The Journal of Experimental Biology 205, 2799–2802 (2002)
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JEB4182



# Mechanism allowing an insect to survive complete dehydration and extreme temperatures

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*Accepted 17 June 2022*

## Summary

**Cryptobiosis describes the state of an organism whose body water is completely dehydrated and metabolic activity has become undetectable. Our study aimed to elucidate the physiological mechanism of cryptobiosis in the highest cryptobiotic invertebrate, *Polypedilum vanderplanki*. Larvae of this insect rapidly accumulated a large amount of the carbohydrate, trehalose, (18% of dry body mass) during desiccation for 2 days, suggesting that a high level of trehalose accumulation contributed to the successful induction of cryptobiosis in *P. vanderplanki* as well as in other lower cryptobiotic organisms. When larvae deprived of the brain, suboesophageal ganglion (SG) and thoracic ganglia (TG) were completely dehydrated and then rehydrated, they were able to recover and move actively. During desiccation, such larvae also accumulated trehalose, although only about half as much as the intact larvae. It is concluded that the brain, SG and TG do not affect the induction and termination of cryptobiosis, and hence in this higher multicellular animal cryptobiosis is independent of brain, SG and TG regulation, just as in plants or in unicellular organisms.**

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

## Introduction

Organisms have evolved various mechanisms for adaptation to adverse environmental conditions such as lack of water (cryptobiosis or anhydrobiosis), freezing temperatures (cryobiosis) and lack of oxygen (anoxia) (Hochachka and Somero, 1984). In plants or unicellular organisms that do not have a clearcut nervous system, individual tissues or cells express stress tolerance in response to a change in the environmental condition (Sakai and Larcher, 1987; Ingram and Bartels, 1996). However, vertebrates and higher invertebrates are believed to regulate stress tolerance directly or indirectly through the brain and become dormant when those stresses are severe and sustaining (Lyman et al., 1982; Denlinger, 1985; Danks, 2000).

A chironomid, *Polypedilum vanderplanki* Hint., is the largest multicellular animal known to tolerate almost complete dehydration without ill effect (Hinton, 1951, 1960a). Cryptobiotic larvae show extremely high thermal tolerance from –270°C to +106°C and can recover soon after prolonged dehydration of up to 17 years (Hinton, 1960a,b, 1968). However, the underlying molecular and metabolic mechanisms largely remain a mystery. Here we show that rapid accumulation of trehalose plays a key role in the successful induction of cryptobiosis, and that, surprisingly, cerebral regulation is not involved in the process of cryptobiosis.

## Materials and methods

### *Insects*

Cryptobiotic larvae of *Polypedilum vanderplanki* Hint. were collected from rock pools in Nigeria in 2000. They were transferred to the laboratory and put into a plastic container (200 mm×300 mm×100 mm) containing water (depth, 20–30 mm) on autoclaved soil (depth, 20–30 mm). The rearing water was aerated continuously. The container was covered with a nylon-mesh cage (200 mm×300 mm×250–300 mm). The larvae were reared for successive generations under controlled light (13 h:11 h light:dark) and temperature (27°C).

### *Desiccating procedure*

Groups of 3–5 larvae were placed on pieces of filter paper with 0.44 ml of distilled water in a glass Petri dish (diameter 65 mm, height, 20 mm). Two or three of these dishes were immediately transferred to a desiccator (<5% relative humidity) at room temperature (24–26°C) and gradually dried over a period of 48 h (0.22–0.23 ml day$^{-1}$).

### *Desiccation and recovery of intact and treated larvae*

After ligation (applied behind the head or thorax), the head or head and thorax segments were severed from final instar larvae of a similar body mass (approximately 1 mg) in iced water. The remaining body parts were incubated in distilled

