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Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae)

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Abstract

Overwintering larvae of the rice stem borer, *Chilo suppressalis* accumulate glycerol and are freezing tolerant to about -25°C. However, non-diapausing larvae cannot accumulate glycerol and are killed by freezing. We compared the extent of tissue damage, the effects of glycerol concentration, and the transport of glycerol and water in fat body tissues from these larvae at selected freezing temperatures. Tissues from overwintering larvae, but not non-diapausing larvae, survive when frozen at -20°C with 0.25M glycerol, but the protection afforded by glycerol is offset by the water-channel inhibitor mercuric chloride. Glycerol in higher concentration (0.75M) affords some protection even to the fat body of non-diapausing larvae. Radiotracer assays of overwintering larvae show that water leaves the tissues during freezing while glycerol enters, and that mercuric chloride disrupts this process. Transport is also disrupted after lethal freezing at -35°C. Therefore, membrane transport of water and glycerol is involved in the avoidance of freezing injury to fat body cells of the rice stem borer, apparently by mediating the replacement of water with glycerol in freezing-tolerant tissues.

Keyword: Freeze tolerance; Glycerol; Water; Membrane transport; *Chilo suppressalis*

1. Introduction

Insects living in the temperate and arctic zones must be able to withstand the severe cold of winter. Insect cold tolerance has been divided for practical purposes into two main categories. Freeze-tolerant species survive ice formation in their bodies, and freeze-intolerant species die if frozen but survive by supercooling (reviews by Storey and Storey, 1992; Danks, 1996; Sømme, 1999; Duman, 2001; Bale, 2002).

According to the species, various elements of cold hardiness may or may not depend on the diapause state (Denlinger, 1991; Hodkova and Hodek, 2004; for a recent brief review see Danks 2005, p. 200). In the rice stem borer, *Chilo suppressalis,* the relationship between cold tolerance and diapause is complex. For example, the larvae can survive freezing only in the diapause state. Cold acclimation is necessary to increase the cold tolerance of diapausing larvae (Tsumuki, 1990). The cold tolerance of non-diapausing larvae does not increase even if they are acclimatized at cold temperatures.

Overwintering larvae of the rice stem borer are freeze-tolerant and like many other species accumulate the cryprotectant glycerol in the haemolymph and tissues in autumn to winter (Tsumuki and Kanehisa, 1978; Tsumuki, 1990). Glycerol concentrations are about 0.25M in the haemolymph of overwintering larvae, which survive freezing to about -25°C, but non-diapausing larvae cannot accumulate glycerol even when cold acclimatized, and are killed by freezing. In addition, ice nucleators are produced in the muscle and epidermis of overwintering larvae. These peptide nucleators are present on the outside surface of cell membranes of these tissues and directly induce freezing of the haemolymph (Tsumuki, unpublished observations). They induce extracellular freezing at about -15 ˚C and so avoid the very rapid freezing that would take place after cooling to even lower temperatures. Thereby, they prevent intracellular freezing and allow the larvae to survive in the frozen state (Tsumuki and Konno, 1991; Hirai and Tsumuki,

1995). However, this nucleation temperature is lower than in many other freezing-tolerant species (Duman, 2001).

Recently we identified the tissues showing the least tolerance to freezing in overwintering and non-diapausing larvae of the rice stem borer using trypan blue staining methods (Izumi et al. 2005). Significant differences in the degree of freezing injury in the different tissues were shown between overwintering and non-diapausing larvae; the fat body is one of the most susceptible tissues in non-diapausing larvae and the gut is most susceptible in overwintering larvae. However, we do not yet understand what mechanisms are responsible for these differences.

