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Physiological response to low temperature in the freshwater apple snail, *Pomacea canaliculata* (Gastropoda: Ampullariidae)

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SUMMARY

Cold hardiness of the freshwater apple snail, *Pomacea canaliculata*, varies seasonally. We investigated lethal factors and physiological changes arising from exposure of *P. canaliculata* to low temperatures. Snails did not survive freezing. The supercooling point of cold-acclimated (cold tolerant) snails (–6.6±0.8°C) did not differ significantly from that of non-acclimated ones (–7.1±1.5°C) under laboratory conditions. Furthermore, snails died even under more moderately low temperatures approaching 0°C. These results indicate that indirect chilling injury is a factor in the death of *P. canaliculata* at low temperatures. Regardless of whether the snails were acclimated to low temperatures, all of the dead, and even some of the snails still alive at 0°C, had injured mantles, indicating that the mantle may be the organ most susceptible to the effects of low temperatures. The concentration of glucose in the posterior chamber of the kidney and concentration of glycerol in the digestive gland were significantly higher in cold-acclimated snails than in non-acclimated ones, suggesting carbohydrate metabolic pathways are altered in snails during cold acclimation.

Key words: mollusks, cold hardiness, indirect chilling injury, supercooling point, glucose, glycerol.

INTRODUCTION

Low temperature during winter causes serious injuries to invertebrates inhabiting cold to temperate regions (Block, 1982; Lee, 1991; Oswood et al., 1991). Cold injuries can be classified into two categories: freezing injury and chilling (non-freezing) injury. The mechanism of freezing injury is thought to be the osmotic stress to cells and the mechanical damage of cells caused by intracellular ice (Lee, 1991). Chilling injury can be further classified into two types based on duration of exposure to low temperature (Lee, 1991). Injuries caused by rapid cooling are called cold shock (direct chilling injury) whereas those caused by long-term cold exposure are called indirect chilling injury.

Invertebrates have several physiological strategies to avoid cold injuries. Some species can survive freezing by inhibiting lethal intracellular ice formation when exposed to freezing temperatures (Leather et al., 1993). Freeze intolerant species often have a supercooling ability that decreases the temperature at which crystallization of the body occurs by elimination of ice nucleators from the body and production of antifreeze agents such as polyols and proteins (Leather et al., 1993). Increased glycerol and heat shock protein are known to be effective for avoiding cold shock in insects (Denlinger et al., 1991).

Physiological response to low temperature in intertidal and terrestrial mollusks has also been studied (Ansart and Vernon, 2003), although not as extensively as in insects. For instance, increased freeze tolerance in the intertidal pulmonate gastropod, *Melampus bidentatus*, is associated with an increase in glycerol and proline contents within the body (Loomis, 1985). The strategy of the land snail, *Helix aspersa*, to cope with low temperature is known to change from freezing avoidance to freezing tolerance as body size increases (Ansart and Vernon, 2004). However, there are only a few reports that have focused on the response of freshwater mollusks to low temperature (Olsson, 1984; Frisbie and Lee, 1997; Ansart and Vernon, 2003).

The freshwater apple snail, *Pomacea canaliculata* (Lamarck), is an invasive species that originated from South America (Martin et al., 2001; Cowie, 2002). It was introduced into Asia, including Japan, as a human food mainly in the early 1980s. However, it has become a serious pest of rice in invaded countries (Wada, 2004; Cowie et al., 2006; Hayes et al., 2008). In temperate Japan the snails hibernate in paddy fields, irrigation canals, ponds and other bodies of water. In paddy fields the snails multiply during the summer crop season. When farmers drain fields for harvest in September or October, snails bury themselves into the soil and enter dormancy. The snails overwinter in drained paddy fields and remain dormant until the fields are irrigated for rice planting in June. The mortality during winter is quite high (approximately 65–95% in Kyushu, South Japan) every year (Oya et al., 1987; Wada and Matsukura, 2007). The snails also overwinter under flood conditions such as in sediments of canals and ponds.

