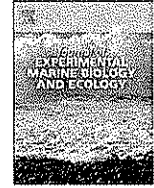




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## Seasonal freezing adaptations of the mid-intertidal gastropod *Nucella lima* from southeast Alaska

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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<sup>-1</sup>), and glycine (43 mol.Kg wet<sup>-1</sup>).

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### 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) methylammonium 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 *Saxidomus giganteus*, are typically dominated by taurine, betaine, and  $\alpha$ -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  $<0^{\circ}\text{C}$ , for those days when the air temperature fell below  $0^{\circ}\text{C}$ , the hours per day when the air temperature was  $<0^{\circ}\text{C}$ , the number of days when probe air temperatures were  $<-4.94^{\circ}\text{C}$  which is the maximum average snail supercooling point throughout the experiments, and the degree hours when the ambient air temperature was below  $-4.94^{\circ}\text{C}$ . This value was determined by multiplying the difference between the negative temperature value below  $-4.94^{\circ}\text{C}$  every time it occurred times 5 min divided by 60 min. The result of these calculations represents the  $^{\circ}\text{h}$  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 =  $2.2^{\circ}\text{C}$ ), March 30, 2007 ( $4^{\circ}\text{C}$ ), July 19, 2007 ( $14.5^{\circ}\text{C}$ ), October 8, 2007 ( $9.0^{\circ}\text{C}$ ), January 7, 2008 ( $3.5^{\circ}\text{C}$ ), March 12, 2008 ( $2.8^{\circ}\text{C}$ ), and June 2, 2008 ( $9.8^{\circ}\text{C}$ ).

*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  $0^{\circ}\text{C}$  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  $\text{LT}_{50}$  temperature. The  $\text{LT}_{50}$  temperature for each sampling date was calculated by the Spearman Karber method. Statistical comparisons among  $\text{LT}_{50}$  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  $0.1^{\circ}\text{C}$ . 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

at 2.5 °C until snail temperature dropped to 6–7 °C after handling the snail. The bath temperature was then dropped from 2.5 °C to -15 °C at approximately 0.5 °C/min. The external air temperature dropped linearly and the snail temperature dropped until the supercooling temperature was reached, this temperature was the lowest temperature reached before the exothermal spike occurred. The number of snails sampled per sampling date was 9. Supercooling point data across the seasons was compared by performing one-way ANOVA analysis with Tukey's test applied if significant variation was found with the ANOVA test. Differences were considered significant at  $P < 0.05$  level.

In order to determine compatible free amino acid concentrations it was necessary to determine percent tissue water values for summer and winter collections. Snails were dissected into gonad-digestive gland, remaining visceral mass, and foot components. The wet weight of each component was determined and the tissues were placed in a 1.4 ml cryotube and frozen between blocks of blue ice and stored at -80 °C. Frozen tissues were shipped to LSU for determination of dry weight at 94 °C in a drying oven. Percent body water values were then determined for each component. *N. lima* samples from July 27, 2007 and January 7, 2008 and percent tissue water values, after arc-sin

transformation, were compared with unpaired Student's t-tests. Only foot percent tissue water values are reported in this paper.

Wet tissue samples stored at -80 °C at LSU were homogenized with a Tekmar homogenizer in 7% PCA then neutralized with 5 M  $K_2CO_3$ . Samples were centrifuged at 15,000 at 4 °C for 15 min and the supernatant of each sample was stored at -80 °C before an aliquot was sent to the Texas A&M University Protein Chemistry Laboratory for analysis. Samples were prepared from snails collected on the January 30, 2007 and July 19, 2007 sampling dates. In addition, egg capsules were collected from the intertidal zone during the October 2007 sampling and processed for free amino acid analyses.