A variety of studies shows that for successful cell cryopreservation it is important that water and cryoprotectants are transported across the cell membrane. Water and cryoprotectants move across the membrane by two main mechanisms, passive osmotic pressure, and active transport by water channels. Water channel proteins called aquaporins are found in all higher animals and plants, and serve especially to transport water across cell membranes (Maurel, 1997; Benos et al. 2001; Chaumont et al. 2001). They have been characterized from some insects (e.g. Beuron et al. 1995; Le Caherec et al. 1996a,b; Pietrantonio et al. 2000). Aquaporins have been divided into two types: one subgroup is highly selective for the passage of water, and the other subgroup transports water as well as small neutral solutes such as glycerol. Experimental studies on the transport properties are facilitated by the fact that the water-conducting properties of these channels are inhibited by mercuric chloride, $HgCl_2$ (Preston et al., 1992), as also shown for an insect aquaporin (Le Caherec et al. 1996b). This inhibition apparently is due to binding of the mercury molecule with the aquaporin protein in a narrow part of the pore, so occluding the aqueous pathway (Murata et al. 2000).

A relationship of aquaporins with freeze tolerance has already been reported in some organisms. In baker’s yeast, studies of freeze-resistant and freeze-sensitive strains have

revealed a correlation between freeze resistance and the presence of aquaporin. Deletion of the relevant genes raised sensitivity to freezing, whereas their overexpression improved freeze tolerance (Tanghe et al. 2002). In mammals, artificial expression of aquaporin-3 improves the survival of mouse oocytes after cryopreservation using glycerol-based solution (Edashige et al. 2003). Previously, there had been no report of mouse oocytes surviving after cryopreservation using glycerol-based solutions because of the low permeability of mouse oocytes to glycerol. However, relationships between freeze tolerance and aquaporin have not yet been reported in insects.

The fat body is important in metabolism and synthesizes cryoprotectants such as glycerol, and moreover is the tissue most easily injured by freezing in non-diapausing larvae of the rice stem borer (Izumi et al. 2005). We hypothesized that water and glycerol transport in fat body tissue might contribute to the avoidance of freezing injury. Therefore, we assessed fat-body viability (visualized by the trypan-blue staining of injured cells) in diapausing and non-diapausing larvae exposed to low temperatures; we tested the effects on viability when glycerol levels were different, and when aquaporins were inhibited with mercuric chloride; and we measured water and glycerol transport directly in overwintering larvae under different conditions through the use of radioactive tracers.

2. Materials and methods

2.1. Insects

Rice stems including overwintering larvae of the rice stem borer were collected from paddy fields in October 2002 and kept in a mesh wire cage at our Institute in Kurashiki,

Japan (34.35 °N, 133.46 °E) until use in experiments. Non-diapausing larvae were reared on rice seedlings (exchanged weekly) at 25°C under a long photoperiod (16L: 8D) as described by Tsumuki and Kanehisa (1978). Last-instar larvae about 10 days after moulting into the fifth instar were used for experiments.

2.2. Fat body tissue viability

Non-diapausing and overwintering larvae were dissected in Grace's insect medium (Gibco). Fat body tissues dissected from whole larvae were moved to a 1.5ml tube with 1ml the medium. After 1h incubation at 20°C, the fat body tissues were incubated at 5°C for 1h in the medium with or without 0.25M glycerol. The tissues were then placed in refrigerators, cooled to -20°C at a rate of 0.2°C/min and held at this temperature for 2h. Observations of such samples on a microscope cryostage show that freezing is initiated in the medium, not in the cells (fat body cells of *C. suppressalis* have a supercooling point of –20.6°C according to Tsumuki and Konno, 1991), and so their freezing is extracellular (unpublished observations).

Following freezing treatments, the tissues were thawed at room temperature and tissue viability was assessed using trypan blue dye (Izumi et al. 2005). After trypan blue dye solution (final concentration of trypan blue dye, 0.1%) was added to the medium containing the fat body tissues, the tissues were incubated for 2 min at room temperature and then washed with the medium. The stained tissues were examined with a digital microscope (KEYENCE VH-7000) at 100 x. The degree of freezing injury was estimated from the area and density of the blue staining (Izumi et al. 2005). Cells with an intact cell membrane exclude the dye; dying or dead cells incorporate the dye, which stains those cells blue. The area stained blue was measured using NIH image

analysis software (developed at US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.3. Transport assays

