Adaptations to environmental extremes by multicellular organisms

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Abstract: Microbes, particularly Archaea, are well-known for their superb adaptation to extreme environments. However, amazing adaptations to extreme stresses do not only occur in microbes, but also in many multicellular organisms. Examples include tardigrades and their capability to survive freezing to near absolute zero, the Pompeii worm withstanding temperatures of up to 105 °C, the Crucian carp remaining active in anoxic conditions for several months, and resurrection plants, which can survive near-complete desiccation. Here, we review some of the cryptobiotic strategies and adaptations exhibited in multicellular organisms, and point out their relevance to astrobiology.

Introduction

The term 'extremophile' usually evokes images of prokaryotes living in acidic hot springs or in the Antarctic Dry Valleys, environments very different from the human environment. However, the taxonomic range of extremophilic organisms spans all three domains, including multicellular organisms and vertebrates (Rothschild & Mancinelli 2001; Clegg & Trotman 2002). For example, hydrothermally formed chimneys harbour dense and diverse microbial and multicellular communities, including luxuriant populations of clams (e.g. *Calyptogena magnifica*), mussels (e.g. *Bathymodiolus thermophilus*), tube worms (e.g. *Riftia pachyptila*, *Tevnia jerichonana*) and a variety of grazers, which are supported by the high chemosynthetic primary production of the hydrothermal vents (Turner 1886). These systems are usually hosted in peridotites, an environment which is not only high in temperature and in pressure, but also very reducing and associated with acidic pH conditions. Other extreme environments useful to understand the range of environments in which terrestrial life can thrive include studies of winter sea-ice (Krembs *et al.* 2002), active sulphide chimneys (Kelley *et al.* 2002) and the acidic, iron-rich river, Rio Tinto, in southern Spain (Zettler *et al.* 2002). The study of these types of environments and an associated survey of the full biodiversity will provide an essential learning tool for the exploration of extraterrestrial life on other planetary bodies and shed light on evolutionary trajectories towards multicellular complexity (Schulze-Makuch & Irwin 2004). In addition, the study of extremophiles will contribute to our understandings of mechanisms of organismic survival, the origin of life and the limits for life in the universe (Rothschild 2006).

Adaptation strategies to environmental stresses by multicellular organisms

Adaptation in a biological sense is a genetically controlled characteristic that increases an organism's ability to survive and reproduce in its environment. Stresses to which organisms have evolved adaptations include dehydration (anhydrobiosis), extreme temperature (thermobiosis) including freezing temperatures (cryobiosis) and lack of oxygen (anoxia) (Hochachka & Somero 1984, 2002). In 1702 van Leeuwenhoek observed inactive 'animalcules' (tardigrades and rotifers) in dry sediments from the gutters of roofs of houses (Keilin 1959; Wright 2001). Shortly after the organisms came into contact with water they started to move. A similar ability of organisms to tolerate dehydration was reported by Needham (1743), Spallanzani (1776) and Doyère (1842). This phenomenon is referred to here as cryptobiosis (latent or hidden life) and anhydrobiosis is a special case of adaptation to dehydration. It is defined as the state of an organism when the organism shows no visible signs of life and when its metabolic activity is hardly measurable or comes reversibly to a standstill (Keilin 1959; Takamatsu *et al.* 1997).

Cryptobiosis is a form of quiescence, which is induced and maintained directly by the occurrence of adverse conditions (such as desiccation, freezing and oxygen deficiency) for active life, and promptly broken once the adverse conditions are removed (Hand 1991; Keilin 1959). Cryptobiosis is one particular adaptation against environmental stresses, but should not be confused with diapause. The latter at the larval, pupal and adult stages is arrested development resulting from reduced hormonal activities, which may persist even when favourable environmental conditions prevail (Tauber & Masaki 1968; Danks 1987).
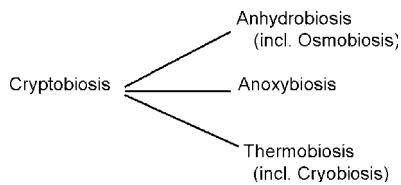
Fig. 1. Classification of cryptobiosis based on environmental stresses to be overcome. Note that some other authors (Keilin 1959; Wright *et al.* 1992; Takamatsu *et al.* 1997) list osmobiosis and cryobiosis as separate processes.

Cryptobiosis can be used as a generic term for ametabolism, and further subdivided into five categories based on the factors that induce it (Fig. 1): cryobiosis (induced by freezing), thermobiosis (low and high temperatures), osmobiosis (extremely high osmolarity), anoxybiosis (lack of oxygen) and anhydrobiosis (lack of water) (Wright *et al.* 1992; Takamatsu *et al.* 1997). At the organismic level, the distinction between anhydrobiosis, cryobiosis and osmobiosis is often unclear because more than one process may be involved in the entry of cryptobiosis. For instance, low temperatures inducing extracellular freezing in the organism will also have a desiccating effect on the cells (Jönsson 2003).

