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Population divergence in cold tolerance of the intertidal gastropod *Littorina brevicula* explained by habitat-specific lowest air temperature

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ABSTRACT

Temperature is a fundamental element determining the distribution patterns of ectotherms, and it is generally recognized that physiological tolerances of ectotherms change according to thermal gradients in their habitats. However, little is known about the variation in cold tolerance of marine invertebrates among local populations of the same species. In this study, three experiments were conducted to demonstrate inter-population variation in the cold tolerance of the intertidal gastropod *Littorina brevicula*, and to explore factors driving the variation. First, seasonal changes in the freezing temperature (the supercooling point, SCP) and the median lower lethal temperature (LLT50) of snails collected from a single population were examined to identify the cold tolerance strategies of this species. Seasonal fluctuation was clear in only the LLT50 which was lower than the SCP in winter, indicating that cold tolerance strategy of this species was freeze tolerant. Second, the cold tolerance of snails from four populations was compared. These populations were distributed at latitudinally different locations, and the comparisons were performed before winter when their cold tolerance was not required and in winter when their cold tolerance increased. In this experiment, while the cold tolerance of snails collected in winter was higher than that of snails collected before winter in all four populations, there were differences in tolerance among the populations. Furthermore, to estimate their ability of acclimation to cold environments, snails that were collected from the northernmost and southernmost populations in the four populations before winter were acclimated to a constant low temperature in the laboratory for a long period (120–130 days). This long period of acclimation led to an increase in the cold tolerance of both populations, but the latitudinal difference was maintained. Additionally, after imposing the long period of acclimation to snails that were collected at 12 locations before winter, factors that might cause local variation in their cold tolerance were explored. This experiment clearly showed that local variation in cold tolerance was explained by the lowest local air temperature. Our study demonstrated inter-population variation in the cold tolerance of an intertidal mollusc, and that the variation is explained by thermal gradients in winter.

1. Introduction

Climate change is anticipated to have a profound impact on the biogeography of ectotherms, because temperature is a fundamental element determining their distribution patterns (e.g. Helmuth et al., 2002; Hampe and Petit, 2005; Hoffmann and Sgrò, 2011; Sunday et al., 2011). While studies on ecological and physiological responses to temperature extremes mainly focus on heat extremes in the face of recent climate change, low temperatures in winter at the other end of temperature extreme have received less attention. Even though increases in the mean air temperature generally induce a poleward shift in the distribution of ectotherms, the success of their redistribution at new locations would relate to their cold tolerance, because unpredictable occurrences of low winter temperatures can offset the shift to the general warming trend (Firth et al., 2011; Wethey et al., 2011).

The distribution of marine invertebrates inhabiting intertidal zones is often affected strongly by temperatures of air and substrate surface of their habitats (Loomis, 1995; Helmuth et al., 2002; Somero, 2002, 2010; Marshall et al. 2010; Wethey et al., 2011). Intertidal ectotherms are exposed to cold extremes during winter low tides, and this cold tolerance has been documented for several molluscs (Loomis, 1991, 1995; Ansart and Vernon, 2003) and a crustacean (Ronges et al., 2012). As for vertical distribution along the shore in temperate regions, higher cold tolerance was observed in species that were distributed in the upper

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intertidal zone and have low mobile ability (Murphy, 1979a, 1979b; Stickle et al., 2015), indicating that low air temperature acts as an evolutionary driving force on the pattern for vertical distribution.

Cold tolerance of intertidal ectotherms may vary between populations owing to genetic differences, since local thermal adaptation has often been documented within a species in ectotherms (Conover and Schultz, 1995; Angilletta, 2009; Franks and Hoffmann, 2012). Geographic variation in cold tolerance has at least been compared among related species in the family Littorinidae (Sinclair et al., 2004) and in the genus *Melampus* (Dennis et al., 2014). Both studies showed that temperate species exhibited higher cold tolerance than related tropical species, indicating that their cold tolerance adapted to the local cold temperature. However, studies on inter-population variation in cold tolerance are limited, and factors and mechanisms causing the variation remain unclear. Although Hilbish (1981) found higher cold tolerance in northern populations of *Melampus bidentatus*, a recent detailed study by Dennis et al. (2014) revealed that the geographic variation was affected by multiple local abiotic factors such as salinity and desiccation rather than local temperature alone. In addition, few reports are available on the interaction between plastic and genetic responses of intertidal organisms to cold environments. Sokolova and Pörter (2003) reported that the metabolic response of *Littorina saxatilis* to changes in temperature varies between temperate and sub-arctic populations. This study suggests that the cold tolerance of the snail can be regulated by both an acclimation and a genetic adaptation to different thermal environments. While it is reasonable that geographic thermal gradient causes population divergence in their physiological tolerance, there has been little empirical evidence for it.