Characteristics of glycerol and water transport were studied using radioisotopes. Incubations at various temperatures were done in a temperature-controlled chamber. In the glycerol transport experiment, fat body tissues (fw 10mg) dissected from single larvae were transferred to a 1.5ml tube with 1ml Grace's insect medium. After 1h incubation at 20°C, the fat body tissue was incubated at 5°C for 2h in the medium containing 0.25M unlabeled glycerol, 37KBq [^{14}C]glycerol and with or without 0.2mM $HgCl_2$. In the freezing experiment, after 1h incubation at 20°C fat body tissues were incubated at 5°C for 1h in Grace's insect medium containing 0.25M unlabeled glycerol, 37KBq [^{14}C]glycerol, and 3.7KBq [^{3}H]water. For the mercuric chloride treatment 0.2mM $HgCl_2$ was added after that. The fat body tissues were then incubated at -20°C or -35°C for 2h. Following various treatments, fat body tissues were washed in the medium without radio isotopes and transferred into a glass homogenizer. Five ml of Scintisol EX-H (Dotite) was added to the homogenate and the radioactivity of the homogenate was determined with a liquid scintillation counter (Wallac 1410E, Pharmacia). The level of radioactivity in the washed tissues corresponds with the degree of uptake of labelled glycerol, which can be quantified from the ratio of unlabelled to labelled glycerol. These results were consistent with HPLC measurements (data not shown).

2.4. Statistical analysis

Data from both the study of tissue viability by staining and the transport assays were analyzed using ANOVA, and multiple comparisons of the means between cold treatments were evaluated with the Tukey-Kramer test. (Original data were normally distributed according to the Kolmogorov-Smirnov test.) Statistical analyses were done using the statistical software package Stat View Version 5.0 (SAS Institute Inc., 1998).

3. Results

3.1. Viability of fat body tissue

The viability of fat body tissues dissected from whole larvae and frozen at various temperatures for 2h in Grace's insect medium with or without the addition of 0.25M glycerol are shown in Table 1. At -20°C, more than 60% of the fat body tissues of overwintering larvae frozen in Grace's insect medium alone were stained. In contrast, when fat body tissues from overwintering larvae were frozen in the same way but in medium with 0.25M glycerol it was not stained. However, when the tissues were frozen at -20°C in Grace's medium containing not only 0.25M glycerol but also 0.2mM $HgCl_2$, which blocks aquaporin, the tissues were stained about 50% (Fig. 1). At -35°C, more than 60% of fat body tissues were stained after freezing in Grace's insect medium containing 0.25M glycerol.

Fat body tissues dissected from non-diapausing larvae and frozen at -20°C in Grace's insect medium with 0.25M glycerol were stained more than 60%. However, when the medium contained 0.75M glycerol the stained area was reduced for these non-diapausing larvae (Table 1).

3.2. Transport assays

At 5°C, the fat body tissues dissected from overwintering larvae took up glycerol, reaching a maximum after 30min (about 160ng of glycerol / mg of tissue) (Fig. 2). However, when 0.2mM $HgCl_2$ was added to the medium, only 50ng of glycerol / mg of tissue was taken up into the tissues after 120min incubation. Fat body tissue from non-diapausing larvae took up only 50ng of glycerol / mg of tissue, the same level as in overwintering larvae with 0.2mM of $HgCl_2$ (Fig. 2).

The amount of water in fat body tissue of overwintering larvae decreased as freezing proceeded (Fig. 3a). However, when $HgCl_2$ was added to the medium, the amount of water in fat body tissues did not decrease even after freezing. In fat body tissues from non-diapausing larvae, the amount of water increased with time (Fig. 3a). The amount of glycerol in fat body tissue of overwintering larvae increased for the first 30 minutes of incubation and then decreased, but such an increase was not observed with added $HgCl_2$. In fat body from non-diapausing larvae, the amount of glycerol increased with time (Fig. 3b), in a similar way as for the water.