For our samples, the naturally occurring amino acids found in protein were reported along with asparagine, glutamine, citrulline, B-alanine, taurine, ornithine, and tryptophan. The AminoQuant method analysis of all samples was performed by pre-column derivatization of the amino acids with o-phthalaldehyde (OPA) and 9-fluoromethylchloroformate (FMOC). OPA reacts with primary amino acids and FMOC with secondary amino acids (proline). Both reagents react rapidly and quantitatively and give highly fluorescent and UV-absorbing isoindole derivatives. The derivatized amino acids are separated by reverse phase HPLC and detected by UV absorbance with a diode array detector or by

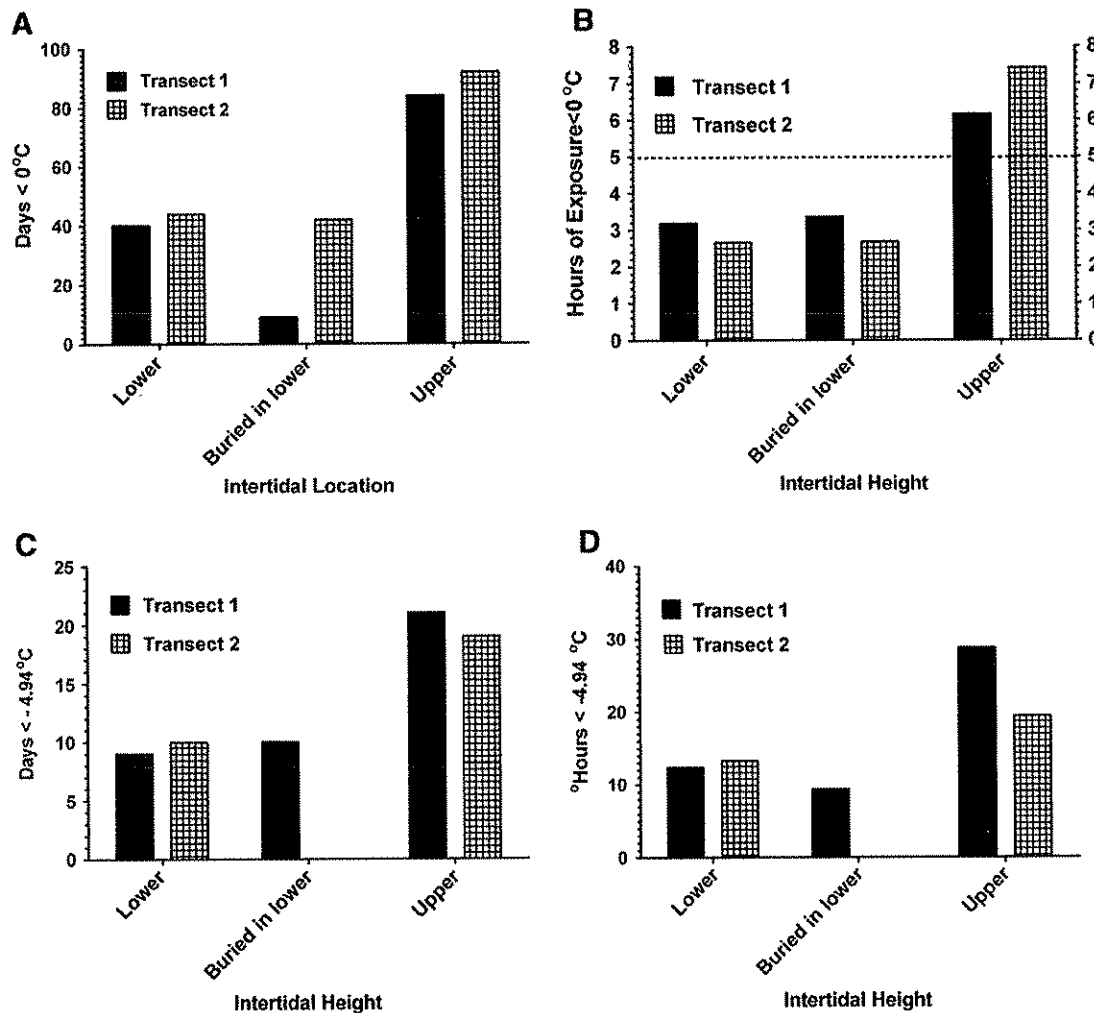


Fig. 1. Freezing temperature measurements were recorded every 5 min along two transects of the *Nucella lima* zone in the mid rocky intertidal zone at Bridget Cove Alaska between May 2007 and September 2008. A– Days when air temperature fell below 0 °C in the lower edge, and upper edge of the zone. Also shown are data from one probe which was loosely buried in particulate matter at the base of rocks (Transect 1), and one which was firmly surrounded by particulate matter. B– Average hours of emersion to air temperatures <0 °C for days when air temperature was <0 °C. The dashed line at 5 h aerial exposure indicates that our experimental design of a 5 h exposure period falls within the exposure period in the mid-intertidal zone occupied by *N. lima*. C– Days when air temperature fell below -4.94 °C at the same locations. D– Degree hours below -4.94 °C at each location.

fluorescence using an in-line fluorescence detector. Every sample was analyzed in duplicate and the usual sample size was 4–5.

Free amino acid data expressed as mmol. Kg wet weight<sup>-1</sup> were analyzed with two-way ANOVA with season and free amino acids being the main effects and an interaction term was also calculated. The Bonferroni post hoc test was also used to test for significant differences between individual free amino acid concentrations as a function of season.