Gastropods belonging to the family Littorinidae represent one group of common intertidal species in the world (Reid, 1996), and they are one of the few intertidal animals for which cold tolerance has been examined (Murphy, 1979b; Sinclair et al., 2004; Stickle et al., 2015). *Littorina brevicula* (Phillipi, 1844) is a littorinid species that inhabits the upper intertidal zone of rocky shores in the northwestern Pacific (Reid, 1996). Although this species is also found in subtropical regions such as Hong Kong (22° N), they are mainly distributed in temperate regions at a range from 30° N to 45° N (Ohgaki, 1983; Reid, 1996; Kurihara, 2007). Several studies have reported on the population genetics of *L. brevicula*, and their conclusions are slightly different. Some reports concluded that there was substantial gene flow among local populations (Tatarenkov, 1995; Zaslavskaya and Takada, 1998; Kim, et al., 2003). There may be high gene flow among populations due to passive dispersal during the planktonic larval period of at least 10 days (Kojima, 1957; Golikov, 1976; Son and Hong, 1998). However, Zaslavskaya and Takada (1998) detected a limited gene flow between populations in Far East Russia and Japan, and Tatarenkov (1995) reported on genetic differences between samples collected from the same shore in Far East Russia. Analysis using a mitochondrial DNA marker also showed a shallow genetic divergence among 12 populations in Japan (32–45° N) and a north–south population structure (Azuma and Chiba, 2016). To sum up these results, while genetic differentiation among populations of *L. brevicula* seems to be generally small, there is still room for argument about the possibility of population divergence in phenotypic traits related to a slight genetic difference, which molecular markers failed to find. It is therefore worthwhile to verify variations in the response of *L. brevicula* to the thermal environment throughout their distribution from south to north in temperate regions.

For this study, three experiments were conducted to demonstrate population divergence in the cold tolerance of the intertidal gastropod *L. brevicula*, and to explore factors causing the variation. In the first experiment, seasonal changes in the freezing temperature (the supercooling point, SCP) and the lower lethal temperature (LLT) of snails collected from a single population were examined to identify the cold tolerance strategies of this species, given that seasonal variation in cold tolerance is known in many ectotherms including some molluscs (Loomis, 1991, 1995; Rako and Hoffmann, 2006; Stickle et al., 2010; Ronges et al., 2012; McGaughran and Sommer, 2014; Peck et al., 2014). In the second experiment, the cold tolerance of snails was compared among four populations that were distributed at different latitudinal locations. For this experiment, snails were collected from each location before winter when their cold tolerance was not required, and in winter when their cold tolerance increased. Moreover, in order to evaluate their cold acclimation ability, snails were collected from the northernmost and southernmost populations of the four populations before winter, and were acclimated to a constant low temperature in the laboratory for a long period (120–130 days). In the third experiment, snails that were collected at 12 locations before winter were acclimated for a long period, following the same method as that used for the second experiment, and factors that might cause local variation in their cold tolerance were explored.

2. Materials and methods

2.1. Experiment 1: Seasonal changes in cold tolerance

This experiment was conducted to identify the cold tolerance strategy – freeze tolerant or not – of *L. brevicula* by observing seasonality in freezing temperature and low lethal temperature (Ansart and Vernon, 2003; Loomis, 1995). About 50–100 individuals of size-matched (10–14 mm in shell length, SL) *L. brevicula* were collected at a rocky shore in Abashiri (44° N, population no. 2 in Table 1 and Fig. 1), Hokkaido, Japan, in February, April, June, August, October, November of 2006, and January of 2007, from winter to winter of the following year. After each collection, the snails were placed in a plastic bottle (2 L) with papers that were wetted with seawater to maintain humidity and to reduce any rolling of the snails in the bottle. The bottle was then put in a box that was kept at about 4–10 °C and delivered to a laboratory at Tokyo University of Agriculture in Abashiri. In the laboratory, the snails were moved to a round tank (40 cm in diameter (D) × 30 cm, 30 L) filled with 1 L of seawater (salinity approximately 30) that was filtered by fine sand to remove as many unnecessary substances as possible, and they were acclimated without food for 7–14 days in an incubator at about 4–5 °C under a light:dark photoperiod of 12:12 h. The tank was covered with a lid to minimize water evaporation, and the water was changed every week.

After acclimation, each individual was picked up from the tank with tweezers and continued to be pinched until it was placed on a plastic tray (14 × 10 × 2 cm) that was on crushed ice to prevent any rapid increase in the body temperature of the snail until the operculum opened. When the snails extended their foot, the tip of a sensor cable (1.8 D × 500 mm) from a temperature logger (TR52, T & D Corporation) was slipped into their shell from a space around the outer lip. Since the cable could be flexibly bent following the shape of the shell and the whorl of the shell became gradually narrower than the diameter of the sensor, we could assume that the tip of the sensor was always in touch with the foot muscle. Each snail and cable were then gently wrapped with Scotch tape (Scotch 600-1-18C, 3 M). Each wrapped snail was placed into a small bottle (17 D × 40 mm), and the small bottle was covered with a medium-size bottle (31 D × 50 mm) and then a large bottle (47 D × 89 mm) to moderate the effect of changes in outside temperature on the snail's body temperature. Although a hole through which the cable passed was opened in the cap of each bottle, since the diameter of the hole was only 1.8 mm, each bottle was effectively sealed. The three-layer structure of bottles was placed upright in a deep freezer (MDF-C 8V, SANYO) at −40 °C, and the body temperature of the snail in the bottle was checked via the monitor of the logger that was left outside the deep freezer. The snail's body temperature dropped from about 5 °C to a given temperature at 1.85 ± 0.4 °C min^{-1}. Although this cooling rate was faster than changes of air temperature in the wild, we assumed no effect in comparing relative ability of cold tolerance among seasons. The exotherm released by the latent heat of crystallization was identified as a single peak (Fig. 2), and the supercooling point

Table 1
Summary of studied populations.
Both 'Lowest air temperature' and 'Mean air temperature' were shown in the mean value between 1979 and 2013. BW and W used in the Collection date column mean that snails were collected before winter and in winter, respectively. S and L used in the Collection date column mean that snails were acclimated for a short (7–14 days) and a long (120–130 days) periods, respectively.