Larvae thawed following 2h freezing showed different responses after exposure to -20°C (non-lethal) and -35°C (lethal) (Fig. 4). Water and glycerol decreased during freezing at -20°C but were restored to initial levels after thawing (Fig. 4). In contrast, after thawing from -35°C, water and glycerol in the cells were at higher concentrations than after -20°C exposure (Fig. 4).

4. Discussion

This study demonstrates that both the presence of glycerol and the transport of water

across the cell membrane is required to avoid freezing injury in fat body tissues of the rice stem borer. Even tissues from diapausing larvae are killed by exposure to -20°C if glycerol has been removed. When fat body dissected from the larvae was incubated in Grace's medium without glycerol, almost all endogenous glycerol was transported from the fat body tissues into the medium after 1h incubation at 20˚C (data not shown). When the resultant fat body tissue was frozen *in vitro* at -20˚C in Grace's insect medium without glycerol, the surface of the tissue became tattered (Fig1.a). In contrast, the fat body tissue of overwintering larvae frozen at -20˚C in the medium with 0.25M glycerol (the normal level in overwintering larvae) survived.

When fat body tissue was frozen in the medium with 0.25M glycerol and 0.2mM $HgCl_2$ which inhibits water transport by water channels, the tissue was seriously injured by freezing at -20˚C, even though its surface remained smooth (Fig.1c) unlike the tissue frozen without glycerol (Fig. 1a). These results suggest that destruction of the cell membrane by freezing is avoided or reduced by the presence of glycerol.

Fat body tissues of non-diapausing larvae did not survive even when frozen with 0.25M glycerol. Freezing injury of fat body tissues was reduced when the concentration of glycerol in the medium was increased by two or three times apparently because the amount of glycerol in the tissue increases with increasing glycerol concentration in the medium (data not shown). Moreover, in non-diapausing larvae the level of injury of the fat body was the same even when $HgCl_2$ was added to the medium. These results suggest that a high concentration of glycerol can reduce freezing injury even when the transport system for water and glycerol is absent, as in non-diapausing larvae.

Our radiotracer experiment shows that in fat body from overwintering larvae water leaves the tissue and, at the same time, glycerol enters. This suggests that free water in the tissue might have been replaced by glycerol. Moreover, the water channel appears to be related to this change, because the change does not occur when $HgCl_2$ is added to the

medium, and glycerol uptake by fat body tissue is inhibited by $HgCl_2$ at 5 ℃. It has already been reported that some water channels, such as human aquaporin 3, transport not only water but also glycerol (Ishibashi, *et al*. 1994). Moreover, success in cryopreservation when aquaporin 3 is artificially expressed has been reported for mouse oocytes (Edashige *et al.* 2003) and zebrafish embryos (Hagedorn *et al*. 2002).

Fat body tissue of non-diapausing larvae takes up only 1/3 of the amount of glycerol taken up in overwintering larvae at 5 ˚C, and this amount did not change with added $HgCl_2$. Moreover, the amount is almost the same as in overwintering larvae with $HgCl_2$ which inhibits glycerol transport. These results suggest that glycerol is taken into the fat body tissue of non-diapausing larvae only by osmotic pressure. If so, non-diapausing larvae may be unable to survive freezing not only because glycerol cannot protect the cells but also because the cells cannot transport and accumulate the glycerol.

Fat body tissue of overwintering larvae cannot survive at -35 ˚C even with added glycerol. Nor do whole larvae survive at -35 ˚C, and most of the tissues including the fat body are then stained by trypan blue dye (Izumi *et al.* 2005). Down to -25˚C, which is the LT50 (temperature of 50% freezing death) for whole larvae, water and glycerol movements in the fat body were almost same as those at -20℃, but below -25˚C the amount of water and glycerol in the tissue increased (data not shown). In the present experiment, the fact that after thawing water and glycerol were high in fat body tissue frozen at -35 ˚C but not at -20˚C confirms that the cell membrane does not function normally after lethal freezing.