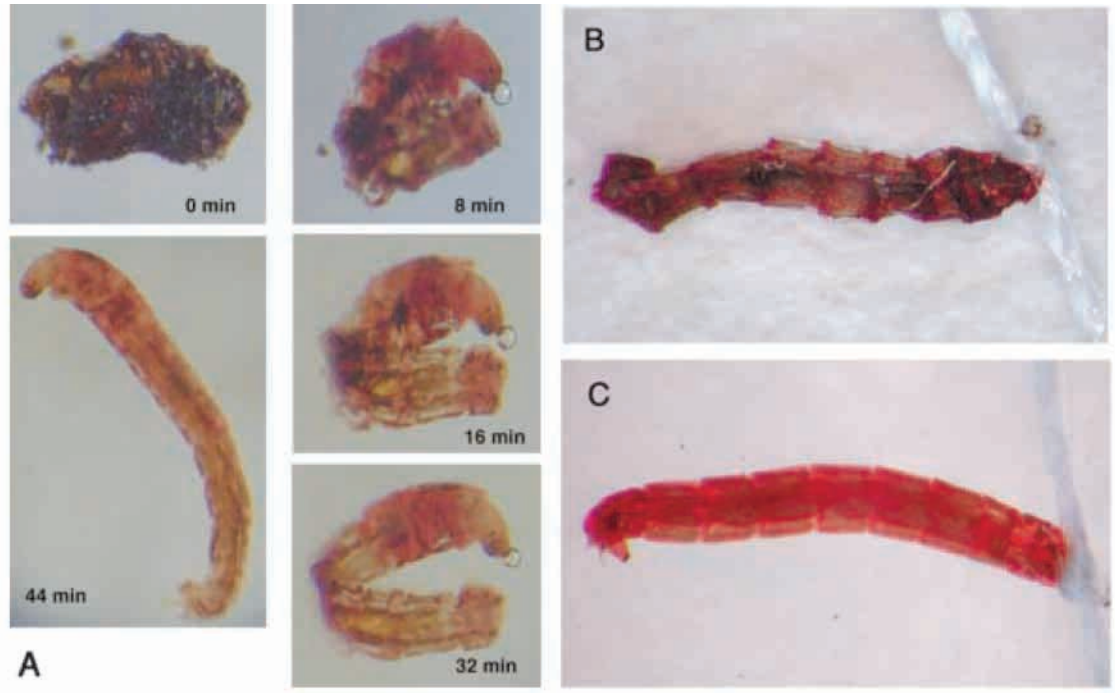


Fig. 1. *P. vanderplanki* during recovery from cryptobiosis. (A) Larva recovering from cryptobiosis at 0, 8, 16, 32 and 44 min after rehydration. (B) Decapitated cryptobiotic larva. (C) Decapitated larva on the second day after rehydration.

water for one day, and then completely dried over 3 days in the desiccator. Intact larvae were transferred directly to the desiccator. Subsequently, after being rehydrated by immersion in distilled water, the larvae were observed closely every 0.5–1 h to check their recovery. Larvae were judged to survive if they could repeatedly contract their abdomen. Because insects have an open circulatory system, radical treatments such as ligation and decapitation are routinely used, particularly in the field of insect endocrinology (Wigglesworth, 1972).

### *Sugar and polyol measurements*

Each group of 3–5 intact or operated larvae was placed in a desiccating Petri dish or in distilled water for 12, 18, 24, 30, 36, 42, 48 or 72 h, and then homogenized individually with 0.1 mg of sorbitol as an internal standard in 0.2 ml of 90% ethanol. After membrane filtration (pore size 0.45 μm), the supernatant was dried under a stream of nitrogen gas at 60°C and the dried residue dissolved in 500 μl of MilliQ water (Millipore). The samples were analysed on a Shimadzu HPLC system (LC-10A system, Shimadzu, Japan) equipped with a guard column (Shim-pack SCR-C, 4.0 mm×50 mm; Shimadzu, Japan) connected to an analytical column (Shim-pack SCR-101C, 7.9 mm×300 mm; Shimadzu, Japan) and a reflective index detector (RID-6A; Shimadzu, Japan). The columns were heated to 80°C, and MilliQ water as the mobile phase was allowed to flow at the rate of 0.8 ml $min^{-1}$. The injection volume was set at 10 or 20 μl. Standard trehalose and sorbitol solutions were prepared in MilliQ water in the range of 1–5,000 μg $ml^{-1}$. From the HPLC profile, trehalose and sorbitol could be quantified, at least in the higher range.

## Results and discussion

*P. vanderplanki* lives in the temporary rock pools of tropical Africa. When the pool dries up, the larva in its mud nest becomes dehydrated and remains desiccated until the next rain. Recently, we succeeded in the successive rearing of this species in the laboratory, and established a short-term (2 day) desiccating method for induction of cryptobiosis. Using this method, we examined the process and degree of larval dehydration.

Cryptobiotic larvae of this species were crumpled and sometimes folded in the middle, as is typical for larvae found in mud nests in the field. When given water, all of them recovered within half an hour and moved actively (Fig. 1A, Table 1). During the process of desiccation, the larvae accumulated a large amount of the sugar trehalose (average of approximately 18% of dry body mass: Intact, dry in Fig. 2). Other sugars and polyols were not detected.

Cryptobiotic organisms usually contain a high concentration of disaccharides at the dry state (Crowe, 2002). In general, sucrose is found in seeds and higher plants. Lower plants, lower animals and microorganisms, such as *Artemia* cysts (Clegg, 1965), nematodes (Madin and Crowe, 1975; Womersley and Smith, 1981), fungus (Sussman and Lingappa, 1959) and bacteria (Payen, 1949; Clegg and Filosa, 1961), all accumulate trehalose at a high concentration of approximately 20% of the dry mass. It has been suggested that trehalose provides the most effective protection against dehydration because of its high ability for water replacement and glass formation (Crowe et al., 1987, 1992; Green and Angell, 1989). Similarly, in *P. vanderplanki* as a higher invertebrate, a high

Table 1. *Effect of head and thorax removal on induction and termination of cryptobiosis in larvae of* P. vanderplanki

| | *N* | Larvae recovered (%) | Time for recovery (h) |
|---|---|---|---|
| Intact | 25 | 25 (100) | 0.5±0[a] |
| –Head | 20 | 19 (95) | 5.6±1.5[b] |
| –Head and thorax | 8 | 4 (50) | 6.7±2.2[b] |

The intact or treated larvae were completely dried over 3 days after incubation in water for 1 day. They were judged as surviving when they could move their body after rehydration.