Anhydrobiosis

Anhydrobiosis is the most common form of cryptobiosis, and anhydrobiotic organisms are able to survive almost complete dehydration; 95–99% of the water content is lost in many cases (Drinkwater & Crowe 1991; Danks 2000). Desiccation tolerance is exhibited by many life forms and found in many taxa ranging from unicellular organisms to higher invertebrates and plants, such as plant seeds, vegetative tissues of higher plants (resurrection plants), yeasts, bacteria, fungal spores, protozoans, eggs of turbellaria, nematodes, rotifers, tardigrades, springtails, cysts of primitive crustaceans including the brine shrimp (*Artemia salina*) and larvae of a chironomid midge (e.g. *Polypedilum vanderplanki*); see Takamatsu *et al.* (1997), Ricci (1998), Scott (2000), Clegg (2001) and Wharton (2002). Anhydrobiosis is commonly identified by morphological change. The coiling of the body of soil nematodes such as *Scottnema lindsayae*, *Eudorylaimus antarcticus* and *Plectus antarcticus* accompanying the physiological changes have been used to identify anhydrobiotic individuals in field studies (Townshend 1984; Freckman *et al.* 1987; Treonis *et al.* 2000) and in the laboratory (Higa & Womersley 1993a). One of the common features of anhydrobiotic stages is limited size, and most of the invertebrates are less than 1 mm in size. No anhydrobiotic species have been found in vertebrates (Takamatsu *et al.* 1997). However, in many anhydrobiotic plants, this stage is not restricted to small size.

Concerning the issue of size variations between anhydrobiotic animals and plants, Alpert (2006) has demonstrated that desiccation-tolerant animals may be small in size because of physical stresses associated with drying. Animal cells shrink as a result of drying and the whole animal must shrink with it. All animals that tolerate anhydrobiosis or desiccation as adults adopt distinctive or curled shapes as they dry. Rigid external or internal skeletons would prevent the curling. Some tolerant animals have exoskeletons, but their tolerance is restricted to juvenile stages before skeletons form.

Plant communities have a greater ability than animals to combine tolerance and rigidity. The leaves of desiccation-tolerant plants often shrink or fold as they dry, but the length of the stems appears to remain unaffected. This is possible because each plant cell has its own exoskeleton and a rigid cell wall. Physical stress probably does not compound across groups of cells as readily in plants as in animals. Some tolerant plants perform various specialized traits that reduce the shrinkage of cells away from their walls or increase the ability of the wall to fold or bend as the cell shrinks (Schneider *et al.* 2000).

Longevity in the anhydrobiotic state varies with the type of organism, but many species can remain viable for significant time periods. For example, there have been reliable demonstrations of the longevity of anhydrobiotic invertebrates after laboratory preservation including a 39-year-survival of the nematode *Tylenchus polyhypnus* (Steiner & Albin 1946). The longest records of recovery from the anhydrobiotic state for insects is 17 years, for crustaceans 16 years, and for tardigrades and rotifers 9 years (Baumann 1922; Clegg 2001; Bertolani *et al.* 2004). However, survival periods may be much longer. There are reports that tardigrades and rotifers were recovered from a dry moss sample taken from an Italian museum after 120 years of preservation, although the individuals underwent 'quivers in several zones of their body' (Franceschi 1948; Jönsson 2003).

Anhydrobiotic organisms can express extremely high tolerances against various kinds of stresses. Gavaret (1859) showed that rotifers and tardigrades could survive being kept in a vacuum. *Artemia* cysts are often preserved in a vacuum and nitrogen gas packs to maintain high recovery rates. *Artemia* cysts have also been exposed to the conditions of outer space with negligible mortality (Gaubin *et al.* 1983). Anhydrobiotic tardigrades can tolerate extreme temperatures down to $-270\,^{\circ}$C, immersion in saturated brine and organic solvents, and extremely high hydrostatic pressures (600 MPa) (Rahm 1921; Keilin 1959; Seki & Toyoshima 1998).

For more information and recent results on anhydrobiosis, the reader is referred to the published proceedings of a symposium on 'anhydrobiosis', which appeared in *Integrative and Comparative Biology* in 2005 (**45**, 683–820) with a total of 16 individual contributions.

Biochemical changes during anhydrobiosis

Cells of organisms that are naturally adapted to survive severe stress usually contain large amounts of low-molecular-mass 'compatible solutes' (Yancey *et al.* 1982; Somero & Yancey 1997), which will also play important protective roles. Among these solutes, trehalose, the disaccharide of glucose, plays a considerable role (Drinkwater & Crowe 1991; Eleutherio *et al.* 1993; Clegg 2001). Trehalose accumulates in anhydrobiotic organisms, such as unicellular organisms

(bacteria, yeast and spores of fungi), invertebrates (chironomids, tardigrades, nematodes and encysted *Artemia*) and resurrection plants (Yancey *et al.* 1982; Ingram & Bartels 1996; Takamatsu *et al.* 1997; Clegg 2001; Tunnacliffe & Lapinski 2003), whereas other disaccharides, mainly sucrose, are present in seeds and pollen grains of higher plants (Drinkwater & Crowe 1991; Behm 1997; Alpert 2000). Most of these organisms accumulate a large amount of trehalose during dehydration, about 10–20% of their dry body weight (Takamatsu *et al.* 1997).