We conclude that membrane transport of water and glycerol is involved in the avoidance of freezing injury to cells. In particular, water channels appear to be involved in replacing water with glycerol in competent tissues, such as the fat body of overwintering larvae but not of non-diapausing larvae of the rice stem borer. To understand the mechanisms by which freezing injury is avoided, we are making further

studies of aquaporins and transport through cell membranes at low temperatures.

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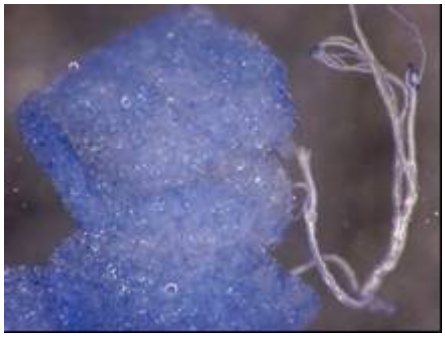
Table 1

Blue stained area of fat body tissues in overwintering and non-diapausing larvae by trypan blue under different medium after freezing at -20, -35 ºC for 2 h.

Temperature	Medium	ue colored area in fat body tissues (%	
		Overwintering	Non-diapausing
5℃	Grace's medium	3.8±0.8	4.2±1.0
	Grace's medium with 0.25M glycerol	4.0±0.7	4.0±0.8
	Grace's medium with 0.25M glycerol and 0.2mM HgCl2	3.9±0.9	3.5±0.8
-20℃	Grace's medium	63.3±9.4b	68.5±8.4b
	Grace's medium with 0.25M glycerol	3.3±0.4	65.3±7.5b
	Grace's medium with 0.25M glycerol and 0.2mM HgCl2	50.4±7.8a	67.4±9.8b
	Grace's medium with 0.5M glycerol		59.0±9.6ab
	Grace's medium with 0.75M glycerol		48.2±7.6a
	Grace's medium with 0.75M glycerol and 0.2mM HgCl2		49.5±8.5a
-35℃	Grace's medium with 0.25M glycerol	64.56±7.1b	

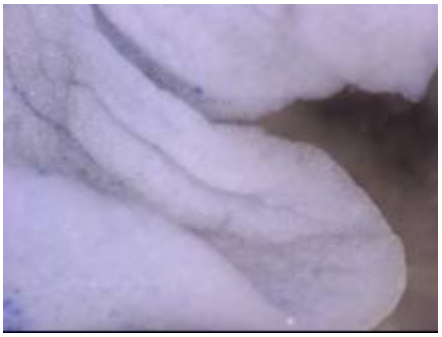
Data are expressed as mean ± SE, n=30 replicates. Results with different letters are significantly different (p<0.05). Results without a letter are not significantly different from control (5 ºC) values.

