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Increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in larvae of *Polypedilum vanderplanki*

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Summary

Larvae of an African chironomid, *Polypedilum vanderplanki*, which live in temporal rock pools, are completely dehydrated when the pools dry up and undergo anhydrobiosis until the next rain comes. During the dehydration process, larvae accumulate large amounts of trehalose, which provides effective protection against desiccation because of its high capacity for water replacement and vitrification. As the occurrence of rapid trehalose synthesis coincides with loss of body water, changes of osmolarity in the body are thought to be a cue for trehalose synthesis. Indeed, exposure to high salinity triggers rapid and efficient accumulation of trehalose even without desiccation treatment. As this rapid production occurs mainly in high concentrations of salt solutions, we conclude that an increase in internal ion concentration triggers trehalose synthesis associated with cryptobiosis in this species.

Key words: *Polypedilum vanderplanki*, cryptobiosis, anhydrobiosis, trehalose, ion.

Introduction

Organisms often suffer from water stress, e.g. after dehydration or exposure to high salt under drought or freezing conditions. They have evolved various physiological and biochemical mechanisms to survive such water stresses. The most unique and ultimate strategy against drought stress is cryptobiosis (=anhydrobiosis), which is the state in which an organism can tolerate complete desiccation without ill effects and can survive for an extended period (Clegg, 2001).

Accumulation of disaccharides at an extremely high level is a common physiological trait among cryptobiotic organisms. Trehalose is often found in cryptobiotic fungi (Sussman and Lingappa, 1959), *Artemia* cysts (Clegg, 1965), nematodes (Madin and Crowe, 1975; Loomis et al., 1980), yeast (Panek et al., 1986) and bacteria (Payen, 1949). The mechanism of induction for trehalose production may differ in the wide array of organisms; for example, *Saccharomyces cerevisiae* accumulates trehalose in response to various external conditions, such as starvation, heat shock and osmotic stress (e.g. Lillie and Pringle, 1980; Ribeiro et al., 1994). Intracellular trehalose has an important role to enable high desiccation tolerance in human cells (Guo et al., 2000). This compound provides the most effective protection against desiccation among sugars and polyols because of its high ability for water replacement and vitrification (Crowe et al., 1987, 1998; Green and Angell, 1989; Sano et al., 1999). On the other hand, trehalose is rarely used in higher plants, where sucrose, together with other sugars, seems to substitute for trehalose (Ingram and Bartels, 1996).

An African chironomid, *Polypedilum vanderplanki*, is the highest and largest multicellular animal with cryptobiotic ability. The cryptobiotic larva can tolerate not only exposure to extremely high (106°C) and low (–270°C) temperatures but also submersion in pure ethanol (Hinton, 1960a,b, 1968). Recently, we reported that this chironomid also accumulates a large amount of trehalose (approximately 20% of the dry body mass) in the state of cryptobiosis, and that cerebral regulation is not involved in the process for induction of cryptobiosis (Watanabe et al., 2002). However, the factor(s) that triggers explosive synthesis of trehalose during desiccation remains unknown. In this study, we demonstrate that elevation of internal ion concentration is an important factor for triggering trehalose synthesis associated with cryptobiosis in larvae of *P. vanderplanki*.

Materials and methods

Insects

A stock culture of *Polypedilum vanderplanki* (Hinton) was established from cryptobiotic larvae collected from rock pools in Nigeria in 2000. They were reared for successive generations under controlled light (13 h:11 h light:dark) and temperature (27°C). The detailed methods for rearing are described in Watanabe et al. (2002).

Procedures for desiccation and incubation in solutions

Groups of 5–7 final-instar larvae (approximately 1 mg wet body mass) were placed on pieces of filter paper with 0.44 ml

or 1.5 ml of distilled water in a glass Petri dish (diameter, 65 mm; height, 20 mm). These dishes were immediately transferred to a desiccator (<5% relative humidity) at room temperature (24–26°C), where they were gradually dehydrated over 2 days (0.44 ml distilled water) or 7 days (1.5 ml distilled water).