Population	(Abbreviation)	Latitude	Longitude	Distance from meteorological station (km)	Lowest air temperature	Mean air temperature	Mean shell length	Collection date for BW-S in Exp. 2	Collection date for W-S in Exp. 2	Collection date for BW-L in Exp. 2 and Exp. 3
1. Rebun, Hokkaido	(RBN)	45° 26	141° 00	2 or 15*1	−13.46	6.69	12.49			3 Jul. 13
2. Abashiri, Hokkaido	(ABS)	44° 02	141° 15	5	−17.56	6.54	11.34*2	28 Jun 06	3 Jan 07	10 Sep 13*4
3. Usujiri, Hokkaido	(USJ)	41° 56	141° 56	4	−11.17	8.41	9.71			5 Jun 13
4. Matsumae, Hokkaido	(MTM)	41° 24	141° 12	10	−8.51	10.31	11.09			17 Jun 12
5. Miyako, Iwate	(MYK)	39° 41	141° 58	5	−9.73	10.57	8.60			25 Jul 13
6. Kesennuma, Miyagi	(KSN)	38° 54	141° 37	6	−8.85	10.89	11.02			27 May 12
7. Shichigahama, Miyagi	(SGH)	38° 19	141° 03	5	−6.91	11.41	–*3	28 Aug 06	19 Jan 07	
8. Teradomari, Niigata	(TRD)	37° 38	138° 46	<1	−4.16	13.66	11.17			25 Apr 12
9. Himi, Toyama	(HM)	36° 53	136° 59	3	−6.28	13.15	13.06			9 Jun 12
10. Hitachinaka, Ibaraki	(HTN)	36° 23	140° 37	24	−4.32	13.96	11.97			22 May 12
11. Miura, Kanagawa	(MUR)	35° 08	139° 39	5	−1.50	15.80	–*3	19 Aug. 06	22 Nov 06	
12. Sagazeki, Ooita	(SGZ)	33° 13	131° 52	24	−3.00	16.38	10.82			22 Jun 12
13. Saiki, Ooita	(SIK)	32° 57	131° 56	3	−2.33	16.44	11.59			13 Jun 12
14. Makurazaki, Kagoshima	(MKR)	31° 15	130° 18	<1	−1.13	18.02	9.99*3	5 Aug 06	1 Feb 07	2 May 13*4

*1 Air temperatures of 1979–2002 and those of 2003–2013 were separately recorded at two stations of Rebun, Hokkaido.
*2 Only mean shell lengths (SL) in Experiment 3 were shown, although snails used in this study were chosen to be within an error range of 2 mm in SL at each population.
*3 Mean SLs were not calculated although their SLs were between 10 and 12 mm and between 9 and 11 mm at SGH and at MUR, respectively.
*4 Data were used in both Experiment 2 and Experiment 3.

(SCP, the temperature at which ice formation begins) inside the shell was significantly higher than that outside the shell (Supplementary material S1 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.), indicating that the snails froze from the inside.

Between 5 and 10 individuals were cooled to a given temperature (hereafter, target cold temperature), at 2 °C intervals until all individuals survived or died at the adjacent two or more temperature treatments, and the interval was narrowed to ±1 °C when the number of surviving snails largely changed between 2 °C intervals (Supplementary material S2 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.). Irrespective of being frozen, when the snails' body temperature reached each target cold temperature, the nested bottles were removed from the deep freezer, and the cooled snails were immediately placed in a small clear bottle (80 mL) that was filled with seawater at 4–5 °C in an incubator. The condition of the snails was observed 24 h after each cooling treatment ended, and a snail was classified as survived if it was attached to the wall or the bottom of the clear bottle, or if its foot was partially extended and the foot quickly moved back into the shell when it was gently picked with tweezers.

In this experiment, the SCP and the median of the lower lethal temperature at which mortality of the snail was 50% (LLT50) were examined. Both values are commonly used as an index of cold tolerance in ectotherms (Ansart and Vernon, 2003; Sinclair et al., 2015). The mean of the SCP was calculated from 40, 52, 44, 16, 22, 19, and 21 individuals in February, April, June, August, October, November, and January, respectively, and normality of data distribution of the SCP was observed in all months by Kolmogolov–Smirnov test ($P > 0.05$). LLT50 was calculated from the logistic regression model below:

$$\mathrm{logit}(E[y_i]) = \alpha_0 + \alpha_1 \, \mathrm{TT}_i \quad (1)$$

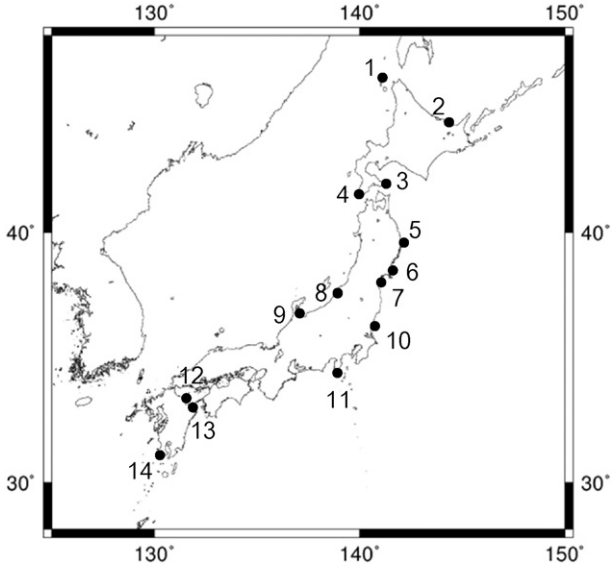

Fig. 1. Map of study sites. Studied populations are numbered from the north.