a



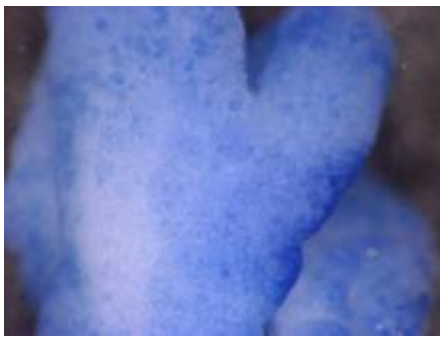
Grace's medium

b



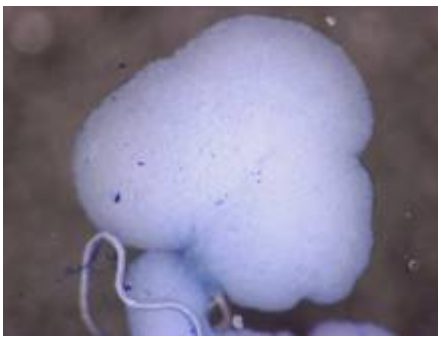
Grace's medium + 0.25M glycerol

c



Grace's medium + 0.25M glycerol +$HgCl_2$

d



Grace's medium + 0.25M glycerol +$HgCl_2$ at 5 ºC

Fig. 1

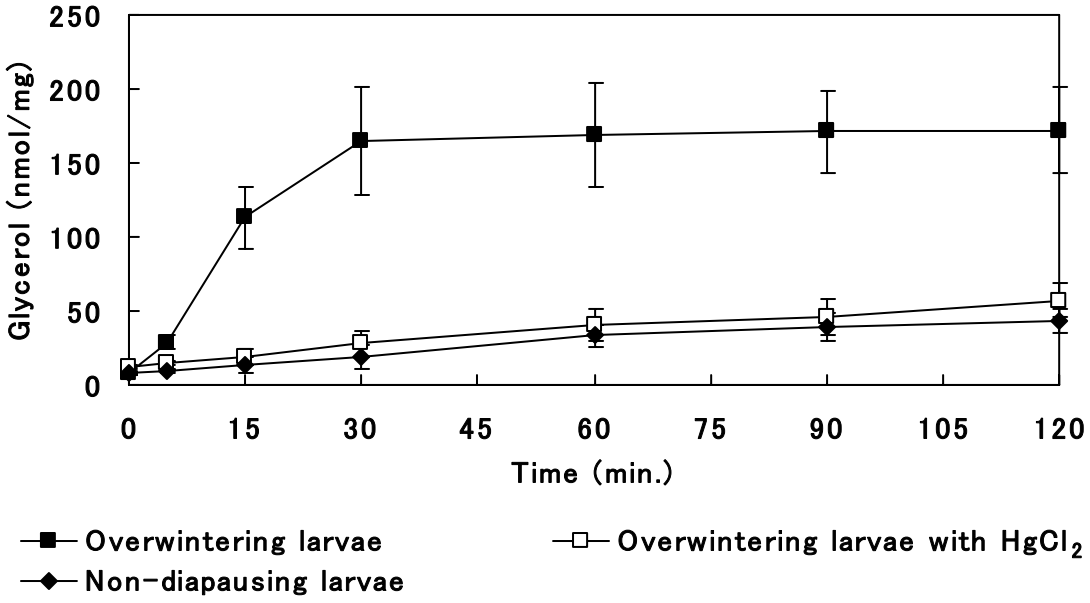

250
200
150
100
50
0
Glycerol (nmol/mg)
0
15
30
45
60
75
90
105
120
Time (min.)
Overwintering larvae
Overwintering larvae with HgCl2
Non-diapausing larvae

