Physiological traits of invertebrates entering cryptobiosis in a post-embryonic stage

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Abstract. Cryptobiosis is the state when the metabolic activity of an organism is hardly measurable or is reversibly at a standstill. Many groups of invertebrates have this ability, and can be divided into two types according to the developmental stage in which it occurs; embryonic (eggs) or post-embryonic stages (larvae and adults). The latter must be able to reversibly regulate the physiology and biochemistry of development and cryptobiosis. There are several reviews on cryptobiosis and its regulation, but none on the physiological mechanism of cryptobiosis in chironomids. The present paper reviews the physiological traits of invertebrates entering cryptobiosis in a post-embryonic stage. These unique phenomena, which occur in a post-embryonic stage of three groups of cryptobiotic invertebrates (insects, tardigrades and nematodes) are discussed with particular reference to; 1) the behavioural and physiological adaptations of cryptobiotic invertebrates, 2) role of trehalose in cryptobiosis and 3) regulation of cryptobiosis.

INTRODUCTION

In 1702, Leeuwenhoek observed inactive animalcules (tardigrades or rotifers) of an oval shape in the dry sediments in the gutters of roofs of houses. The animalcules started moving shortly after coming into contact with water. Such a phenomenon is termed cryptobiosis, and is the state in an organism when its metabolic activity is hardly measurable, or reversibly at a standstill (Keilin, 1959).

Keilin (1959) noted that cryptobiosis could be divided into several categories; cryobiosis (freezing), osmobiosis (extremely high level of solutes), anoxybiosis (lack of oxygen) and anhydrobiosis (desiccation). The last category, anhydrobiosis, is common and found in many taxa ranging from unicellular organisms to higher invertebrates and plants.

Cryptobiosis is found in many groups of invertebrates including insects (chironomids), tardigrades, nematodes, rotifers, collombolans and primitive crustaceans (brine shrimp, Artemia) (Somme, 1995). Cryptobiotic organisms can be divided into two types according to the developmental stage in which cryptobiosis occurs; embryonic or post-embryonic. In the former, as in Artemia and collombola, female parents prepare the molecules necessary for cryptobiosis in their progenies (eggs) to some extent in response to external conditions (Hochacha & Guppy, 1987). In contrast, in the latter case, as in chironomids, most tardigrades, nematodes and rotifers, parents do not affect induction of cryptobiosis in their progenies at all, and individuals themselves must be able to reversibly switch their physiology and biochemistry between development and cryptobiosis. That is, in this case more complex physiological mechanisms are associated with cryptobiosis.

An African chironomid, Polypedilum vanderplanki, is the most advanced and largest multicellular animal with cryptobiotic ability in a post-embryonic stage. Hinton reported extremely high tolerance of various stresses by larvae of this chironomid between 1951 and 1968 (Hinton, 1951, 1960a, b, 1968), but the mechanisms of cryptobiosis in this unique chironomid have not been investigated. In 2000, we successfully reared this chironomid continuously in the laboratory and started to use it to study the physiology of cryptobiosis.

There are reviews on cryptobiosis and its regulation (Hochacha & Guppy, 1987; Crowe et al., 1992; Somme, 1995; Clegg, 2001). However, they do not include an account of the physiological mechanism of chironomid cryptobiosis. The present paper reviews the physiological traits of invertebrates that enter cryptobiosis in a post-embryonic stage, including the recently obtained data on chironomids. We mention and discuss this unique phenomenon in the three main groups of invertebrates (insects, tardigrades and nematodes), which enter cryptobiosis in a post-embryonic stage, in particular; 1) the behavioural and physiological adaptations of cryptobiotic invertebrates, 2) role of trehalose in cryptobiosis and 3) regulation of cryptobiosis.

CHIRONOMID

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This species breeds in small temporary pools formed in shallow hollows in unshaded rocks in Nigeria and Uganda (Hinton, 1951, 1960a). The cryptobiotic larvae show an extremely high thermal tolerance from –270 to 103°C, and can revive and grow after submersion in pure ethanol or glycerol (Hinton, 1960a, b, 1968). The longest record of dry preservation for this species is 17 years (Adams, 1985).

In the field, larvae usually live in soil nest tubes in pools (Hinton, 1951). When the pools dry up, the larvae gradually dry out and fold in the middle. The soil nest tubes decrease the rate of loss of water from the larvae. However, the folded larval form is not essential for inducing cryptobiosis in this insect; larvae that were induced to enter cryptobiosis on wet filter paper were often unfolded (Watanabe et al., 2002).

Larvae in water usually contain a small amount of trehalose in their blood. During the process of desiccation, larvae accumulate trehalose to around 20% of the dry body weight (Fig. 1). Other sugars and polyols were not detected. On the other hand, larvae dehydrated within 1 day do not accumulate as much trehalose and do not recover after rehydration (Watanabe et al., 2003).