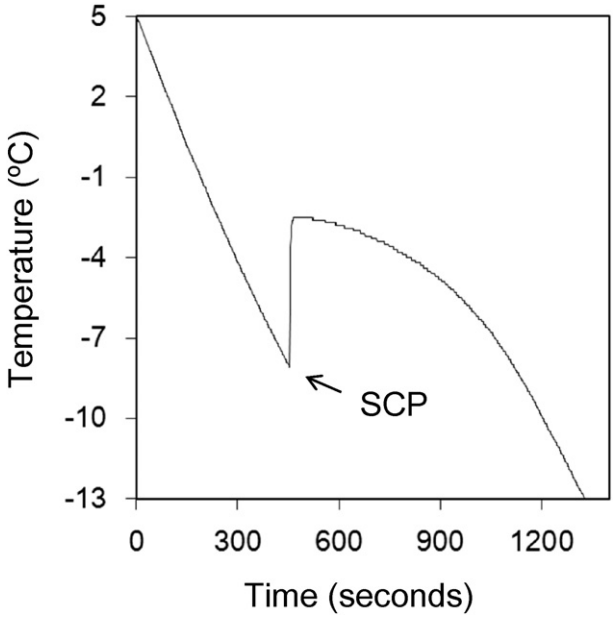

Fig. 2. Example of observed changes in body temperature of a snail that was cooled in Experiment 1. SCP denotes the temperature at which the snail froze, i.e. the supercooling point.

where $E[y_i]$ denotes the expectation of survival (y) of the snail at a target cold temperature (TT) i in each sampling month. Parameters in this model were determined using the maximum likelihood method with the generalized linear model functions of R 3.1.1 (R Development Core Team, 2014).

2.2. Experiment 2: Changes in cold tolerance in different latitudinal populations

In this experiment, we examined whether the seasonality of the cold tolerance observed in Experiment 1 was common in *L. brevicula* that were distributed at latitudinally different locations, and whether there was any variation in tolerance among local populations. *L. brevicula* were collected from populations in four locations, two of which were near the southern and northern limits of their main distribution area. About 100 individuals were collected in summer of 2006 and in the following winter of 2006–2007 at each location: Abashiri, Hokkaido (44° N, population no. 2 in Table 1 and Fig. 1); Shichigahama, Miyagi (38° N, population no. 7); Miura, Kanagawa (35° N, population no. 12); Makurazaki, Kagoshima (31° N, population no. 15). Size-matched snails (8–11 mm in SL) were chosen in each population for this experiment to remove the effect of size variation among populations. The methods of collection, rearing, and the measurement of cold tolerance for the snails in this experiment were the same as those in Experiment 1(Supplementary material S2 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.), although the cooling rate was slightly changed (1.14 ± 0.3 °C min^{-1}), because the air temperature was kept at −30 °C in a different deep freezer (SC-DF25L, TWINBIRD). We assumed that the SCP and the LLT of *L. brevicula* were not affected by the difference in cooling rate between Experiments 1 and 2 based on the results of the supplementary experiments (Supplementary materials S1 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009. and S3 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.).

Moreover, in order to evaluate their cold acclimation ability, approximately 100 individuals of this species were collected from the northernmost (ABS) and southernmost (MKR) populations before initiating cold acclimatization of *L. brevicula* in each local environment in 2013 (Table 1; >20 °C air temperature), and the snails were reared at 4–5 °C for 120–130 days. Although the snails were not provided with specific food such as seaweed during the rearing period, all individuals survived, and they did not appear to be weakened since they actively crawled on the wall of the tank whenever the water was exchanged. They might have consumed fine particles such as phytoplankton in the sand-filtered seawater, because faeces were found on the bottom of the tank until the end of the rearing period. Their LLT50 was also calculated from the logistic regression model shown in Eq. (1).

2.3. Experiment 3: Factors causing variation in cold tolerance among populations

Since the results of Experiment 2 showed that the cold tolerance of *L. brevicula* varied among populations (see Results), factors causing the variation were explored in Experiment 3. In 2012 and 2013, *L. brevicula* was collected from 12 populations along the coast of Japan (31–45° N, Table 1 and Fig. 1). To remove any seasonal effects with latitudinal gradient, snails were collected irrespective of latitudinal order before initiating natural cold acclimation (Table 1). Since we collected size-matched snails that were the most frequently found in each location, the mean SL varied among the populations (Table 1). Snails were reared at 4–5 °C under a light:dark photoperiod of 12:12 h for 120–130 days in the same manner as in the long acclimation method used in Experiment 2. Most of the snails (>99%) survived during the rearing period, indicating that this long period of acclimation did not cause population-specific mortality. Other methods of rearing and measuring cold tolerance in this experiment were the same as those in Experiment 2 (Supplementary material S2 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.).