We have investigated the ecological and physiological mechanisms of cold hardiness of this snail in Japan. Cold hardiness is cued prior to the onset of winter by environmental factors: cold-acclimation enhances cold hardiness and dry conditions promote it to some degree (Wada and Matsukura, 2007; Matsukura and Wada, 2007). From a physiological perspective, we found decreased glycogen and increased glycerol and glucose contents within the whole body to be associated with enhanced cold hardiness (Matsukura et al., 2008). This observation suggests carbohydrate metabolism may be involved in changes to cold hardiness.

The purpose of the present study was to examine specific factors that may be associated with the lethal effects of low temperature

and the possible mechanisms used by *P. canaliculata* for increased cold tolerance. We examined the effect of freezing on survival, changes in the supercooling point and organ injury possibly caused by low temperature. In addition, we compared the contents of carbohydrates in specific body organs between cold tolerant and intolerant snails.

MATERIALS AND METHODS

Snails

A stock culture of *P. canaliculata* originally collected from Kikuchi, Kumamoto Prefecture (32.6°N, 130.4°E) and maintained within the laboratory for two or three generations was used in this study. In a previous paper (Matsukura et al., 2008), we found that the physiological response of laboratory-reared snails to cold acclimation is partially different from that of field snails. However, we used only laboratory-reared snails in this study because we required large numbers of homogeneous samples.

Hatchlings from several egg masses were mixed and reared in a plastic container (20 cm×30 cm, 30 cm deep) with ample water at 25°C and 16 h:8 h light:dark photoperiodic conditions. A few grains of carp food (Hikari, Kyorin, Hyogo, Japan) were provided daily as basic food, and a small amount of oyster shell powder was occasionally provided as a source of calcium. After approximately one month, snails that had grown to an appropriate size (12.5–17.5 mm shell height) were used for the experiments.

Cold acclimation

The cold hardiness of *P. canaliculata* was experimentally induced by cold acclimation under moist conditions as reported in Matsukura and Wada (Matsukura and Wada, 2007). Twenty snails were removed from a rearing container, wrapped in a moist towel and confined in a plastic cup (10 cm diameter, 4 cm deep). The temperature was decreased by 5°C every five days from 25°C to 10°C, and then maintained at 10°C for four weeks. The snails developed their cold hardiness in response to this procedure. Photoperiod does not affect the induction of cold hardiness in *P. canaliculata* (Matsukura and Wada, 2007) and was fixed at 16 h:8 h light:dark.

Determination of temperature of crystallization (T_c)

Temperature of crystallization (T_c) was determined for both non-acclimated and acclimated snails. Non-acclimated snails were kept in a moist towel for one day at 25°C immediately after water deprivation. To measure T_c for the whole body, snails were attached to copper–constantan thermocouples that were connected to a multichannel temperature recorder (Thermodac 5001A, Eto Denki, Japan). A thermocouple probe tip was inserted into an opening of the shell so that the tip was fixed between the operculum and the foot. In a preliminary experiment, the probe tips were fixed on the shell surface with cellophane adhesive tape. However, T_c did not differ significantly from that measured by insertion of the tip into a soft part. Thus, we used the method of direct insertion through an opening in the shell. The snails were cooled in a freezer at a rate of 0.1°C per minute. When the entire body of a snail freezes, latent heat of crystallization is released. Therefore, a rapid increase in monitored temperature was regarded as freezing of the snail.

T_c was also estimated for six specific body organs and tissues (blood, foot muscle, mantle, gut, posterior chamber of kidney and digestive gland) and gut contents. T_c of gut contents and each body organ except blood were determined as follows. After a shell and an operculum were removed, each body organ was dissected from the soft part in a physiological salt solution for *P. canaliculata* [0.5% salt solution (K.M., H.T. and Y.I., unpublished data)]. We also measured T_c of gut contents collected from the rectum. The dissected organ and gut contents in physiological salt solution were packed into pre-calibrated Vitrex pipettes (Modulohm A/S, Herlev, Denmark) that had been cut to approximately 3 cm. The thermocouple probe tips were fixed on the surface of each pipette with adhesive tape, the pipettes were cooled and T_c was determined as described above.