In a separate experiment, groups of 5–20 larvae were submerged in approximately 20 ml of various kinds of solution in a glass Petri dish and incubated for 3–24 h at room temperature. Solutions of NaCl, mannitol, glycerol and dimethyl sulfoxide (DMSO) were applied at various osmotic pressures (205–547 mosmol l^{-1} NaCl, 164–412 mosmol l^{-1} mannitol, 217–1628 mosmol l^{-1} glycerol and 128–768 mosmol l^{-1} DMSO). In addition, eight different salt solutions (KCl, $NaNO_3$, KNO_3, Na_2SO_4, K_2SO_4, NaH_2PO_4, KH_2PO_4 and $CaCl_2$) were prepared at 342 mosmol l^{-1} equivalents to a 1% NaCl solution. Larval activity was checked just after 1 day of treatment with each solution and classified into four categories as follows: category 3, all or most larvae moved actively in the same way as untreated larvae; category 2, most larvae moved their bodies slowly only when they were stimulated by tweezers; category 1, most larvae were moribund; category 0, all larvae were dead.

Measurements for water content

Larvae desiccated for 8 h, 16 h, 24 h, 32 h or 48 h in the 2-day desiccation treatment or for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days or 7 days in the 7-day desiccation treatment were individually heated at 100°C for 1 day. Water content was calculated from the difference in mass before and after the heat treatment.

Pre-treatment with 1% NaCl solution before desiccation

10–20 larvae were incubated for 3 h, 6 h or 9 h in 20 ml of 1% NaCl solution in a glass Petri dish at room temperature and were then desiccated with 0.1 ml or 0.44 ml of distilled water. The completely desiccated larvae were used for determining trehalose content and for examining recovery after rehydration with distilled water.

Sugar and polyol measurements

Larvae incubated in solutions and/or desiccated for various periods were homogenized individually with 0.1 mg of sorbitol as an internal standard in 0.5 ml of 90% ethanol. After centrifugation (1500 ***g***, 20–30 min), the supernatant was desiccated completely by using a vacuum concentrator and was stored at room temperature. The dried residue was dissolved in approximately 500 μl of MilliQ water (Millipore, Bedford, MA, USA). After filtration through a 0.45 μm membrane, the amount of low-molecular-mass carbohydrates and polyols was measured as described by Watanabe et al. (2002).

Results

Body water loss and trehalose accumulation

Larvae were dehydrated in a Petri dish with either 0.44 ml or 1.5 ml of distilled water. Larvae in the former treatment were desiccated within 48 h and those in the latter dehydrated over 7 days. All the desiccated larvae in both treatments were able to recover after rehydration due to enough trehalose accumulation (Fig. 1). Larvae in the 2-day desiccation treatment exhibited abrupt trehalose accumulation when they had lost about 25% of their body water (Fig. 1A). Interestingly, larvae in the 7-day desiccation treatment also started accumulating a great amount of trehalose on day 6 after treatment when the fresh body mass decreased by about 25% (Fig. 1B).