2.4. Statistical analyses

For the results in Experiment 2, the effects of the collection season and local population on survival (y) of *L. brevicula* under low temperatures were examined, and a full model using the generalized linear model was designed:

$$\mathrm{logit}\left(E\left[y_{i,j,k}\right]\right) = \beta_0 + \beta_1 \mathrm{TT}_i + \beta_2 \mathrm{S}_j + \beta_3 \mathrm{P}_k + \beta_4 \mathrm{TT}_i \times \mathrm{S}_j + \beta_5 \mathrm{TT}_i \times \mathrm{P}_k + \beta_6 \mathrm{S}_j \times \mathrm{P}_k + \beta_7 \mathrm{TT}_i \times \mathrm{S}_j \times \mathrm{P}_k \quad (2)$$

where $E[y_{i,j,k}]$ represents the expectation of survival of the snail at a target cold temperature (TT_i) in collection season (S_j) in population (P_k), and a cross denotes an interaction. To test the null hypothesis of a parameter in the full model, a log-likelihood ratio test was performed under the assumption that the difference in the deviance of two models with and without the parameter followed a χ^2 distribution. The parameters for the higher-level interaction were tested prior to those for the lower-level interaction (Dobson and Barnett, 2008).

The generalized linear model was also used for the results in Experiment 3 to explore factors that caused variation in the cold tolerance of *L. brevicula* among local populations. As explanatory variables that might reflect local environment, we chose the average of the lowest air temperature because it can be assumed that latitudinal gradients in air temperature are the principal determinants of distribution for intertidal organisms (Loomis, 1995; Helmuth et al., 2002; Somero, 2002, 2010; Wethey et al., 2011). The lowest air temperature was recorded every year between 1979 and 2013 at the nearest meteorological station to each sampling location (Japan Meteorological Agency, http://www.data.jma.go.jp/obd/stats/etrn/, last accessed 12 October 2015; Table 1). The mean of body size (i.e. SL) of the snails used in this experiment was also considered, because their body size varied among local populations (Table 1). This model is expressed as:

$$\mathrm{logit}\left(E\left[y_{l,m,n}\right]\right) = \gamma_0 + \gamma_1 \mathrm{TT}_l + \gamma_2 \mathrm{SL}_m + \gamma_3 \mathrm{AT}_n + \gamma_4 \mathrm{TT}_l \times \mathrm{SL}_m + \gamma_5 \mathrm{TT}_l \times \mathrm{AT}_n + \gamma_6 \mathrm{SL}_m \times \mathrm{AT}_n + \gamma_7 \mathrm{TT}_l \times \mathrm{SL}_m \times \mathrm{AT}_n \quad (3)$$

where $E[y_{l,m,n}]$ denotes the expectation of survival (y) of the snail with a body size (SL_m) at a target cold temperature (TT_l) in the lowest air temperature at each location (AT_n). A log-likelihood ratio test was also conducted as in Experiment 2.

Statistical analyses in this study were conducted using the generalized linear model functions of R 3.1.1 (R-Development-Core-Team, 2014).

3. Results

3.1. Experiment 1: Seasonal changes in cold tolerance

Fig. 3 shows seasonal changes in the mean of the observed SCP and LLT50 of *L. brevicula* collected at Abashiri (ABS, population no. 2 in Table 1 and Fig. 1). Seasonal fluctuation was clear in only LLT50. The highest and lowest values of the mean of SCP were recorded in April and August, respectively, and the difference between the values was 1.20 °C. LLT50 reached its peak in August and decreased by November. The difference between the highest and lowest values in LLT50 was 7.76 °C. LLT50 was higher than the mean of SCP in August (summer), indicating that this species died because of a chilling injury, since no latent heat due to ice formation within the body was observed in the dead specimens. By contrast, between November and April (winter), LLT50 was over 5 °C lower than the mean of SCP, and the survival of individuals that were cooled to −8 and −10 °C was 100% and 75–90%, respectively (Supplementary material S2 in the online version at http://dx.doi.

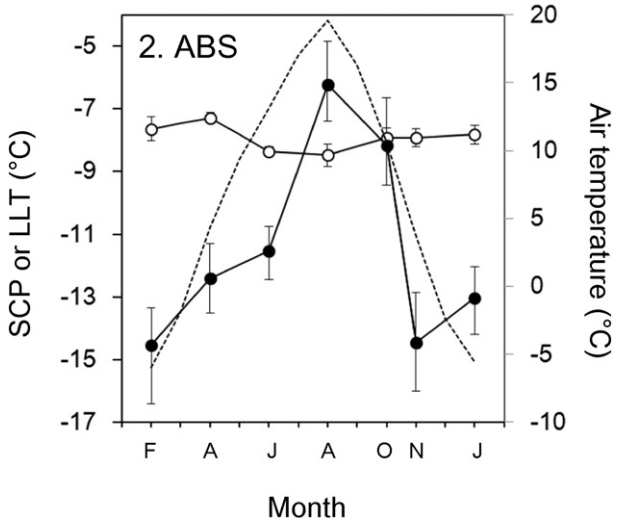

Fig. 3. Seasonal changes in the supercooling point (SCP) and lower lethal temperature (LLT) of *L. brevicula* collected in Abashiri (Experiment 1). '2. ABS' denotes the population number and abbreviation of Abashiri (Table 1, Fig. 1). Open circles denote the mean of the observed SCP, and solid circles denote the LLT at which 50% of the snails (LLT50) were estimated to die. Vertical bars with each plot show the 95% confidence interval. Air temperature is shown as the mean value for each month between 1981 an d2013, recorded at the meteorological station at Abashiri (Table 1).

org/10.1016/j.jembe.2016.04.009.), indicating that the snails survived tissue freezing.