### 3. Results

*Nucella lima* was emersed for a significant number of days when air temperature was below 0 during our study (Fig. 1A). The number of hours per day to which snails were exposed to air temperatures below 0 °C increased from an average of 2.91 h at the lower end of the range of *N. lima* to 6.78 h at the upper end of its range (Fig. 1B). The days when the intertidal air temperature during emersion fell below -4.94 °C occurred in December 2007 and January and February 2008 (Fig. 1C). The earliest date when mid-tidal air temperature fell below 0 °C was October 20, 2007 and the latest date was April 20, 2008. None of the freezing air temperatures in October or November 2007 or in March and April 2008 fell as low as the maximum supercooling point of *N. lima* which was -4.94 °C. The lowest ambient air temperature recorded was -12.42 °C in December, -12.71 °C in January, and -12.79 °C in February 2008. As expected, the number of freeze days increased with intertidal height in the mid-intertidal range of *N. lima*. Ambient air temperature also never fell below the supercooling point of *N. lima* when particulate material was tightly packed around the temperature probe at the lowest end of the snails intertidal range; nor did the air temperature fall below the supercooling point of snails regularly when the particulate material was very loosely packed around the temperature probe.

The degree hours of emersion exposure to freezing air temperatures below the supercooling point of -4.94 °C exceeded the experimentally determined 5 h LT<sub>50</sub> degree hours for snails located in the upper end of the intertidal distribution of *N. lima* at Sunshine Cove, AK on a number of days (Fig. 1D). The experimentally determined °h values at the LT<sub>50</sub> for 5 h on January 7, 2008 (-18.2°h) and March 12, 2008 (-13.3°h) collections were warmer than those observed for the intertidal emersion values at the upper end of the intertidal range of *N. lima* which was -28.8°h for transect 1 and -19.4°h for transect 2 on days when emersion temperatures fell below -4.94 °C.

A survey of the distribution and density of *N. lima* at regular intervals along both temperature probe transects indicated that snail distribution along both transects exhibited an increase in visible snails during the summer months and a decrease during the winter (Fig. 2).

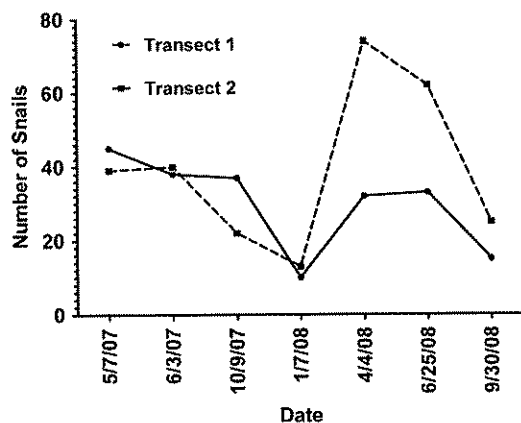


Fig. 2. Swath survey data for visible *Nucella lima* 1 meter on either side of transects 1 and 2. The substrate was not disturbed when surveying visible snails.