T_c of blood was estimated using a similar method except blood was drawn directly from the heart. The heart of *P. canaliculata* can be observed on the surface of the soft part and, therefore, when we inserted a tip of the Vitrex pipette into the heart, blood spontaneously rose into the pipettes. Approximately 20 µl of blood was sampled from each snail.

Investigation of freezing tolerance

Cold-acclimated snails were used to examine freezing tolerance. Thirty snails were individually attached to copper–constantan thermocouples connected to the multichannel temperature recorder, and cooled in the same manner as for determination of T_c (see above). All snails were removed from the freezer when approximately half of the snails (approximately 15) had frozen. The snails were categorized as frozen or unfrozen, immersed in tap water for one day at room temperature (17–22°C) and the number of survivors was counted. Snails that extended their foot from the shell were considered survivors.

Investigation of injured organs at 0°C

Both non-acclimated and acclimated snails were used. Snails were wrapped in a moist towel and exposed to 0°C. Non-acclimated snails were sampled at three and five days, and acclimated snails were sampled at five and 11 days after the beginning of 0°C exposure. The sampled snails were immersed in tap water at room temperature and, after 3 h, they were categorized as either survivors or non-survivors using the method previously mentioned. Injured organs were examined by Trypan Blue staining following Izumi et al. (Izumi et al., 2005b) with some modifications. This method often underestimates the number of dead cells (Krause et al., 1984); Trypan Blue, however, stains only injured organs regardless of cell type, unlike some other staining methods, because the stain enters cells through membranes that have been injured. Both live and dead snails were individually removed from the shell, cut open on the right side of mantle and soaked in 0.4% Trypan Blue solution for 1 min to stain the injured organs. These staining conditions are suitable in this case because a lower density of Trypan Blue solution would not stain any organs due to a large amount of mucus around the snail's body, and longer stain periods caused excessive osmosis to healthy organs (K.M. and H.T., unpublished). After rinsing the entire body with distilled water, coloring of foot muscle, head, mantle, lung, inner mantle fold, gut, digestive gland and posterior chamber of kidney were observed through a microscope.

Chemical analysis

Glycogen, glucose and glycerol contents were determined in three types of snails: non-acclimated, quasi-acclimated and acclimated. Quasi-acclimated snails were exposed to 10°C for two weeks whereas acclimated snails remained at 10°C for four weeks. The cold hardiness of the quasi-acclimated snails is as high as that of acclimated snails (Matsukura et al., 2008).

We investigated the chemical contents of five body organs (N=5 in each organ): foot muscle, mantle, gut, posterior chamber of kidney

and digestive gland. These organs were dissected from snails using the same method for determination of T_c (see above).

The three chemical components were measured as described in Matsukura et al. (Matsukura et al., 2008). The dissected organ was individually homogenized in 2 ml of 80% ethanol. After purification of the extract, as described in Izumi et al. (Izumi et al., 2005a), the water solution from the extraction was used to measure glucose and glycerol, and the precipitates were used to measure glycogen. The amount of glucose was measured by the mutarotase–GOD method, glycerol was quantified by the colorimetric method and glycogen was quantified by anthrone (for details, see Matsukura et al., 2008).

Statistical analyses

All statistical analyses were done with R 2.2.1 (R Development Core Team, 2005). For freezing tolerance, minimum cooling temperatures were compared with a Student's *t*-test and survival rates between frozen and unfrozen snails were compared by Fisher exact probability test. T_c of non-acclimated and acclimated snails was compared with a Student's *t*-test. T_c for each organ were compared with that of whole body by Dunnett's test after log transformation of absolute values of T_c. Fisher exact probability tests were used to compare frequency of organ injury between alive and dead snails after the same cold treatment and organ injury of non-acclimated and acclimated snails after five days exposure to 0°C. Tukey's HSD was used to test for differences in chemical analysis within organ type from non-acclimated, quasi-acclimated and acclimated snails.