Watanabe et al. (2003) demonstrated that the increase in the internal ion concentration triggers the rapid trehalose synthesis in *P. vanderplanki*. Trehalose accumulates in larvae kept in highly concentrated salt solutions (Fig. 2) depending on ion in solution (Fig. 3). It does not accumulate when larvae are kept in solutions of DMSO or glycerol. The yeast, *Saccharomyces cerevisiae*, has an osmosensor that activates glycerol synthesis in response to high osmolarity regardless of solute (Maeda et al., 1994; Posas & Saito, 1997; Maeda, 1999). Unlike in yeast...
the rapid accumulation of trehalose in P. vanderplanki larvae is not a simple osmotic response.

Many temperate insects enter diapause in various developmental stages in order to survive adverse conditions. Although the endocrines regulating larval, pupal and adult diapause differ, the brain is the common prime regulator of diapause (Denlinger, 1985). Watanabe et al. (2002) have demonstrated that cryptobiosis can occur in P. vanderplanki larvae without a brain. Larvae without a head accumulate relatively large amounts of trehalose during desiccation (Fig. 4) and recover after rehydration like intact larvae (Fig. 5). Furthermore, we recently found that an isolated fat body can synthesize a large amount of trehalose during desiccation and after desiccation for a week can recover on rehydration (Watanabe et al., unpubl. data). Therefore, it is likely that the central nervous system is not involved in the induction of crypotbiosis in this chironomid.

TARDIGRADES

There are 600 species of Tardigrada in the world (Pilato, 1979). They usually live in fresh water or humid soil. Anhydrobiosis is widespread in this phylum and occurs mainly in the adult stage.

There is an abundance of literature on anhydrobiosis in tardigrades covering more than 200 years, which has been reviewed by Ushatinskaya (1990), Wright et al. (1992), Sømme (1995, 1996). Spallanzani (1769) showed that tardigrades could survive being kept in a vacuum. In the anhydrobiotic state, tardigrades can tolerate extreme temperatures from –270°C to 151°C, exposure to X-rays (570,000 R) and high hydrostatic pressure (600 Mpa) (Rahm, 1937; Crowe & Cooper, 1971; Seki & Toyoshima, 1998). The revival rate gradually decreases with increase in the dehydration period; the maximum period for which anhydrobiotic Anguillulina tritici can survive may be 10 years (Keilin, 1959).

Anhydrobiotic tardigrades always contract into a structure resembling a small tun when dehydrated (Somme, 1995). Tun formation is essential for the successful induction of anhydrobiosis in tardigrades. The rates of water loss and transpiration gradually decrease as the surface area is reduced during tun formation (Wright et al., 1992). Both rates rapidly decline just after completion of tun formation, finally to an undetectable level. When tardigrades are desiccated at a low relative humidity or under anoxia they cannot form tun and be revived (Crowe, 1972).

Trehalose accumulation is also observed in the tardigrade, Adorybiotus coronifer, during dehydration (Westh & Ramløv, 1991). Trehalose in active individuals of this species makes up 0.1% of the dry body weight. During dehydration, this tardigrade completes tun formation in 3 h and accumulates trehalose up to 2.3% of the dry weight within 7 h. The level of trehalose accumulation is much lower than that often found in other anhydrobiotic organisms (10–20%) (Somme, 1995; Clegg, 2001).

Protein synthesis resumes 2 h after rehydration and the trehalose content decreases to the normal level 6 h after rehydration (Westh & Ramløv, 1991). CO₂ induction of aerobic acidosis in rehydrating tardigrades caused a reduction in anabolic and catabolic activities, trehalose degradation and protein synthesis.

NEMATODES

Most anhydrobiotic animals are nematodes (Womersley, 1987). A number of anhydrobiotic nematodes are widely distributed from deserts to the Antarctic area (Ellenby 1969; Crowe & Madin, 1975; Womersley, 1987). Anhydrobiosis in nematodes occurs in juvenile and adult stages, but sometimes in the egg cysts within protective shells (Somme, 1995). Cryptobiotic nematodes can be revived after exposure to extremely low temperatures (Pickup & Rothery, 1991; Wharton & Barclay, 1993). Anguina tritici and Tylenchus polyhypnus recovered from the anhydrobiotic state after 32 and 39 years, respectively (Steiner & Albin, 1946; Womersley, 1980).

Anhydrobiotic nematodes can be divided into two groups; slow-dehydration and fast-dehydration strategists (Womersley, 1987; Wharton, 2002). The former need to be dehydrated slowly at high relative humidity (RH) to successfully enter anhydrobiosis. On the other hand, the fast-dehydration strategists can survive relatively high rates of dehydration or possess adaptations that slow down the rate of water loss.

Slow rate of water loss is achieved by behavioural and/or physiological mechanisms (Wharton, 2002). The
most common behavioural strategies for decreasing the rate of water loss are coiling and clumping. Coiling in *Aphelelenchus avenae* decreases the rate of water loss by decreasing the external surface of the body, enabling these nematodes to enter cryptobiosis (Crowe & Madin, 1975). Clumping (forming of an aggregation) by nematodes also decreases water loss, especially of the nematodes in the center of the aggregation, and increases their survival in the desiccated state (Ellenby, 1969).