It has been demonstrated that this sugar protects the desiccation-linked damage in organisms (Drinkwater & Crowe 1991; Allison *et al.* 1999; O'Hara *et al.* 1999). Trehalose has a high solubility, a low reactivity and a low tendency to crystallize. It is a non-reducing sugar, and so is less harmful to cells and tissues than reducing sugars such as glucose, even at extremely high concentrations (Drinkwater & Crowe 1991; Pannewitz *et al.* 2003). It substitutes for bound and free water, and thus maintains the structures of cell membranes and proteins. At the same time trehalose leads to metabolic shutdown.

On the other hand, anhydrobiosis without trehalose has been reported in bdelloid rotifers by Tunnacliffe & Lapinski (2003). The rotifers *Philodina roseola* and *Adineta vaga* gave surprising results as a response to desiccation stress concerning sugar accumulation. No trehalose at all was observed in the analytical results and no other simple sugar was produced in detectable quantities during dehydration. Even trehalose synthase genes were not found in the rotifer genomes. This first observation of animal anhydrobiosis without trehalose thus challenges our present understanding and requires a re-thinking of existing models.

Higa & Womersley (1993b) reported that the accumulation of trehalose in itself is not sufficient for the induction of anhydrobiosis in *Aphelenchus avenae*. Also, species of *Di. myceliophagus* varying in trehalose content ranging from 3 to 16% dry weight were not able to survive direct exposure to low humidity (Womersley *et al.* 1998).

Thus, it is likely that other or additional biochemical changes are required for anhydrobiosis. Heat shock proteins (Hsps) are good candidates, because they can serve as molecular chaperones. The Hsp70 family and the Hsp60 chaperon complexes (the classical molecular chaperones) are commonly perceived as 'heat shock proteins'. They are up-regulated by heat stress, although they also play a vital role in folding nascent proteins under non-stress conditions (Ellis & Hartl 2003). These molecules were reported to participate in unfolding and relocalization of proteins damaged by stresses, to assist the folding of newly synthesized proteins, to protect them from denaturation and aggregation and aid in their renaturation, and to influence the final intracellular location of mature proteins (Ellis & Hartl 1999; MacRae 2000). Hsps are also involved in tolerance against various other stresses such as low and high temperatures, oxidation, anoxia and heavy metals.

The function of Hsps as stress combatants is to potentiate refolding of polypeptide structures which have become partially denatured. Besides such 'folding' chaperones, both the eukaryotic and prokaryotic cytoplasm also contains 'holding' and 'disaggregating' chaperones. Holding chaperones include small Hsps which form large multimeric 'windowed' complexes capable of passively stabilizing protein species in a partially unfolded state, preventing aggregation until stress has abated, and refolding by Hsp70 and Hsp60 teams (e.g. Haslbeck *et al.* 1999). Disaggregating chaperones include the Hsp100 family, which is thought to disentangle protein aggregates and to serve as clients for folding chaperones (Ben-Zvi & Goloubinoff 2001).

In fact, one type of small Hsp, p26 and artemin, may be involved in stress resistance in *Artemia* cysts (Willsie & Clegg 2002; Chen *et al.* 2003; Collins & Clegg 2004; Warner *et al.* 2004), and p26 also appears to act synergistically with trehalose *in vitro* (Viner & Clegg 2001). Goyal *et al.* (2005a, b, c) characterized genes in the nematode *Aphelenchus avenae*, which requires a period during which water loss is slow and limited before the animal becomes fully desiccation tolerant. This is probably to allow time to switch on a critical set of genes, which can be identified by their dehydration-responsive nature. During this preconditioning period, trehalose synthase genes are induced (Goyal *et al.* 2005a) and trehalose biosynthesis occurs (Madin & Crowe 1975). Of other up-regulated genes identified to date, two encode 'hydrophilins' (highly hydrophilic proteins; Garay-Arroyo *et al.* 2000): a novel protein called anhydrin, and a polypeptide, Aav-LEA-1, related to plant Group 3 late embryogenesis abundant (LEA) proteins (Browne *et al.* 2002, 2004).

The widespread occurrence of LEA and other hydrophilic proteins in desiccation-tolerant or -resistant systems is consistent with combating damage due to water loss. Other functions for LEA proteins, besides that of preventing protein aggregation, are also possible and it has been suggested that they might be multi-functional, with one potential role as storage proteins in plants (Goyal *et al.* 2005b). Some LEA or LEA-like proteins are known to associate with cell membranes and have been shown to prevent liposome leakage on desiccation (Sales *et al.* 2000). LEA proteins could also act as 'molecular shields' to physically prevent or reduce the formation of protein aggregates (Goyal *et al.* 2005c). It has been proposed that folding of LEA proteins on membranes occurs in a manner similar to that proposed for alpha-synuclein, which has a role in vesicle management (Koag *et al.* 2003). LEA protein accumulation has been reported in plant seeds and tissues of resurrection plants, associated with the acquisition of desiccation tolerance during maturation (Ingram & Bartels 1996; Cuming 1999; Scott 2000; Clegg 2001). Expression of LEA is induced by drought, low temperature and high salt content (Ingram & Bartels 1996; Cuming 1999), and confers increased osmotic or freezing tolerance to the yeast *Saccharomyces cerevisiae* (Imai *et al.* 1996; Zhang *et al.* 2000). A barley LEA improves drought tolerance in transgenic plants including rice and wheat (Xu *et al.* 1996; Sivamani *et al.* 2000). A LEA-like protein (Hsp12) is also suggested to be involved in protection of membrane proteins (Sales *et al.* 2000). Group 3 LEA protein homologues were