Snails were feeding during the summer months and were observed in crevices, and partially buried in particulate matter at the base of rocks during the winter. Finally, egg capsules were observed during snail collections from October 2007 through the March collection of 2008.

The freeze tolerance of *N. lima* varied seasonally from -11.6 (95% C.I. = -12.3–11.0) °C on January 30, 2007 to -5.1 (-5.6–4.7) °C on July 19, 2007 (Fig. 3A). There was a seasonal trend in the freeze tolerance of *N. lima* in 2007 and 2008 with much less seasonal variation in 2008.

The supercooling point of *N. lima* was higher than the 5 h LT<sub>50</sub> and did not vary significantly (ANOVA) across the seven sampling dates. Supercooling points only varied between  $-4.20 \pm 0.60$  (n=9) in July 2007 and  $-4.94 \pm 0.89$  (n=9) in March 2008.

The pattern of activity of *N. lima* 24 h after emersion under freezing conditions varied seasonally with snails being more active at lower temperatures during the winter and spring than during the summer and fall. Data for the sampling dates in 2007 are presented as representative for the entire study (Fig. 3B). The 0.5 activity coefficient (foot extended and irritable) of *N. lima* exhibited the following seasonal variability: 1/30/07 = -8.3 °C, 3/30/07 = -7.55 °C, 7/19/07 = -4.85 °C, 10/8/07 = -6.35 °C, 1/7/08 = -7.45 °C, 3/12/08 = -6.5 °C, and 6/2/08 = -6.6 °C.

There were significantly higher concentrations of total free amino acids, taurine, and glycine in the foot tissue of *N. lima* in the winter than in the summer of 2007 (two-way Anova with season, free amino acids, and the interaction term all being significant at  $p < 0.0001$ ). The

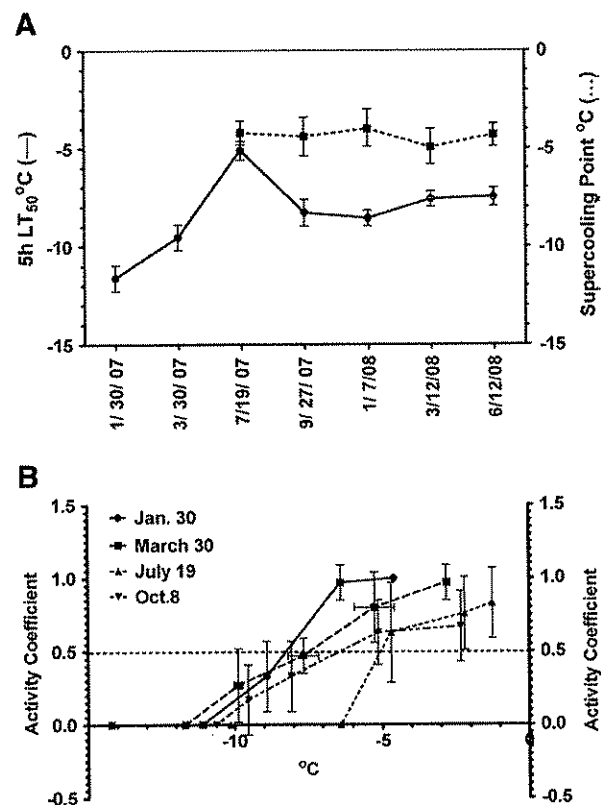


Fig. 3. Seasonal change in the freeze tolerance, supercooling point, and activity coefficients 24 h after the freeze tolerance experiments of *Nucella lima*. A– Data are plotted for the supercooling point and 5 h LT<sub>50</sub> of snails collected seasonally from Sunshine Cove, AK. Seawater freezing point at 30 psu is -1.64 °C. B– The activity coefficients of *N. lima* were determined for each experimental temperature 24 h after snails were exposed to freezing conditions. Data are presented here for freezing experiments conducted in 2007. The dashed line at an activity coefficient of 0.5 indicates the level of activity where the average activity indicates irritable snails. Activity coefficients varied seasonally and were higher at lower temperatures during the winter than during the summer.