3.2. Experiment 2: Changes in cold tolerance in different latitudinal populations

In all four populations, seasonal differences in LLT50 were larger than those in the mean of SCP, and LLT50 dropped below the mean of SCP in winter (Table 2). Survival of *L. brevicula* at low temperatures correlated with three explanatory factors: target cold temperature, collection season, and population (Table 3, Supplementary material S4 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.). Survival at a given target cold temperature was always greater for the snails collected in winter than those collected in summer (Tables 2 & 3, Fig. 4). Table 2 shows that LLT50 in both summer and winter collections was lowest in Abashiri (ABS, population no. 2 in Table 1 and Fig. 1), followed in order by Shichigahama (SGH, population no. 7), Miura (MUR, population no. 12), and Makurazaki (MKR, population no. 15). Moreover, the coefficients for population (β_3) that were shown as relative values to ABS in Table 3 show that survival at low temperatures decreased in the same order: highest in ABS, followed by SGH, MUR, and MKR. LLT50 that was calculated after exposure to cold environment for a long period (120–130 days) was lower than that for a short period (7–14 days) in both populations of ABS and

Table 2
The median of SCP and temperature at which 50% of *Littorina brevicula* (LLT50) in Experiment 2. Numeric number with the abbreviation of population are correspond with the number in Table 1 and Fig. 1.

Population	Collection SEASON	SCP	LLT50
2. ABS	Before Winter	−8.39	−6.21
2. ABS	Winter	−7.76	−13.02
7. SGH	Before Winter	−8.41	−5.50
7. SGH	Winter	−8.13	−12.67
11. MUR	Before Winter	−8.29	1.48
11. MUR	Winter	−8.88	−9.72
14. MKR	Before Winter	−8.58	2.11
14. MKR	Winter	−7.07	−9.00

Table 3
Model explaining survival of *L. brevicula* collected from four local populations in Experiment 2.

	Estimate	S.E.	z value	P value
Intercept	3.538	0.496	7.130	<0.001
Cooling temperature	0.722	0.074	9.806	<0.001
Season				
Winter	6.466	0.719	8.990	<0.001
Population				
SGH	−0.320	0.405	−0.792	0.429
MUR	−3.662	0.553	−6.621	<0.001
MKR	−4.366	0.614	−7.107	<0.001
Residual deviance	49.389			
d.f.	52			

Levels in categorical factors (i.e., 'Season' and 'Population') were analyzed as dummy variables. Estimates in 'Season' and 'Population' are relative values to 'Before Winter' and 'ABS', respectively.

MKR, indicating their cold acclimation initiated in the laboratory (Fig. 5). The latitudinal difference, however, was maintained (Fig. 5).

3.3. Experiment 3: Factors causing variation in cold tolerance among populations

Table 4 shows parameters of the model after performing a log-likelihood ratio test (Supplementary material S5 in the online version at http://dx.doi.org/10.1016/j.jembe.2016.04.009.). In this model, coefficient (γ_5) for the interaction between the target cold temperature (TT) and the lowest air temperature (AT) was 0.035 ± 0.010 (Table 4),

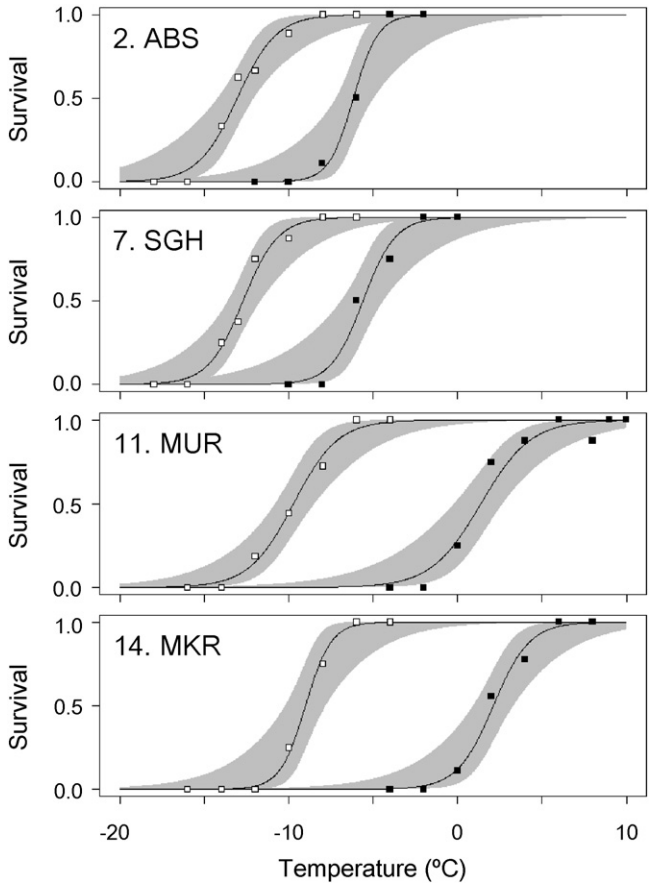

Fig. 4. Survival of *L. brevicula* at low temperature in each population (Experiment 2). '2. ABS', '7. SGH', '11. MUR', and '14. MKR' denote population numbers and abbreviations of Abashiri, Shichigahama, Miura, and Makurazaki, respectively (Table 1, Fig. 1). Solid and open squares represent data from summer and winter collections, respectively. Dark areas denote the 95% confidence interval of each estimated regression line.