found in the nematodes *Caenorhabditis elegans*, *Steinernema feltiae* and *A. avenae*, and in the prokaryotes *Deinococcus radiodurans*, *Bacillus subtilis* and *Haemophilus influenzae* (Postnikova *et al.* 1999; Dure 2001; Browne *et al.* 2002).

All groups of LEA proteins (1, 2, 3 and 6) were suggested to act as molecular chaperones (Imai *et al.* 1996; Wise & Tunnacliffe 2004). Wise & Tunnacliffe (2004) proposed a possible function of LEA proteins (group 3) during induction of anhydrobiosis. Natively unfolded LEA proteins exhibit a strong alpha-helical component during desiccation. The superhelical structures form coils and filaments associated with the cytoskeleton, and finally generate intracellular filament networks within the dehydrating cells. This configuration might increase mechanical strength. LEA proteins also act synergistically with trehalose as an aggregation protectant for several kinds of enzymes (Goyal *et al.* 2005a, b, c). Hsps and LEA proteins have been found in broad taxa of anhydrobiotic organisms from plants to invertebrates, and seem to have important roles for the induction and maintenance of anhydrobiosis. However, the protective roles have not been demonstrated in invertebrates. Further analysis of the behaviour of proteins during water stress is required to elucidate the function of LEA proteins *in vivo* during anhydrobiosis. Thus, anhydrobiosis appears to be a complex physiological response requiring the interplay of several different gene products and metabolites, which needs to be further elucidated by future molecular studies.

Induction of anhydrobiosis

Although the brain is the common prime regulator of larval, pupal and adult diapause in insects (Denlinger 1985), this does not seem to be the case for anhydrobiosis. In *P. vanderplanki* larvae, anhydrobiosis occurs without the brain: larvae without a head accumulate relatively large amounts of trehalose during desiccation and recover after rehydration like intact larvae (Takamatsu *et al.* 1997). Furthermore, body parts mainly consisting of fat body tissues can synthesize a large amount of trehalose during desiccation, and the fat body can recover after long-term dry preservation (Takamatsu *et al.* 1997). Therefore, the central nervous system is not involved in the induction of anhydrobiosis, and individual cells and tissues themselves could enter the anhydrobiotic state in the chironomid. Trehalose content in anhydrobiotic organisms ranges from 2% in tardigrades to 40% in yeast. Trehalose cannot easily enter cells and tissues because of its relatively large molecular size. Recently, it has been shown that the presence of intracellular trehalose at a high level contributes to an increase of desiccation tolerance in vertebrate cells: human primary fibroblasts intracellularly producing trehalose (due to a recombinant adenovirus vector) could be maintained in a completely dry state for 3 days (Guo *et al.* 2000). These cells accumulated only 1 mM of trehalose intracellularly. Freeze-dried human and mammalian platelets (apyrene) could recover after rehydration only when they were loaded intracellularly with a relatively high concentration of trehalose (20 mM) before freeze-drying (Wolkers *et al.* 2001, 2002). On the other hand, mouse cells containing

10% trehalose, through expressing trehalose phosphate synthase intracellularly, could not survive complete desiccation, although they had increased tolerance to high osmolarity (Garcia-de-Castro & Tunnacliffe 2000). It is still not determined whether the importance of intracellular trehalose reported in vertebrate cells is applicable to cells, tissues and individuals of invertebrates in the anhydrobiotic state. Physiological processes involved in the induction and emergence of anhydrobiosis in invertebrates are described in detail in Womersley *et al.* (1998) and Takamatsu *et al.* (1997).

Ma *et al.* (2005) have compared cellular viabilities following drying and rehydration, and demonstrated that trehalose and p26 improved cellular viability. They reported that the 293H cells lacking p26 and trehalose indicated the greatest sensitivity to water loss, decreasing viability at high water content. E11'L cells expressing p26, but lacking trehalose, showed a negligible improvement at the lowest water content. The survival rate of trehalose-loaded 293H cells was higher at a lower water content. E11'L cells expressing p26 and loaded with trehalose indicated major improvement in survival (at least a 20% increase in viability) over all other treatments. This phenomenon positively indicated that both trehalose and p26 produce significant advantages in the ability of the cells to survive drying. Optimal protection was achieved in the presence of both trehalose and p26, suggesting a synergistic mechanism.