Bonferroni post hoc test indicated significant differences in total free amino acids ( $P < 0.001$ ), taurine ( $P < 0.001$ ), and glycine ( $P < 0.001$ ) (Fig. 4). Nineteen amino acids and derivatives were identified in the tissue samples but only taurine, glycine and alanine were present at concentrations above  $10 \text{ mmol.Kg wet}^{-1}$ . The total pool of free amino acids was 54.6% higher in the winter than in the summer while percent tissue water varied significantly by season but not by much quantitatively; the mean percent tissue water was  $74.4 \pm 0.5$  (SEM)  $n = 8$  in the winter and  $72.1 \pm 0.5$   $n = 8$  in the summer (unpaired  $t$ -test  $P < 0.003$ ,  $t = 3.550$ ,  $df = 14$ ). The concentration of taurine was  $119.2 \pm 9.0$  ( $n = 5$ )  $\text{mmol.Kg wet}^{-1}$  in the winter versus  $87.4 \pm 6.2$  ( $n = 5$ )  $\text{mmol.Kg wet}^{-1}$  in the summer while values for glycine were  $43.0 \pm 4.8$  and  $2.0 \pm 0.3$   $\text{mmol.Kg wet}^{-1}$ . When calculated on a Kg water basis instead of wet weight basis, all free amino acid concentrations would be 34.4% higher for the winter samples and 38.7% for the summer samples because of dry tissue in the wet weight of tissue. Taurine comprised 55.6% of free amino acid pool in the winter and 63.0% in the summer while glycine represented 20.1% of the total pool in the winter but only 1.4% in the summer. The total free amino acid pool, taurine, and glycine are clearly adaptive compatible osmolytes in enhancing winter freeze tolerance of *N. lima* in the winter.

#### 4. Discussion

Multiple freeze events, as low as  $-12.79^\circ\text{C}$  which were below the maximum supercooling temperature of *Nucella lima* occurred in the mid-intertidal zone throughout the snails vertical distributional range at Bridget Cove, AK during the winter of 2007–08. These freezing events were significantly colder than at Cattle Point and Collins Cove on San Juan Island, WA (Helmuth et al., 2006). As predicted, the duration of emersion in air increased with intertidal height. The only freeze events that occurred on the outer coast of the United States and at Bamfield on the outer coast of Vancouver Island during a five year period were at Boiler Bay (November 2003), Hopkins (December 1998), and Lompoc (November 2003). The upper intertidal zonation limit of *N. lima* and other rocky intertidal species may be set by their freeze tolerance to aerial emersion during the winter. *N. lima* at Bridget Cove were exposed to a number of days when the degree hours below the experimentally determined 5 h  $LT_{50}$  occurred during the winter. Likewise, in January 1989 *Mytilus trossulus* suffered nearly total mortality in the rocky upper mid-intertidal zone at Kachemak Bay in south Central Alaska over three weeks of exposure to recorded air temperatures as low as  $-31^\circ\text{C}$  (Carroll and Highsmith, 1996). Behavioral escape by *N. lima* burrowing into particulate matter, moving into crevices or crawling under boulders likely reduced freeze exposure during emersion by minimizing radiation and convection

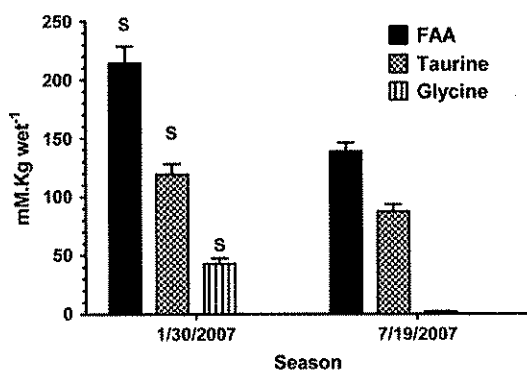


Fig. 4. Free amino acids in the foot tissue of *N. lima* on January 30, 2007 and July 19, 2007. Total free amino acids, taurine, and glycine were significantly higher on January 30, 2007.

from the air and amelioration of the temperature gradient between the snail and rocks or particulate matter through conduction. In addition, since snails were not feeding in the winter, partial retraction into their shells would not have an impact on their annual energy budget.