Effect of various solutions on trehalose synthesis and larval activity

As the occurrence of rapid trehalose synthesis coincided

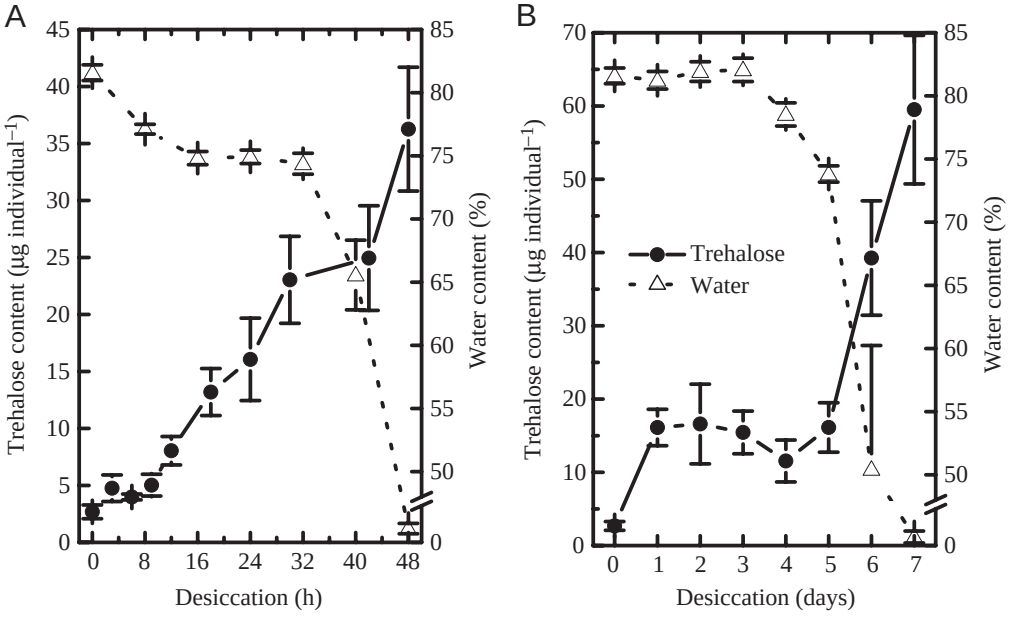

Fig. 1. Changes of water and trehalose content in *P. vanderplanki* larvae during desiccation for (A) 2 days and (B) 7 days. Solid lines with filled symbols represent trehalose content; broken lines with open symbols represent water content. *N*=6–10 for trehalose measurements and *N*=12 for water measurements.

with a decrease in body water loss (Fig. 1), changes of osmolarity in the body were thought to be a cue for trehalose synthesis. It was therefore postulated that we might induce trehalose synthesis by increasing internal ion concentration even without the desiccation treatment. Trehalose content was thus compared among larvae treated for 1 day in solutions of NaCl, mannitol, glycerol or DMSO at various osmotic pressures. Larvae incubated for 1 day in NaCl solution at around 350 mosmol l^{-1} (342 mosmol l^{-1}=1% NaCl solution) accumulated quite a large amount of trehalose (approximately 35 μg individual^{-1}; equal to approximately 20% of the dry body mass; Fig. 2A). Most of these larvae were moribund or dead just after the treatment. All larvae died even when they were transferred into distilled water or rehydrated after desiccation over 48 h. Larvae treated at less than 300 mosmol l^{-1} of NaCl solution moved actively but did not accumulate as much trehalose. Larvae treated with solutions of mannitol and DMSO increased trehalose content slightly (approximately 5 μg individual^{-1}) only when osmotic pressure of the solution was extremely high (Fig. 2B,D). Thus, the level of trehalose accumulation depended upon the kinds of solutes and was not a simple osmotic response.

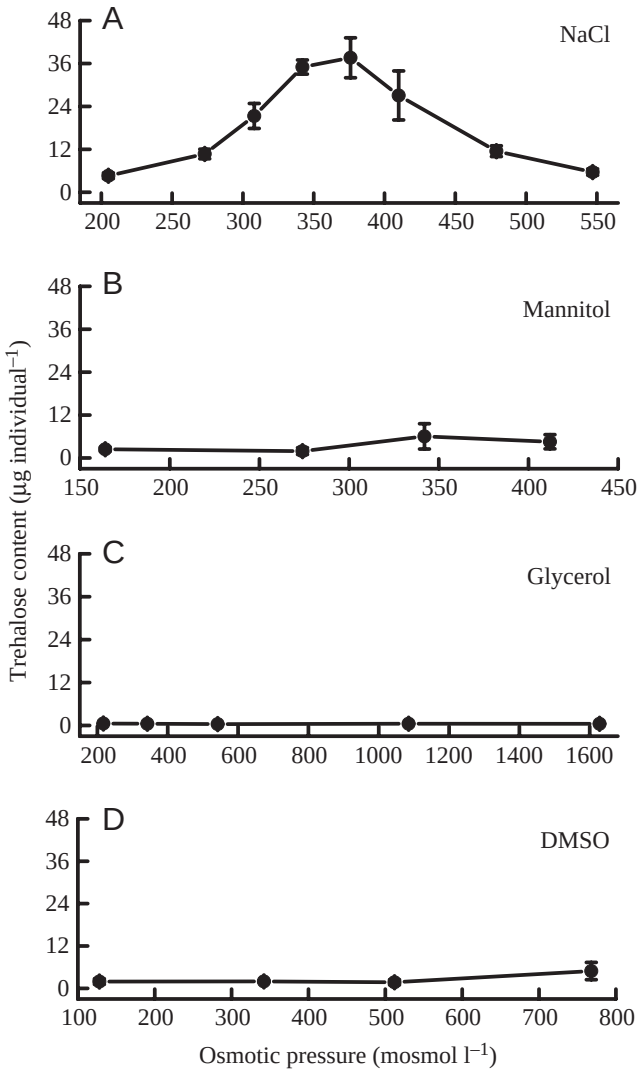

Fig. 2. Trehalose content of *P. vanderplanki* larvae incubated for 1 day in (A) NaCl, (B) mannitol, (C) glycerol and (D) dimethyl sulfoxide (DMSO) solutions at various osmotic pressures. A 1% NaCl solution is 342 mosmol l^{-1}. *N*=4–10 for each solution.