Resurrection plants as an example organism using anhydrobiosis

A group of higher plants known as poikilohydric or resurrection plants possesses a uniquely effective mechanism for coping with drought stress. All plant species are at least partially tolerant to desiccation (i.e. seeds and pollen; Bewley 1979), but the ability of mature tissue, such as roots and leaves, to survive virtually complete desiccation is very rare. Resurrection plants survive the loss of most of their tissue water content until a quiescent stage is achieved. Upon watering the plants rapidly revive and are restored to their former state (Fig. 2). This resurrection capacity is not restricted to the main meristems of these plants. Fully mature leaves can lose up to 95% of their water content and then, upon rewatering, are rehydrated and are fully photosynthetically active within 24 hours (Bernacchia *et al.* 1996). Tissue damage through this drying and rehydration process appears to be minimal to non-existent. The plant is preserved until water becomes available. At this point it is ready to take immediate advantage of the new conditions. Thus, a resurrection plant has a great competitive advantage over other species in certain ecological niches.

Most resurrection plant species are native to arid climates in the world such as southern Africa, southern America and western Australia (Gaff 1977, 1987). Resurrection plants inhabit ecological niches that are subjected to lengthy periods of drought with periods of rain during the year. They are frequently found as denizens of rocky outcrops and grow in shallow, sandy soils (Sherwin & Farrant 1996). Their habitats often take the form of depressions on rock outcrops which
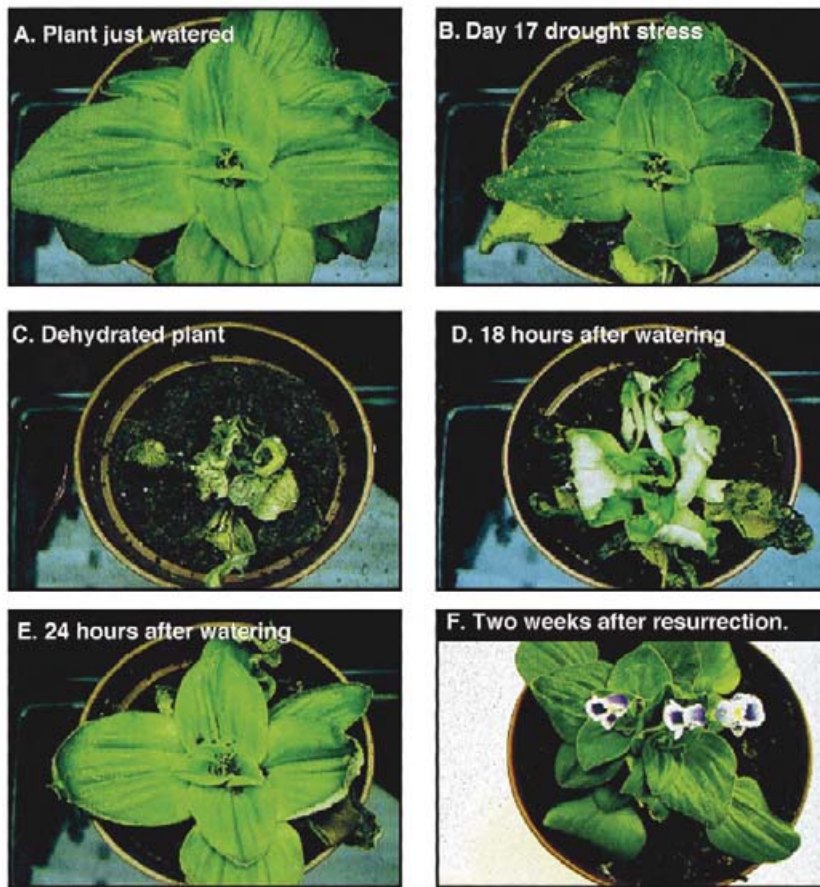
Fig. 2. Dehydration and rehydration phases of resurrection in *Craterostigma plantagineum*. On day 1 watering ceased (A). Photographs were taken of the same plant (Scott 2000).

have the capacity to form ephemeral pools (Gaff 1971; Gaff & Giess 1986). The growth and reproduction of the plants occurs in rainy seasons, but upon drying the plants can remain dormant for considerable time periods. The aquatic plant *Ch. intrepidus* has been known to survive for up to 11 months in the dehydrated state (Hartung *et al.* 1998). However, this period is short compared to species of *Craterostigma*, which have been known to last at least 2 years without water, and probably much longer (Gaff & Wood 1988; Gaff 1989). The survival mechanisms in these plants protecting them from dehydration are frequently also effective against other environmental stresses including high salt content (Gaff & Wood 1988; Gaff 1989) and heat (up to 60 °C; Hartung *et al.* 1998). Some other organisms specialized in utilizing anhydrobiosis are listed in Table 1.

Sherwin & Farrant (1996) have studied the mechanisms of avoidance and protection against light damage in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*. In *C. wilmsii*, a combination of both physical and chemical changes appeared to afford protection against free radical damage. The leaf tissue became purple/brown in colour during dehydration, which coincided with a three-fold increase in anthocyanin content and a 30% decline in chlorophyll content. Thus, light-chlorophyll interactions

were progressively reduced as chlorophyll became masked by anthocyanins in abaxial layers and shaded in the adaxial layers. Ascorbate peroxidase activity increased during this process but declined when the leaf was desiccated. During rehydration, superoxide dismutase and glutathione reductase activities increased markedly, possibly affording free radical protection until full hydration and metabolic recovery had occurred.