Fig. 2

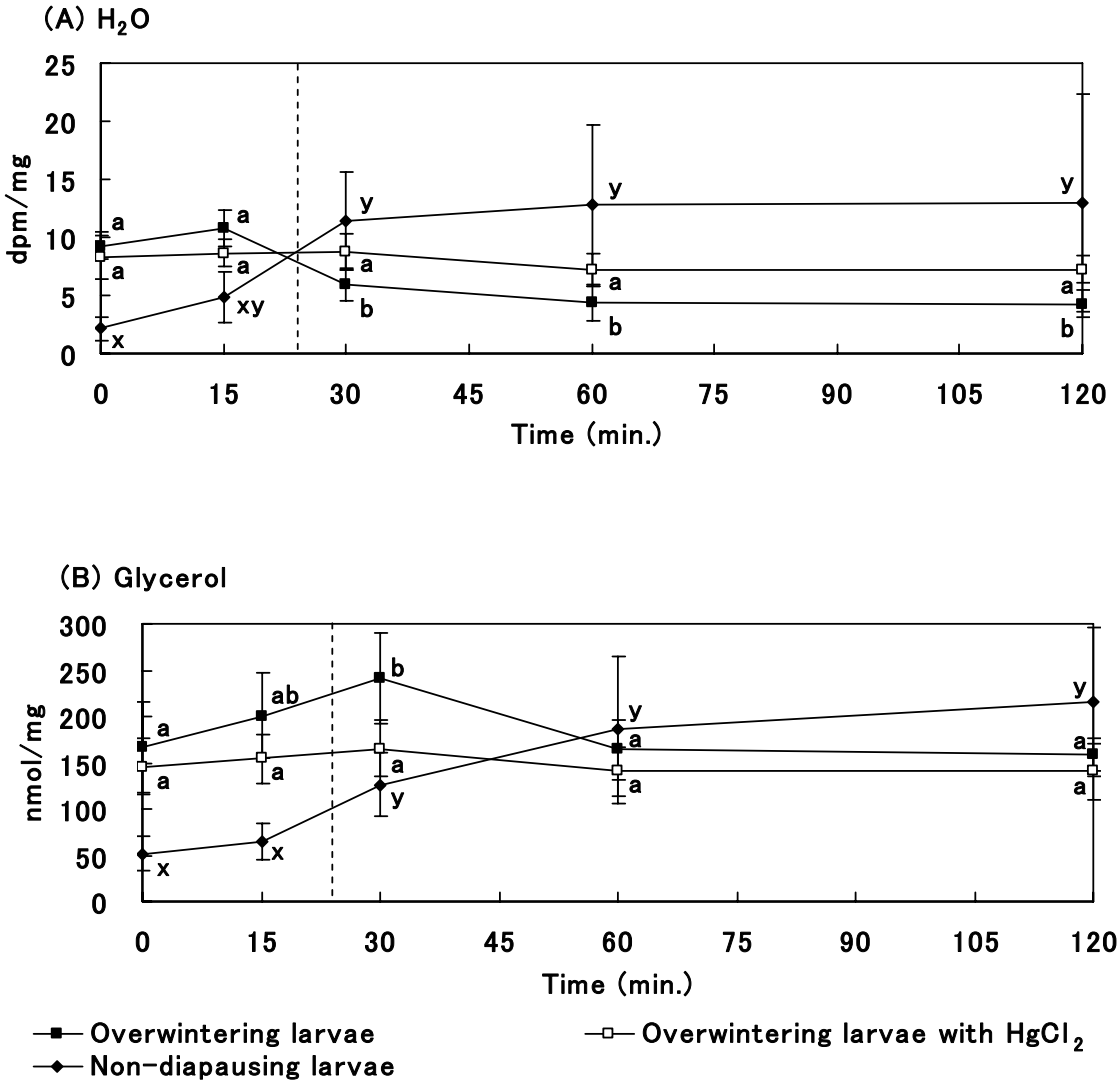

(A) H2O
dpm/mg
Time (min.)
(B) Glycerol
nmol/mg
Time (min.)
Overwintering larvae
Overwintering larvae with HgCl2
Non-diapausing larvae