Comparison of the rate of trehalose synthesis between larvae desiccated and incubated in 1% NaCl

Larvae started accumulating trehalose significantly 3 h after both desiccation and incubation in 1% NaCl (Mann–Whitney *U*-test; $P<0.05$, $N=4–6$; Fig. 3). The desiccating larvae then gradually increased trehalose content for 48 h (at a rate of approximately 0.7 μg h^{-1}), whereas the 1% NaCl-treated larvae accumulated trehalose rapidly between 6 h and 15 h after the beginning of the treatment (at a rate of approximately 4 μg h^{-1}). These results suggest that the treatment with 1% NaCl solution stimulated trehalose synthesis much more efficiently than did desiccation.

Comparison of trehalose content among larvae incubated in various salt and carbohydrate solutions

Fig. 4 shows trehalose content in larvae incubated for 1 day in various salt and carbohydrate solutions at the same osmotic pressures (342 mosmol l^{-1}). There was no significant difference in trehalose content between untreated larvae (control) and those treated in solutions of glycerol or DMSO (Mann–Whitney *U*-test; $P>0.05$, $N=5–8$). All of the salt solutions, with the exception of KCl and K_2SO_4, were more effective in eliciting trehalose accumulation than those of carbohydrates such as mannitol, glycerol and DMSO (Mann–Whitney *U*-test; $P<0.05$, $N=5–8$).

Salt molecules dissociate into cations and anions in solution. We also compared the effect of cations on trehalose content between molecules with the same kinds of anions. Na^+ tended to be more effective in stimulating trehalose accumulation than

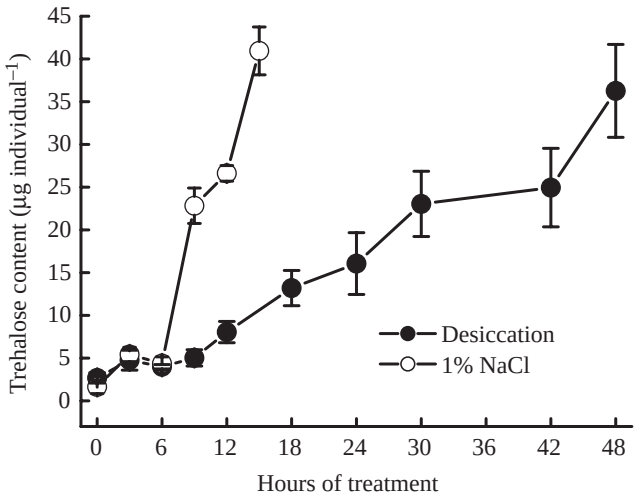

Fig. 3. Changes of trehalose content in *P. vanderplanki* larvae during desiccation (up to 48 h) or incubation (up to 15 h) in 1% NaCl solution. Filled symbols represent desiccation; open symbols represent 1% NaCl. *N*=4–6.