Scott (2000) demonstrated that the roots (being in the soil) are most likely to detect a drop in water availability first. Abscisic acid synthesis and release by roots is a common response in plants to drought stress. Once released, abscisic acid could activate batteries of genes required for metabolic processes such as the accumulation of sucrose from either stored carbohydrates or through an alteration in photosynthetic carbon partitioning. In addition, the synthesis of other proteins would be induced by dehydrins and LEAs, which could help to stabilize the plant cells as they lose water.

As the tissues dehydrate, leaves shrink, chlorophyll is degraded, sucrose accumulates, the xylem cavitates eventually and the plants become desiccated. On addition of water, the xylem refills and cells begin to take up water and expand. Enzymes present in the tissues are activated, sucrose is metabolized and chlorophyll is resynthesized. Within

Table 1. *Some example organisms using anhydrobiosis as a survival mechanism*

Organism	Survival ability
African Lungfish	This fish can survive 4 years without water (Coates 1937; Greenwood 1986) and is an obligatory air-breather during the dry season (Sturla *et al.* 2001). The African Lungfish is not strictly anhydrobiotic since it contains some water internally
Chironomid Midge (*Polypedilium vanderplanki*)	This midge can survive almost complete dehydration without ill effects and its cryptobiotic larvae can tolerate $-270\ ^{\circ}C$ to $+106\ ^{\circ}C$ for up to 17 years (Hinton 1960)
Tardigrades	These organisms are resistant to temperatures near absolute zero and those well above $100\ ^{\circ}C$, and are also resistant to very high hydrostatic pressures and ionic radiation (Rahm 1921; Becquerel 1950)
Nematode (*Ditylenchus dipsaci*)	This plant parasitic nematode can survive exposure to vacuum conditions and possibly exposure to space (Bhatt & Rohde 1970; Wharton 2002)
Resurrection Plants (S. Africa)	These plants can survive exposure to 5% relative water content and can completely recover within 80 hours after a precipitation event (Sherwin & Farrant 1996)
Lotus Plant (China)	This plant holds the record of the oldest seed ever germinated (1288 yrs old) (Shen-Miller *et al.* 1995)

24 hours the plant is restored and is reproductively active within 2 weeks.

Ndima *et al.* (2001) have studied *Xerophyta viscosa*, which is a monocotyledonous resurrection plant that is capable of tolerating extreme desiccation conditions. Upon rewatering, it rehydrates completely and assumes its full physiological activities. They conducted studies on changes in gene expression associated with dehydration stress tolerance. A cDNA library was constructed from mRNA isolated from dehydrated *X. viscosa* leaves. *XVT8* represents one of 30 randomly selected clones that were differentially expressed when *X. viscosa* was dehydrated. Sequence analysis of *XVT8* revealed that it exhibited 45% and 43% identity to dehydrin proteins from *Rabidopsis thaliana* and *Pisum sativum*, respectively, at the amino acid level. *XVT8* encoded a glycine-rich protein (27 kDa), which is largely hydrophilic and contains a hydrophobic segment at the C-terminus. Southern blot analysis confirmed the presence of *XVT8* in the *X. viscosa* genome. *XVT8* transcripts accumulated in *X. viscosa* plants that were exposed to heat, low temperature and dehydration stresses, and to exogenous abscisic acid and ethylene. The high sequence identity observed with known dehydrins, the presence of the conserved serine- and lysine-rich motifs, and the hydropathy analysis data clearly supported the view that *XVT8* is a dehydrin. These results provided direct evidence for the dehydration, low temperature, heat, abscisic acid and ethylene dependent regulation of the *XVT8* gene in *X. viscosa*.

Osmobiosis (*as a special case of anhydrobiosis*)

Osmobiosis is a form of cryptobiosis induced by elevated osmotic pressures. Some intertidal marine species and eury-haline limno-terrestrial species can tolerate variations in salinity. However, most freshwater and terrestrial tardigrades form contracted tuns (barrel-shaped resistant forms) in various salt solutions (Wright *et al.* 1992). Induction of the dormant state by evaporative dehydration is called 'anhydrobiosis' and when water is removed by osmotic dehydration it is called 'osmobiosis' (Perry 1998). Thus osmobiosis can be understood as a special case of anhydrobiosis. It also involves a cessation of metabolism.

Fig. 3. *Artemia franciscana* (from Artemia Reference Center, Ghent, Belgium).