Freeze tolerance of *N. lima* was enhanced in the winter and reduced in the summer as was also observed with *Melampus bidentatus* (Loomis, 1985). The supercooling point of neither species varied seasonally so that variation in freeze tolerance is initially due to toleration of ice formation in the extracellular compartment during the emersion time at a temperature below the supercooling point and also to the synthesis of cryoprotectants (Loomis, 1985; Ansart and Vernon, 2003; Yancey, 2005). Formation of ice in the extracellular fluid compartment when the snail temperature falls to the supercooling point draws water out of the intracellular compartment and prevents ice formation in the intracellular compartment as long as the osmolality of the ICF compartment is tolerable. Seasonal variation in the hemolymph supercooling point (Hayes et al., 1985; Madison et al., 1991) does not correlate with the supercooling point of snails so the *in vitro* observations of hemolymph supercooling point is of minimal significance to intact snails. We also observed that the supercooling point of *N. lima* was similar to the freezing point of seawater in several cases when we did not blot seawater from inside the operculum of the experimental snails prior to the start of supercooling experiments. Therefore seawater inside the snail operculum can seed ice formation in intact snails.

Taurine and glycine are major osmolytes of quantitative significance in the foot of *N. lima* and the seasonal change in the concentration of total free amino acid, taurine, and glycine is adaptive to increased freeze tolerance in the winter. Yancey (2005) indicates that the osmolyte concentrations of the shallow water gastropod *Mitrella carinata*, the clam *Saxidomus gigantus*, and the polychaete worm *Glycera* sp. are dominated by taurine, betaine and  $\alpha$ -free amino acids such as glycine. Taurine also dominates the free amino acid pool of the temperate mid-tidal *Nucella lapillus* from Bovisand, South Devon, in England (Stickle et al., 1985). Taurine was considered to be a poor osmolyte with respect to adaptation of *N. lapillus* to a salinity gradient because its concentration changed very slowly over time along the salinity gradient or in salinity transfer experiments. Consequently the dogwhelk *N. lapillus* was a very poor volume regulator as a function of salinity gradients. High taurine concentrations in the dogwhelk may be an adaptation to freeze tolerance not salinity gradients in this temperate species of gastropod.

Cryoprotectants fall into two distinct classes. Colligative cryoprotectants are present in higher quantitative concentrations and act as significant osmolytes while a second group of cryoprotectants are present in lower concentrations and include proline and trehalose which, in effect, protect cellular structures (Yancey, 2005). Proline was present in much lower  $\text{mM.Kg wet}^{-1}$  concentrations in the winter but was found in high enough concentration to be protective of cellular structures. Loomis (1995) indicated that no specific colligative cryoprotectants have been identified in the pulmonate snail *Melampus bidentatus* but he had identified low levels of glycerol and proline in *M. bidentatus* (Loomis, 1985).

*Nucella lima* from the mid-intertidal zone of Bridget and Sunshine Cove Alaska are exposed to multiple freezing emersion events during the winter. Snails move into crevices or the sediment in the winter. These behaviors may reduce the thermal stress imposed on snails by freezing events. In laboratory experiments, snails supercool below the freezing point of seawater which delays freezing during tidal cycle related emersion as has also been observed in *Littorina littorea* (Murphy, 1979a; Murphy and Johnson, 1980), *Nassarius obsoletus* (Murphy (1979a) and *Thais (Nucella) lapillus* (Murphy, 1979b). The supercooling point of intact *N. lima* does not vary seasonally. However, the 5 h  $LT_{50}$  of *N. lima* varies seasonally and is always below the supercooling point indicating that *N. lima* tolerates freezing. It is likely

that the seasonal synthesis of the quantitatively important osmolytes taurine and glycine are responsible for some of the seasonal variation in freeze tolerance of *N. lima*.

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