Temperatures in the field

We obtained temperature data at Kikuchi, Kumamoto Prefecture (32.9°N, 130.8°E) to infer an ecological significance of the mechanism of cold hardiness in *P. canaliculata*. The temperature data was referred from an Automated Meteorological Data Acquisition System (AMeDAS) point at Kikuchi operated by the Japanese Meteorological Agency. We calculated the daily mean, mean of daily maximum and mean of daily minimum temperatures for five seasons (from 2003–2004 to 2007–2008) from December to April.

RESULTS

Temperature of crystallization (T_c)

There was no significant difference in T_c between non-acclimated and acclimated snails; T_c in the whole body was –6.6±0.8°C (±s.d.) in acclimated snails and –7.1±1.5°C (±s.d.) in non-acclimated ones (Fig. 1). In addition, T_c of gut content and the five body organs (foot muscle, mantle, gut, posterior chamber of kidney and digestive gland) and blood also did not differ significantly between non-acclimated and acclimated snails. The six organs froze at much lower temperatures (from –15.3°C to –10.0°C) than the whole body whereas T_c of gut content did not significantly differ from that of the whole body (Fig. 1).

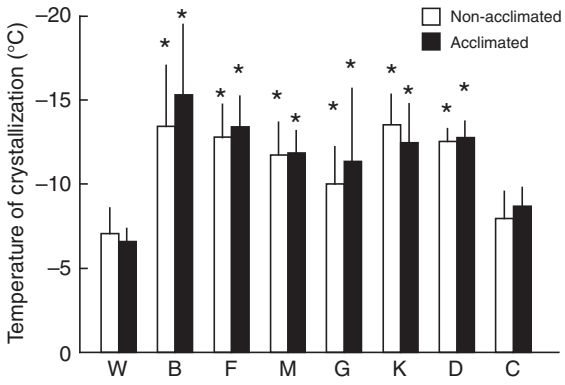

Fig. 1. Temperature of crystallization (T_c) of the whole body, major body organs or tissues and gut content in non-acclimated and acclimated *Pomacea canaliculata* (W, whole body; B, blood; F, foot muscle; M, mantle; G, gut; K, posterior chamber of kidney; D, digestive gland; C, gut content). Sample sizes were: whole bodies, non-acclimated=14; whole bodies, acclimated=15; gut content=6; and other=9. The vertical bars represent s.d. An asterisk indicates a significant difference among T_c of the whole body within snails of the same treatment (Dunnett's test, P=0.05).

Effect of freezing on survival

Freezing of the whole body resulted in death of *P. canaliculata*. All 16 frozen snails died whereas five out of 12 unfrozen snails survived even after cooling to almost the same temperature experienced by the frozen snails (Table 1), indicating that freezing is fatal in *P. canaliculata* under the experimental condition.

Injured organs at 0°C judging from Trypan Blue staining

More than half (11 out of 18) and all (N=14) non-acclimated snails were dead at three and five days after the beginning of exposure to 0°C, respectively. By contrast, 11 out of 29 acclimated snails survived even 11 days after the beginning of exposure to 0°C (Table 2). According to the intensity of color of each organ stained by Trypan Blue, all dead snails and some survivors had injury to the mantle, regardless of whether they were acclimated or non-acclimated (Table 2; Fig. 2). In addition, non-acclimated snails that died within three days of exposure to 0°C suffered injury on a greater proportion of the inner mantle fold (nine out of 11 snails were injured) and posterior chamber of the kidney (six out of 11 snails were injured). On the contrary, there were no organs except the mantle that had a high proportion of injury among acclimated snails that died within 11 days after the beginning of exposure to 0°C (Table 2; Fig. 2).