Chen *et al.* (2005) studied the entomopathogenic nematode *Steinernema feltia*. They discovered a novel accumulated protein, GroEL, and also found that the protein GroES was up-regulated in the osmotically desiccated infective juveniles (IJs) compared with non-desiccated IJs under osmotic stress. These proteins may act to repair damaged proteins or assist in the maintenance of homeostasis under osmotic stress. This hypothesis is consistent with the previous observations that GroEL and GroES are members of the Hsp60 family of chaperons (Hennequin *et al.* 2001). The osmobiotic state of the nematodes was associated with the accumulation of a significant amount of stress-related proteins. The results showed that all changes in protein abundance depended on their synthesis and breakdown, or on the up-regulation of the osmotic process. Genomic and proteomic tools need to be employed to decipher the induction process of osmobiosis.

The brine shrimp *Artemia* is the best example of an organism that can withstand large salt contents (Fig. 3). *Artemia* cysts will survive osmotic stress, ultraviolet radiation, temperature extremes and anoxia. Brine shrimps occur more or less in every continent except Antarctica. However, their habitat range is restricted. Since *Artemia* is highly vulnerable to predation, they are found in habitats where ionic concentrations are high enough to avoid fish and other invertebrates. The general habitats include terminal, inland salt lakes and coastal salterns associated with commercial salt production. *Artemia sp.* survive in a wider range of ionic concentrations than any other metazoan (Browne & Mac-Donald 1982). *Artemia*'s survival abilities are enhanced by a protective cyst wall, the accumulation of trehalose and

glycerol, the protein artemin and the heat-stress proteins, which act as molecular chaperons (Wharton 2002). Encysted embryos of the primitive crustacean *Artemia franciscana* are among the most resistant of all multicellular eukaryotes to environmental stress, in part due to massive amounts of a small heat shock/a-crystallin protein (p26). These embryos also contain very large amounts of the disaccharide trehalose, well known for its ability to protect macromolecules and membranes against damage due to water removal and temperature extremes. Drinkwater & Crowe (1991) suggested that the carbohydrate metabolism, which involves the conversion of trehalose to glycerol and is required for hatching, responds to increasing salinity as reported in *Artemia* species. They concluded that increasing amounts of glycerol must be synthesized as salinity is raised.

Anoxybiosis

Oxygen (O_2) is essential for multicellular life on Earth. Anaerobic energy sources can supply the requisite ATP and maintain cellular function for a limited time only, before substrate depletion, energy shortfall or end-product poisoning threaten the survival of the organism. Anoxia tolerance in most vertebrates is short, of the order of minutes, because of the immediate dependence of the heart and central nervous system for a continuous supply of O_2. The brain of most vertebrates consumes a high intrinsic rate of oxygen and as a result the brain is generally the first organ to fail. However, remarkably, some species have adapted to survive several months in the absence of O_2 and can recover their full functionality at the end of this time when O_2 is available again.

Various aquatic species can survive anoxybiosis ranging from a few hours to 5 days. For example, intertidal *Echiniscoides* specimens have been shown to survive for months in vials of sea water with decaying barnacles (Wright *et al*. 1992). Brine shrimp (*Artemia*) cysts have been stored in anoxic conditions for 4 years and then hatched successfully (Wharton 2002). Their food reserves, i.e. trehalose, glycogen and glycerol, were constant during this period, thus indicating a state of anoxybiosis (Wharton 2002). Other reports indicate that embryos of copepods were isolated and viable when retrieved from anoxic marine sediments after 40 years (Marcus *et al*. 1994) and from anoxic freshwater sediments after 332 years (Hairston *et al*. 1995). The resting embryos were assumed to be in the state of anoxybiosis (Clegg 2001). Encysted embryos (cysts) are encased in a hard capsule, or cyst, so that they are protected and can hatch later when conditions are better. Dry conditions cause the encysted embryo to enter a dormant state, which allows it to withstand complete drying, temperatures over 100 °C or near absolute zero, high-energy radiation and a variety of organic solvents. The dehydrated cyst can be stored for months or years without loss of hatchability (Treece 2000; Snell 2005). While larvae are active, feeding stages in the development of many animals occur after birth or hatching and before the adult form is reached. Larvae and adults are motile and cannot reversibly dehydrate.

The ability of a limited number of ectothermal vertebrates and a substantial number of invertebrates to survive long bouts of continuous anoxia is very impressive. These animals are well-adapted to respond to anoxia by reducing their overall metabolic rates, commonly to between 1 and 10 % of aerobic levels (Storey *et al*. 1981; Hand & Podrabsky 2000). This response, called metabolic rate depression, has been well studied in sessile intertidal invertebrates and involves some interesting modifications in intermediary metabolism. Studies on the encysted Gastrula embryos of the brachiopod crustacean *Artemia franciscana* revealed no evidence of an ongoing metabolism during bouts of anoxia, which lasted for periods of years (Warner *et al*. 1997; Clegg 2001). If metabolic activity occurred in *Artemia* cysts while in anoxybiosis, it was at least 50 000 times slower than the aerobic metabolic rate (Clegg 2001). Long-term anoxybiosis of a nematode species has been reported in which the metabolic rate was reduced to undetectable levels for over 3 months of continuous anoxia (Drinkwater & Crowe 1991). Fresh-water sponge gemmules subjected to anoxia at room temperatures in water survived almost 4 months of continuous anoxia and most of these were still alive when the study was terminated (Reiswig & Miller 1998).