Fig. 3

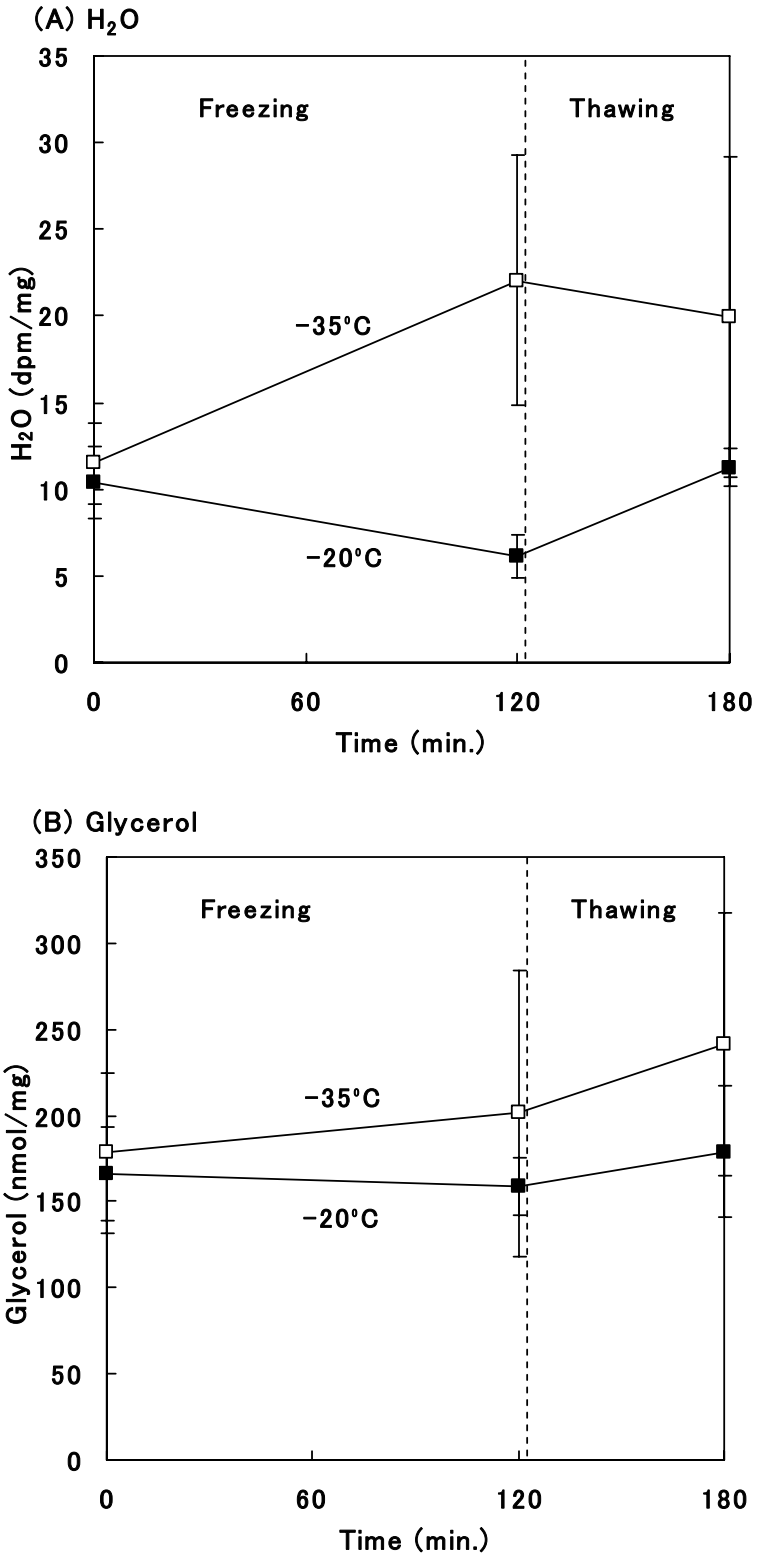

(A) H_2O
Freezing
Thawing
−35°C
−20°C
H_2O (dpm/mg)
0
5
10
15
20
25
30
35
0
60
120
180
Time (min.)
(B) Glycerol
Freezing
Thawing
−35°C
−20°C
Glycerol (nmol/mg)
0
50
100
150
200
250
300
350
0
60
120
180
Time (min.)

Fig. 4

Figure legends

Fig. 1. Damage of fat body tissue of overwintering larvae after freezing at -20 ˚C for 2 h in different media (a-c) and incubation at 5 ˚C for 2 h (d).

Fig. 2. Glycerol uptake at 5 ˚C of fat body tissue of overwintering and non-diapausing larvae in Grace's medium with or without $HgCl_2$. Each value represents the mean (±S.E.) of 20 samples.

Fig. 3. Change in water (A) and glycerol (B) in fat body tissue of overwintering and non-diapausing larvae during the freezing process at -20 ˚C. The vertical dotted line shows the average time to freezing. Each value represents the mean (±S.E.) of 30 samples. Different letters indicate significant differences between values (p<0.05), as shown by the Tukey-Kramer test after ANOVA.

Fig. 4. Change in water (A) and glycerol (B) in fat body tissue of overwintering larvae during 2h of exposure to -20°C or to -35 ˚C, and after thawing. Each value represents the mean (±S.E.) of 20 samples. Freezing time is about 20~25min at -20°C and -35°C (cf. Fig. 3).