When we compare non-acclimated snails (alive and dead) with acclimated ones that were exposed to 0°C for the same duration of five days, there were significant differences in injury ratios of some organs. Proportion of injury to the mantle (all 14 non-acclimated and nine out of 16 acclimated snails were injured), lung (all 14 non-acclimated and one out of 16 acclimated snails), inner mantle fold (all 14 non-acclimated and none of the acclimated snails) and posterior chamber of kidney (all 14 non-acclimated and two out of 16 acclimated snails) was significantly lower in acclimated snails than in non-acclimated snails.

Change of chemical components in organs

Remarkable changes in glycogen, glucose and glycerol concentration in several organs were observed during cold acclimation. Glycogen in the foot muscle and the posterior chamber of the kidney decreased

Table 1. Survival rates of frozen and unfrozen *Pomacea canaliculata*

	N	Minimum cooling temperature* (°C ±s.d.)	No. survived	Survival rate† (%)
Frozen	16	–7.1±1.5	0	0.0
Unfrozen	12	–6.6±0.9	5	41.7

*There is no significant difference between frozen and unfrozen snails (P=0.289, Student's *t*-test).

†There is significant difference between frozen and unfrozen snails (P=0.044, Fisher exact probability test).

Table 2. Percentage of injury to various organs of *Pomacea canaliculata* after exposure to 0°C, estimated by intensity of staining with Trypan Blue

Snails	Exposure period	Viability	Snails used	Foot muscle	Head	Mantle	Lung	Inner mantle fold	Gut	Digestive gland	Kidney
Non-acclimated	3 days	Alive	7	0	0	57.1	0	0	0	0	0
		Dead	11	9.1	18.2	100.0*	18.2	81.8**	27.3	9.1	54.5*
	5 days	Alive	0	–	–	–	–	–	–	–	–
		Dead	14	0	14.3	100.0	78.6	71.4	21.4	0	50.0
Acclimated	5 days	Alive	13	0	0	46.2	0	0	0	0	0
		Dead	3	0	0	100.0	33.3	0	0	33.3	66.7*
	11 days	Alive	11	0	0	27.3	0	0	0	0	0
		Dead	18	0	27.8	100.0***	22.2	27.8	11.1	5.6	5.6

Asterisk in each column indicates a significant difference in injury ratio between live and dead snails of the same treatment (*P<0.05; **P<0.01; ***P<0.001, Fisher exact probability test).

by nearly half (Fig. 3) whereas glucose in the mantle and posterior chamber of the kidney, and glycerol in the foot muscle and digestive gland increased during cold acclimation (Fig. 3).

Field temperatures during the cold season

The maximum decrease in temperature occurred from early January to middle February in Kikuchi (Fig. 4). Daily mean temperatures, however, never went below 0°C. Daily minimum temperature fluctuated from –1.4 to 3.4°C during this period.

DISCUSSION

Our results indicate that indirect chilling injury is a factor contributing to the death of *P. canaliculata* at low temperature. *Pomacea canaliculata* did not survive freezing under the experimental condition, and they died at milder temperatures [0°C or 10°C (Matsukura and Wada, 2007)] than they froze (approximately –7.0°C) (Table 1). Furthermore, obvious differences in the survival ratio between cold-acclimated and non-acclimated snails were observed at 0°C (Table 2) but not at subzero temperatures where both types of snails cannot survive (Wada and Matsukura, 2007). These facts indicate that development of cold hardiness of *P. canaliculata* is not a countermeasure to freezing but serves as avoidance or reduction of indirect chilling injury at moderately low temperatures.