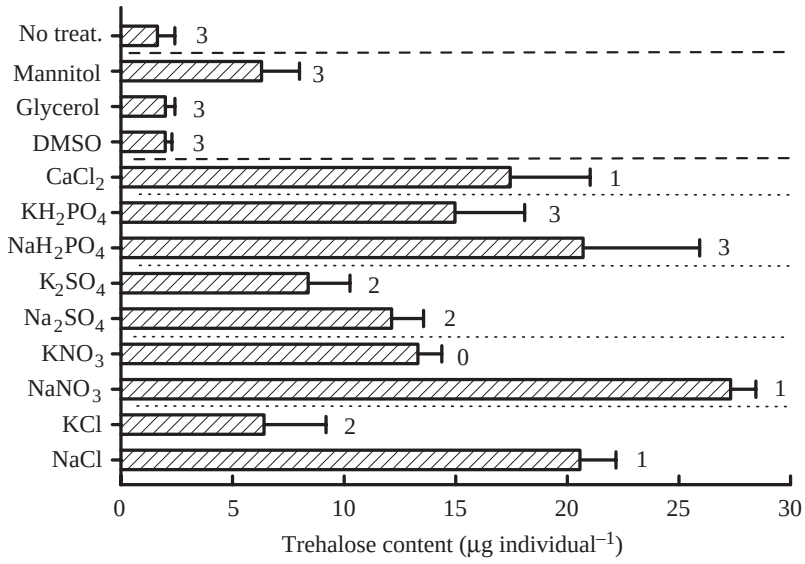

Fig. 4. Effect of various salt and carbohydrate solutions on trehalose content of *P. vanderplanki* larvae. Larvae were incubated for 1 day in each solution; each solution was at the same osmotic pressure (342 mosmol l^{-1}). Control shows the trehalose content of untreated larvae. Numbers to the right of the S.E.M. bar indicate larval activity after 1-day treatment of each solution: 3 – all or most larvae moved actively in the same way as untreated larvae; 2 – most larvae did not move actively, and such larvae moved slowly only when they were stimulated by tweezers; 1 – most larvae were moribund; 0 – all larvae were dead. *N*=5–8 for each solution.

did K^+, especially with Cl^- and NO_3^- (Mann–Whitney *U*-test; $P<0.05$, except for PO_4^-; Fig. 4). Ca^{2+} also appeared to trigger trehalose synthesis effectively.

Effect of pre-treatment with 1% NaCl solution prior to desiccation

We examined how short-term treatment with 1% NaCl solution affects trehalose synthesis and induction of cryptobiosis after the subsequent desiccation treatment. Desiccation with 0.1 ml of distilled water caused trehalose synthesis in larvae to some extent (mean, 23.2 μg) but rarely induced cryptobiosis (Table 1). Pre-treatment with an NaCl solution for 3 h or 6 h prior to desiccation with 0.1 ml of distilled water greatly accelerated trehalose synthesis (80.8 μg for 3 h or 70.1 μg for 6 h) but did not enhance the success rate of induction of cryptobiosis. Desiccation with 0.44 ml of distilled water triggered trehalose synthesis (54.7 μg) more than that with 0.1 ml distilled water (Mann–Whitney *U*-test; $P<0.05$). Pre-treatment with an NaCl solution did not cause the further accumulation of trehalose by desiccation with 0.44 ml but did decrease the rate of recovery after rehydration depending on the time for the pre-treatment (Mann–Whitney *U*-test; $P<0.05$).

Discussion

Larvae of *P. vanderplanki* grow in temporal rock pools of tropical Africa (Hinton, 1960a). Pools sometimes dry up, whereby the larvae also become dehydrated and remain desiccated until the next rain. We can induce cryptobiosis under laboratory conditions that mimic those in the field; i.e. slow desiccation, taking more than 2 days to achieve complete dehydration, in a Petri dish allows the larvae to enter a state of cryptobiosis (Watanabe et al., 2002). By contrast, larvae dehydrated by quick desiccation within 1 day do not accumulate trehalose and die (data not shown). This indicates

Table 1. *Effect of pre-treatment with 1% NaCl solution prior to desiccation on trehalose content and recovery rate in larvae of* P. vanderplanki

Hours of pre-treatment with 1% NaCl	Water volume for desiccation (ml)	*N*	Trehalose content (μg individual^{-1})	*N*	Recovery (%)
0	0.1	10	23.2±4.0	30	3.3
3	0.1	4	80.8±9.0*	35	2.9
6	0.1	4	70.1±9.0*	33	0
9	0.1	–	–	25	0
0	0.44	6	54.7±5.9	29	90
3	0.44	4	63.0±8.7	25	28.0*
6	0.44	6	69.2±13.9	30	16.7*
9	0.44	–	–	28	0*

Larvae were desiccated with 0.1 ml or 0.44 ml of distilled water in a glass Petri dish after treatment for 3 h, 6 h or 9 h in a 1% NaCl solution. Recovery was checked 6 h after rehydration.

Asterisks represent a significant difference ($P<0.01$) between individuals desiccated with the same volume of water with and without pre-treatment with the 1% NaCl solution. Values for trehalose content represent means ± S.E.M.

that a certain time lag is needed between the input of the stimulus for trehalose synthesis and its accumulation. It is of interest to know what the cue is for triggering rapid trehalose synthesis during cryptobiosis in *P. vanderplanki*. The present study shows that rapid trehalose synthesis occurred just before the complete desiccation of the larvae, namely when larvae lost approximately 25% of body water (Fig. 1). These data indicate that changes of osmolarity occurring in the body were the likely cue for rapid trehalose synthesis. Indeed, exposure to high salinity effectively triggered a rapid accumulation of trehalose even without desiccation treatment (Figs 2, 3).