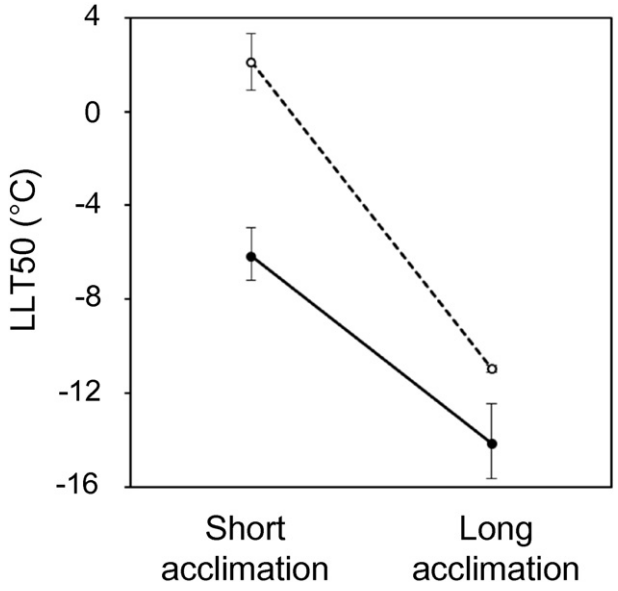

Fig. 5. Changes in the lower lethal temperature at which 50% of the snails were estimated to die (LLT50) with acclimation period (Experiment 2). Snails were collected before initiating cold acclimation in the wild (Table 1) and their LLT50 was measured after acclimating at a low temperature for 7–14 days (short acclimation) and for 130–140 days (long acclimation). Filled and open circles denote Abashiri (2. ABS) and Makurazaki (14. MKR), respectively (Table 1, Fig. 1). Vertical bars with each plot show 95% confidence interval.

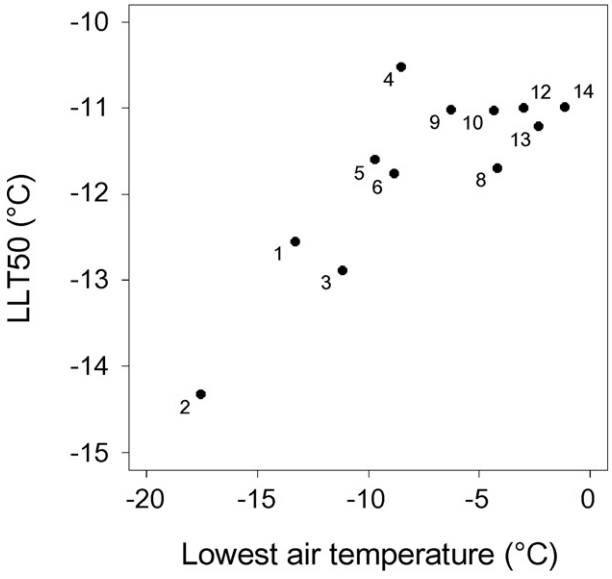

Fig. 6. Relationship between the lowest air temperature at each location and the lower lethal temperature at which 50% of the snails were estimated to die (LLT50) (Experiment 3). Numeric characters at each plot correspond to populations shown in Table 1 and Fig. 1.

indicating that the survival of snails collected from locations where the lowest air temperature was relatively high declined with target cold temperatures more steeply than that of snails from locations where the lowest air temperature was relatively low. Fig. 6 clearly shows that LLT50 in each population decreased with the lowest air temperature in their habitats.

4. Discussion

From the results of Experiment 1, we concluded that the cold tolerance strategy of *L. brevicula* was freeze tolerant. Decreased LLT in winter has been also documented in other freeze tolerant intertidal molluscs (Loomis, 1991; Stickle et al., 2010). In a strict sense, freeze tolerance may be carefully defined after confirming 'complete' freezing, that is when ice formation in the body tissue is completed (Sinclair et al., 2015). Murphy (1979a) demonstrated that other species of *Littorina* (*Littorina obtusala*, *L. littorea*, *L. saxatilis*) were not injured by freezing until 82% of their tissue water froze, and that freezing injury of *L. obtusala*, *L. littorea*, and *L. saxatilis* was mainly caused under −16, −17, and −20 °C, respectively. Although the SCP of those *Littorina* species was not mentioned, their SCP would have been higher than the temperature at which freezing injury occurred because the SCP of most intertidal molluscs is in the range of −4 and −11 °C (e.g. Murphy, 1979b; Murphy and Johnson, 1980; Loomis, 1991; Stickle et al., 2010, 2015; Dennis et al., 2014). Although we did not measure how much *L. brevicula* tissue water froze after reaching the SCP in this experiment, the lethal freezing temperature of this species would also be much lower than the SCP because over 50% of individuals survived until temperatures that were approximately 5 °C lower than the SCP during winter. Therefore, even though their body partially froze in Experiment 1, it is reasonable to assume that *L. brevicula* mortality had not been caused by a freezing injury. Cold tolerance by initiating extracellular ice formation in body fluids is common in intertidal molluscs (Loomis, 1991, 1995; Ansart and Vernon, 2003) except for some cases (Sinclair et al., 2004). Stickle et al. (2015) demonstrated that the concentration of cryoprotectant free amino acids in tissue relates to the seasonality of freeze tolerance in intertidal gastropods *Littorina sitkana* and *Nucella lima*. A similar mechanism may therefore act in response to seasonal changes in the freeze tolerance of *L. brevicula*.