At the histological level, certain organs seemed more susceptible to injury from low temperatures near 0°C relative to other organs. In particular, the mantle seems to be the weakest organ at moderately low temperatures because it was injured in all dead snails and a proportion of live snails (Table 2). The most well-known function of the mantle in snails is the production of a secretion for shell formation (Timmermans, 1968). The mantle is also involved in the control of osmolality (Aunaas et al., 1988), as a barrier to permeance of extrapallial fluid into the body (Albrecht and Cavicchia, 2001) and in pinocytosis of external medium (Zylstra, 1971). Mantle injury after exposure to low temperature may cause a physiological lesion

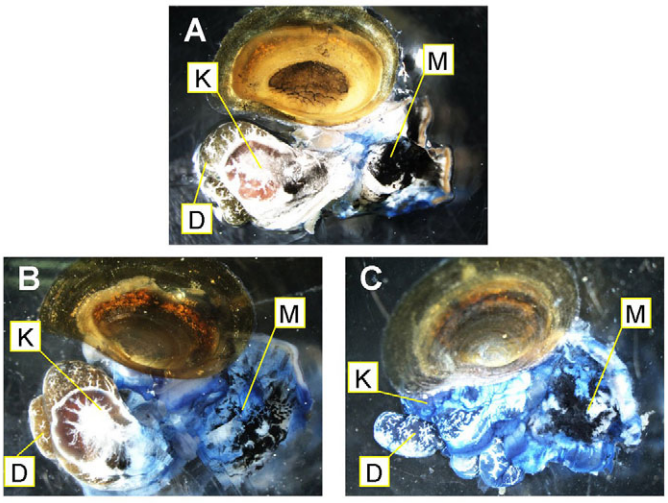

Fig. 2. Examples of coloring of *Pomacea canaliculata* organs stained by Trypan Blue. No organs were stained blue in a non-acclimated live snail before exposure to 0°C (A), blue staining was observed on the mantle but not on the digestive gland and the posterior chamber of the kidney in an acclimated dead snail after exposure to 0°C for 11 days (B) and all organs were stained blue in a non-acclimated dead snail after exposure to 0°C for 3 days (C). M, mantle; D, digestive gland; K, posterior chamber of kidney.

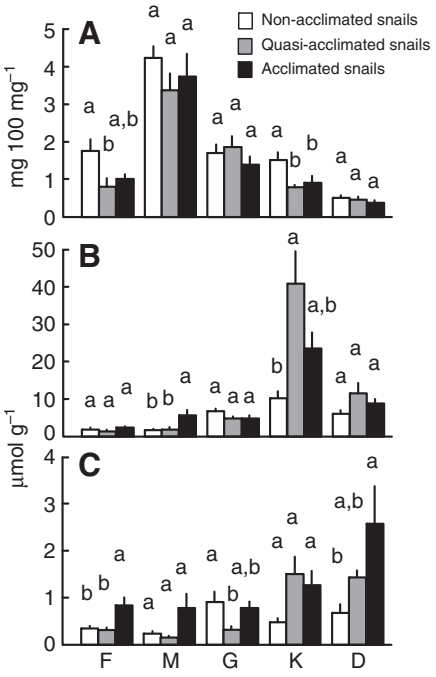

Fig. 3. Glycogen (mg 100 mg^{-1}) (A), glucose (µmol g^{-1}) (B) and glycerol (µmol g^{-1}) (C) contents in five major body organs at different stages in cold acclimation (F, foot muscle; M, mantle; G, gut; K, posterior chamber of kidney; D, digestive gland). Quasi-acclimated snails were exposed to 10°C for two weeks in the cold acclimation treatment whereas acclimated snails were exposed for four weeks. Sample size=5 for all measurements. Vertical bars represent s.e.m. The same alphabetical letter on each vertical bar indicates no significant difference from the same organ of other treatments.