Recently, several groups of researchers partly demonstrated the existence of some components of a general regulatory network governing synthesis of compatible solutes against the stresses of desiccation or high osmolarity. In the yeast *S. cerevisiae*, exposure to high extracellular osmolarity induced a two-component osmosensor (Sln1 and Sho1) to activate the high osmolarity glycerol response (HOG) and mitogen-activated protein (MAP) kinase cascades and finally caused accumulation of glycerol as compatible solutes (Maeda et al., 1994, 1995; Posas et al., 1996; Posas and Saito, 1997; Raitt et al., 2000). This activation response was induced by high osmolarity regardless of the kinds of solute (Maeda, 1999). Also, in the higher plant *Arabidopsis thaliana*, an osmosensor (ATHK1) homologous to Sln1 has already been found (Urano et al., 1999), and signal transduction cascades of the osmotic response similar to those in the yeast have been shown to be genetically conserved and activated under desiccation and high salinity stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Mizoguchi et al., 1998; Miyata et al., 1998; Urano et al., 1998). In the case of *P. vanderplanki* larvae, exposure to both desiccation and solutions containing salt or other substances at high concentrations was able to cause an internal increase of salt and other solute concentrations. The change is caused by gradual evaporation of water from the body in the former and invasion of solutes into the body in the latter. These treatments allowed larval tissues and cells to become exposed to high osmolarity and high concentrations of various solutes. Unlike *S. cerevisiae* and *A. thaliana*, rapid accumulation of trehalose was not a simple osmotic response in *P. vanderplanki* larvae; i.e. the explosive production occurred mainly in high concentration salt solutions and depended on the kind of cation in solution. Thus, increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in this species.

One-day incubation in high concentrations of salt solution triggered effective synthesis of trehalose in larvae, but all of the treated larvae died immediately or after the subsequent desiccation. Pre-treatment with NaCl solution accelerated trehalose synthesis rapidly during the following desiccation with 0.1 ml of distilled water, but such desiccated larvae also failed to recover after rehydration. There appeared to be two possible reasons why individuals containing a large amount of trehalose died in these cases. One could be due to the stresses of osmotic shock and/or invasion of ions into the body. Ion stress often provides detrimental effects on organisms by confusing various physiological states and functions (Hasegawa et al., 2000). This hypothesis is supported by the marked depression of larval recovery rate by treatment of NaCl solution prior to desiccation with 0.44 ml of distilled water. A second explanation could be that the accumulation of trehalose is not the only essential factor. Indeed, additional unknown critical factor(s) and mechanism(s) are often needed for successful induction of cryptobiosis (reviewed in Crowe, 2002; Oliver et al., 2002). A similar phenomenon was also found in this study: namely, larvae desiccated with 0.1 ml of distilled water accumulated a relatively large amount of trehalose (mean, 23.2 μg), although most of them did not recover from the dehydration state. On the other hand, decapitated larvae that contained less than 20 μg of trehalose succeeded in inducing cryptobiosis (Watanabe et al., 2002), indicating that additional factor(s) beside trehalose are needed for successful induction of cryptobiosis. The physiological basis for regulating cryptobiosis remains to be elucidated.

The carbon source for compatible solutes to protect against desiccation and osmotic stresses is uniformly distributed in unicellular organisms and plants; i.e. mainly glucose in the former and sucrose in the latter (Ingram and Bartels, 1996). By contrast, in insects, glycogen is the main source of sugars and polyols and is distributed locally in the fat body (Storey and Storey, 1991). Indeed, the larvae of *P. vanderplanki* had a large amount of glycogen in the fat body (M. Watanabe, T. Kikawada and T. Okuda, unpublished data). We presume that proper distribution of newly synthesized trehalose into all tissues or cells, probably by the fat body through the hemolymph, might be an important task for desiccating larvae. This insect may have more complex physiological and molecular mechanisms for induction of cryptobiosis than those of the osmotic responses found in *S. cerevisiae* and *A. thaliana*.

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