A physiological strategy for decreasing the rate of water loss is to change the permeability of the body surface (Wharton, 2002). *Ditylenchus dipsaci* decreases water loss by rapidly lowering body surface permeability 2 min after the onset of desiccation (Wharton, 1996). This occurs via a phase change in the composition of the epicuticle or a decrease in the thickness of the cuticle (Wharton & Lemmon, 1998).

Many species of nematodes accumulate low-molecular weight carbohydrates in the anhydrobiotic phase (Womersley, 1987). However, the nature and content of these compounds depends upon nematode; larvae of *Ap. avenae* accumulated trehalose (11–13% of the dry weight) and glycerol (5%) (Madin & Crowe, 1975). *An. tritici* increased its inositol (0.8%) and trehalose (9%) content during dehydration (Womersley & Smith, 1981). On the other hand, Higa & Womersley (1993) demonstrated that the accumulation of trehalose in itself is not sufficient for the induction of anhydrobiosis in *Ap. avenae*; the nematode survival was directly related to rates of evaporative water loss rather than trehalose content. Other physiological and biochemical adaptations for surviving anhydrobiosis may exist in nematodes (Womersley, 1987).

**IMPORTANCE OF TREHALOSE IN DEHYDRATION**

Trehalose is a common compatible solute in cryptobiotic organisms, such as unicellular organisms (bacteria, yeast and spores of fungus), invertebrates (chironomid, tardigrades, nematodes and encysted *Artemia*) and resurrection plants, although other disaccharides such as sucrose is present in seeds and pollen grains of higher plants (Ingram & Bartels, 1996; Clegg, 2001). Most of these organisms accumulate quite a large amount of trehalose, around 10–20% of the dry body weight.

It is of interest to discuss why trehalose is the compatible solute in cryptobiotic organisms ranging from unicellular organisms to higher invertebrates and plants. Trehalose, as a non-reducing sugar, is less harmful to cells and tissues than reducing sugars such as glucose even at extremely high concentrations. Among sugars and polyols trehalose provides the most effective protection against desiccation because of its high ability for water-replacement and glass formation (vitrification) (Burke, 1986; Crowe et al., 1987; Green & Angell, 1989; Crowe et al., 1998); it substitutes for bound and free water, and so maintains the structures of cell membranes and proteins. Glassy state of trehalose may fill the spaces in tissues during dehydration and allow the orderly packing of body components, which prevents structural damage, and inhibits aggregation of biological molecules and increase in solute concentration. High and stable viscosity of trehalose glasses may also stop all chemical reactions that require molecular diffusion.

Recently it has been shown that human primary fibroblasts intracellularly expressing trehalose, due to a recombinant adenovirus vector, could be maintained in a completely dry state for a short period (Guo et al., 2000). Freeze-dried human and mammalian platelets can recover after rehydration only when they were loaded intracellularly with a relatively high concentration of trehalose before freeze-drying (Wolker et al., 2001, 2002). This indicates that the presence of intracellular trehalose might be important for desiccation tolerance. The study of the localisation of intra- and extra-cellular trehalose in anhydrobiotic invertebrates may provide important information for elucidating the mechanism of cryptobiosis.

**CONCLUSIONS**

The post-embryonic stages of many species of invertebrates survive complete dehydration by entering cryptobiosis. Such invertebrates have evolved behavioural and physiological adaptations for the successful induction of cryptobiosis. Slow desiccation is necessary for cryptobiosis in most cases. The most common physiological feature is the accumulation of trehalose, although anhydrobiotic rotifers, *Philodina roseola* and *Adineta vaga*, do not have trehalose and any other low-molecular weight carbohydrates during dehydration (Lapinski & Tunnacliffe, 2003). However, the existence of a large amount of trehalose in itself is not sufficient for anhydrobiosis in nematodes and a chironomid (Higa & Womersley, 1993; Watanabe et al., 2003). This may indicate that factor(s) other than trehalose are involved in regulating cryptobiosis. In plants, chemicals such as prolin, betaine and the late embryogenesis abundant (LEA) protein, as well as sugars are thought to protect cells against desiccation (Yamaguchi-Shinozaki, 2003). In fact, the anhydrobiotic nematod, *Ap. avenae* has LEA protein gene (Browne et al., 2002), and the anhydrobiotic rotifers, *P. roseola* and *Ad. vaga*, have a dehydration-regulated protein which is recognized by an antiserum against a nematode LEA protein (Tunnacliffe et al., 2004).

The carbon source for the solutes that protect against desiccation is uniformly distributed in unicellular organisms and plants, i.e., mainly glucose in the former and sucrose in the latter (Ingram & Bartels, 1996). By contrast, in insects, glycogen is the main source, and it is stored in the fat body (Storey & Storey, 1991). *P. vanderplanki* larvae have a large amount of glycogen in their fat body at hydrated state and of trehalose in their hemolymph just prior to complete dehydration (Watanabe et al., unpubl. data). It is likely that the distribution of newly synthesized trehalose to all cells and tissues, probably from the fat body via the hemolymph, is important for dehydrating larvae. Invertebrates that enter cryptobiosis in a post-embryonic stage may have more complex physiological and molecular mechanisms for inducing of cryptobiosis than cryptobiotic unicellular organisms and plants.


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