Experiment 2 showed that a freeze tolerant strategy was maintained in all *L. brevicula* populations that were distributed at different latitudinal locations. In three of four populations, the lowest air temperatures in each location (Table 1) were higher than the SCP of the snails in each location (Table 2). This result indicates that these three populations are capable of freeze tolerance despite the low risk of body tissue freezing in their habitat. It is plausible that this strategy remains in the southern populations if it does not bring any disadvantage for their fitness. Otherwise, *L. brevicula* in the southern populations may be also exposed to a risk of inoculative freezing (Ansart and Vernon, 2003) because the lowest temperature reaches below 0 °C even in the southernmost studied population (Table 1).

Experiment 2 also suggests that their freeze tolerance is genetically fixed as a cold tolerant strategy. The order of both LLT50 in each population (Table 2) and the coefficient for population (β_3) in the model for this experiment (Table 3) implies that the snails collected from northern populations showed higher tolerance to cold stress than those from southern populations. It is especially noteworthy that this latitudinal trend was also maintained in summer when the natural cold acclimation of snails at each location had not yet been initiated, because this local variation would reflect the local variation in the genetic-based response of the snails to cold stress rather than the temporal variation in cold stress at which the snails were collected. In addition, the

Table 4
Model explaining survival of *L. brevicula* collected from 12 local populations in Experiment 3.

	Estimate	S.E.	z value	P value
Intercept	11.275	1.402	8.042	
Target cold temperature (TT)	1.056	0.121	8.708	
Air temperature (AT)	0.293	0.129	2.271	
TT × AT	0.035	0.010	3.421	0.001
Residual deviance	62.694			
d.f.	87			

inter-population difference was maintained after acclimating under low temperature for a long period in Experiment 2, meaning that their ability of cold tolerance cannot be explained by cold acclimation alone. The study on another *Littorina* species, *L. saxatilis*, also appears to support the role of both acclimation and genetic adaptation in contributing to the latitudinal difference in metabolic responses of that species to cold condition (Sokolova and Pörtner, 2003). The capacity for freeze tolerance under extreme cold conditions could thus also be a selective trait in other species of *Littorina*.

Experiment 3 clarified that the response of *L. brevicula* to a low temperature also varied with the latitudinal thermal gradient. The snails collected from locations where the lowest air temperature was relatively low showed higher cold tolerance than those from locations where the lowest air temperature was relatively high, demonstrating that the snails' cold tolerance adapted to the severe winter in each location. Inter-population variation in cold tolerance has also been documented in some intertidal species (Hilbish, 1981; Loomis, 1985) but factors causing such variation remain largely unclear. The salt marsh snail, *Melampus bidentatus*, has been the most thoroughly studied mollusc for inter-population difference in cold tolerance, and it has been shown that the contribution of latitudinal temperature gradient to such difference in this species was masked by other local environmental factors such as salinity and humidity (Dennis et al., 2014). Since interaction between physical environments in upper rocky intertidal zones where *L. brevicula* is distributed is relatively simpler than that in other intertidal zones, the effect of air temperature on cold tolerance would be clearer for this species than for species distributed in salt marsh.

The present study suggests that genetic adaptation in cold tolerance is likely to be present in *L. brevicula* even if it has a planktonic larval phase. Adaptation to a habitat-specific environment can occur even though gene flow is detected by some neutral DNA markers (Brown et al., 2001; Räsänen and Hendry, 2008; Gonzalo-Turpin and Hazard, 2009; Sarup et al., 2009), and local adaptation in marine animals that have high dispersal ability but lack genetic differentiation has been attracting considerable attention in recent years (Clarke et al., 2010; Sanford and Kelly, 2011; Hice et al., 2012; Gleason and Burton, 2013; Teske et al., 2013; Peterson et al., 2014). In *L. brevicula*, while gene flow across a broad range of *L. brevicula* populations has been documented (Kim et al., 2003; Tatarenkov, 1995; Zaslavskaya and Takada, 1998), genetic differentiation among populations and within a population has also been discussed (Takada, 1995, 2003; Takada and Rolán-Alvarez, 2000; Tatarenkov, 1995; Zaslavskaya and Takada, 1998). Recently, north–south genetic differentiation in this species along the coastline of Japan was clarified with a mitochondrial DNA marker (Azuma and Chiba, 2016). It is therefore likely that *L. brevicula* genetically adapted to each local thermal environment, although the direct genetic evidence, the specific gene, or the genetic marker directly linking cold tolerance should be explored in the future, because this study did not investigate factors such as maternal and epigenetic effects. Our study at least indicates that inter-population variation in physiological responses to a local environment cannot be dismissed simply by their high dispersal ability. As for the mechanisms of shore-to-shore variation in the phenotype of a snail that is not genetically isolated, Kurihara et al. (2006) suggested intragenerational selection where only individuals with locally adaptive traits would survive after random recruitment on each shore. We speculate that intragenerational selection is one of the plausible mechanisms affecting local variation in the cold tolerance of *L. brevicula*. If climate change leads to poleward shifts in the distribution of ectotherms, this study suggests that unpredictable occurrences of low winter temperatures could not only offset the shift of the distribution of species (Firth et al., 2011; Wethey et al., 2011) but also cause the extinction of local populations of the same species.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2016.04.009.

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