections by measuring supercooling temperatures of individual snails. A Traceable Control Company dual thermometer connected to two type K thermister leads was connected to a laptop computer with a data capture program to record air and snail temperatures to within 0.1 °C. These temperatures were recorded every 30 s. One thermometer lead was placed in the 50 ml plastic centrifuge tube outside the snail. The other lead was inserted through the snail aperture around the operculum and fixed in place by a rubber band. Excess water was blotted from around the operculum before insertion of the snail into the centrifuge tube. The centrifuge tube was placed in a screw cap 500 ml centrifuge bottle and held upright in a refrigerated water bath