Values are means±S.D.

[a,b]The different letters indicate a significant difference between values (Mann–Whitney *U* test; $P<0.05$).

The larvae that turned black (having presumably died before complete dehydration) were eliminated from the data.

level of trehalose accumulation would contribute to successful induction of cryptobiosis.

To elucidate the possible role of the brain, suboesophageal ganglion (SG) and thoracic ganglia (TG) in cryptobiosis of *P. vanderplanki*, we used larvae deprived of the brain, SG and TG by ligation applied behind the head or thorax followed by decapitation or severance of head and thorax. After a 3-day desiccation, such larvae became crumbled, but not folded (Fig. 1B). When completely dehydrated larvae were later submerged in water, most of the decapitated larvae and half the number of those from which the head and thorax were removed were able to recover (Fig. 1C, Table 1). Indeed, some of the decapitated larvae moved actively and survived for more than 2 weeks after recovery.

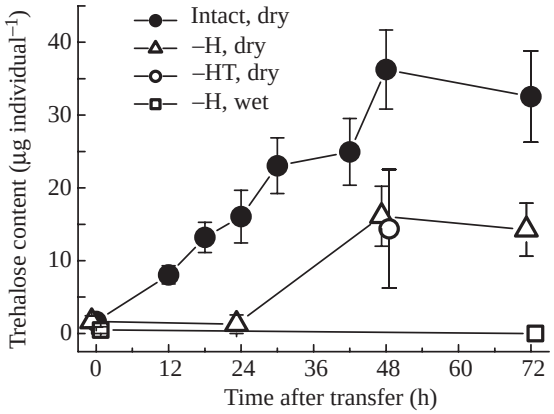


Fig. 2. Change of trehalose level in *P. vanderplanki* larvae during desiccation. Intact, dry: intact larvae (filled circles); –H, dry: decapitated larvae (open triangles); –HT, dry: larvae from which the head and thoraces were removed (open circles); –H, wet: decapitated larvae in water (open squares). There were significant differences in trehalose content 12 h after desiccation in intact larvae, at 48 h and 72 h after desiccation in –H larvae, and at 48 h after dessication in –HT larvae (Mann–Whitney *U* test, $P<0.05$). Values are means ± S.D., $N=3$–9.

The rehydration recovery time of the operated larvae was longer than that of intact larvae (Table 1). Larvae of *P. vanderplanki* are uniformly red due to equal distribution of hemoglobin in their hemolymph. During dehydration, the red color became more intensive in the apical and distal parts of the body (Fig. 1A), suggesting that body water evaporated mainly from the mouth and anus. In the decapitated larvae, both water loss during dehydration and penetration of water during rehydration appeared to occur mainly through the anus. The reduced capacity for water uptake possibly caused the delay in their recovery.

During desiccation, the treated larvae also accumulated a large amount of trehalose, although only about half as much as the intact larvae (–H, dry and –HT, dry in Fig. 2). By contrast, when the decapitated larvae were continuously incubated in water, they did not accumulate trehalose (–H, wet in Fig. 2). It must be stressed that the ligation and subsequent removal of the front body part were carried out in iced water and the larvae had not been exposed to air before transfer to the desiccation dish. Consequently, these larvae received no environmental signal of the forthcoming dehydration before the head and thorax were severed. Paralysis in iced water did not affect the induction of cryptobiosis. Cryptobiosis was successfully induced in decapitated larvae in which ligation and body severing was carried out without ice-water treatment (89%, $N=9$). We concluded that the brain, SG and TG did not affect the induction and termination of cryptobiosis, i.e. cryptobiosis occurs without the regulation of the brain, SG and TG, just as in plants and unicellular organisms. It would be interesting to examine whether such a regulatory system is operative in other cryptobiotic lower invertebrates, such as nematodes, water flea, rotifers and tardigrades (Young, 1985; Sømme, 1995).

Our findings may be greatly relevant to medical science. Currently, relatively long-term storage of living cells can be done only in cryoprotectant solution at extremely low subzero temperatures (Homma and Morimoto, 1997; Wolkers et al., 2002), and live human organs can be stored for only a short period *in vitro* (Cooper, 1991; Novick et al., 1992; Kenmochi et al., 1997). From this study, we suggest that individual organs and cells of cryptobiotic larvae, devoid of cerebral regulatory factors, could be stored at room temperature under dehydrated conditions. Further investigations to elucidate the mechanism of the successful induction and recovery of cryptobiosis in the 'higher' invertebrate *P. vanderplanki* might be of enormous consequence for the field of cell and organ storage.

We thank Drs F. Sehnal, Barbara I. P. Barratt, S. Tanaka and M. Asahina for critical reading of the manuscript. This study was carried out as a part of 'Ground-base Research Announcement for Space Utilization' promoted by the Japan Space Forum.

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