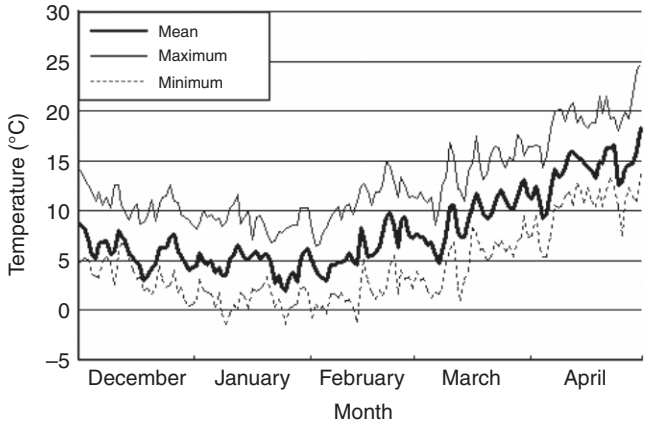

Fig. 4. Daily mean, mean of daily maximum and mean of daily minimum air temperatures from December to April at Kikuchi, Kumamoto, calculated from the data from the past five seasons from 2003–2004 to 2007–2008.

that is lethal to snails. It is uncertain, however, whether mantle injury causes death of *P. canaliculata* directly, because some snails that survived after 0°C exposure also had mantle injuries (Table 2). Further experiments are required to clarify whether mantle injury itself is lethal in *P. canaliculata*.

Metabolic activity of carbohydrates in the posterior chamber of the kidney and digestive gland may be an important possible physiological mechanism involved in cold hardiness of *P. canaliculata*. Remarkable increases of glucose in the posterior chamber of the kidney and glycerol in the digestive gland were observed in cold-tolerant snails (Fig. 3), probably by consumption of glycogen in the foot muscle and posterior chamber of the kidney (Fig. 3). Metabolic disruption related to cellular energetics is considered to be one of the main factors causing indirect chilling injury in animals (Storey and Storey, 1988). Low temperature reduces fluidity of cell membranes on which membrane-associated proteins bind, changes higher-order structure of protein and ionic activities, and disrupts metabolic pathways in the cell (Storey and Storey, 1988). *Pomacea canaliculata* altered metabolic activity of digestive and metabolic organs, including the digestive gland and posterior chamber of the kidney, during cold acclimation; this is similar to observations for other mollusks (Rees and Hand, 1990; Michaelidis, 2002; Ortmann and Grieshaber, 2003) and invertebrates (Storey and Storey, 1990) at hibernation or estivation. Altered metabolic activity may contribute to reduced indirect chilling injury.

Pomacea canaliculata do not have an ability to decrease the supercooling point under cold temperatures. Any body organs and tissues examined in this study did not show a significant decrease in T_c after acclimation. Regardless of their cold tolerance, gut contents seem to act as an ice-nucleating agent that stimulates whole body freezing in insects (Leather et al., 1993) and the land snail *H. aspersa* (Ansart et al., 2002). This finding, however, seems to be meaningless for *P. canaliculata* from an ecological point of view. Temperatures rarely go below freezing at Kikuchi (Fig. 1) and, furthermore, the habitat for overwintering snails (in the soil of drained paddy fields) provides some environmental buffering with temperatures almost remaining above 0°C (Wada and Matsukura, 2007). Nevertheless, winter mortality of snails inhabiting drained paddy fields is high every year. Even temperatures more mild than their freezing point are fatal not only for cold-intolerant snails but also for cold-tolerant snails. This is apparent due to the fact that all the cold-tolerant snails died after incubating them at 0°C for a month (Wada and Matsukura, 2007).

Further studies on cold hardiness of *P. canaliculata* will enhance understanding of the mechanisms of cold hardiness in freshwater mollusks. This species is easily mass-reared and its cold hardiness can be controlled experimentally (Matsukura and Wada, 2007; Matsukura et al., 2009). Moreover, the present study isolated some important cold hardiness-related organs: the mantle, the posterior chamber of the kidney and the digestive gland. Further physiological and molecular biological approaches should be applied to reveal the mechanism of cold hardiness in *P. canaliculata* at the organ level.

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