Biochemical changes during anoxybiosis

While some adaptation mechanisms only slow down metabolism, others require a change in biochemistry. Massive concentrations of the protein p26 have been found in the encysted embryo stage in *Artemia* species (Jackson & Clegg 1996; Liang & MacRae 1999). The protein p26 underwent extensive stress-induced translocation to nuclei and other sites, and these translocations exhibited strong pH dependence (Clegg 2001) in a fashion consistent with intracellular pH changes *in vivo* (Hand 1998; van Breukelen *et al*. 2000). Although the participation of p26 as a major biochemical adaptation in anoxybiosis is now established (Clegg 2001), its participation in the embryos was anticipated earlier based on work on the guanine nucleotide pool (Stocco *et al*. 1972), which is unusually large and diverse (Warner 1992), suggesting that one of these Gp4G molecules (e.g. P1, P4-diguano-sine 5′-teraphosphate) might be utilized during anoxia (Stocco *et al*. 1972). Clegg (2001) suggested that this nucleotide was indeed metabolized during prolonged anoxia, although extremely slowly. Therefore, it seems that the great majority of intermediary metabolism is indeed brought to a reversible standstill by anoxia, but that a specialized and limited guanine polynucleotide pathway continues to provide free energy for these embryos during anoxia.

Warner and Clegg (2001) found that p26 is a GTP-binding protein and demonstrated that this protein is a GTPase. Anoxic embryos undergo a massive and almost complete metabolic shutdown, but have to support the energetic requirements of their molecular chaperone, p26, which they achieve by mobilizing Gp4G and eventually producing GTP and/or ATP. Other endergonic processes in anoxic embryos may exist as well.

Fig. 4. Crucian carp (*Carassius carassius*; photo: J. Bohdal).

Anoxic-tolerant vertebrates as example organisms using anoxybiosis

In aquatic environments, continuous access to oxygen cannot be taken for granted. Water with hypoxic and anoxic conditions includes both ice-covered lakes and ponds (Ultsch 1989) and tropical freshwater systems (Chapman *et al.* 1995). Vertebrates living in habitats with these conditions have evolved physiological mechanisms to cope with such challenges (Lutz & Storey 1997). These mechanisms often involve supplemental air breathing and the evolution of organs (including the brain), which are adapted to survive with little or no oxygen (Nilsson & Lutz 2004).

Turtles are either very anoxia tolerant or rather anoxia intolerant during anoxic submergence at low temperatures. A prominent example of a turtle extremely tolerant to anoxic conditions is the freshwater turtle, *Chrysemys picta*, a widely distributed resident of ponds and streams in the northern United States and southern Canada. This turtle relies on anaerobic glycolysis as an energy source in the anoxic state. Thus, it has to cope with the intrinsic inefficiency of this pathway, and face the dual challenges of depletion of substrate and accumulation of acid metabolites. The turtle spends long periods during the winter in ice-covered ponds without access to the surface, often in water or mud with little or no O_2 (Ultsch 1989). These turtles can survive continuous submergence in nitrogen-equilibrated water at 3 °C for more than 4 months, as has been reported through a simulated hibernation study in the laboratory (Ultsch & Jackson 1982; Jackson *et al.* 2000). The western painted turtle (*Chrysemys picta bellii*) has been shown to survive 4–5 months of anoxic submergence at 3 °C during which high lactate levels were accumulated that resulted in significant acid-base disturbances buffered by mobilized carbonates from the skeleton. Skeletal tissue was also used as a depot for sequestering lactate (Ultsch 1989).

The Crucian Carp *Carassius carassius* with its habitat in northern Europe is possibly the most extreme example of an anoxia tolerant vertebrate (Fig. 4). This fish inhabits small lakes and ponds of northern Europe, which become anoxic as standing water freezes during winter months and thick ice blocks oxygen diffusion and the light necessary for photosynthesis (Holopainen & Hyvarinen 1985). Anoxic survival capacity in Crucian carp varies greatly with temperature. The Crucian carp can survive anoxia for several months at 0 °C, but only a few days at room temperatures (Nilsson & Lutz 2004). Although some turtles such as those described above can survive without oxygen for long periods, they do so by going into 'suspended animation'. The Crucian carp, however, has evolved in a unique way to remain active during anoxic conditions. When water temperatures are dropping, the carp begins to store vast amounts of glycogen in the brain, which enables it to make the switch to an anaerobic metabolism. Stored glycogen in the body of the carp, mostly in the liver, is then broken down into glucose and ethanol providing the Crucian carp with the energy to survive (Vornanen & Paajanen 2006). The same study also showed that the sodium–potassium pump of the Crucian carp is responsible for keeping brain cells functioning, but that it reduced its activity ten-fold in lower water temperatures, decreasing the amount of energy needed for continued brain activity in anoxic conditions. With respect to ion channels, the Crucian carp appears to maintain a well-regulated ion-homeostasis during anoxia (in contrast to mammals) with only a negligible rise in extracellular [K^+] and intracellular [Ca^{2+}] (Sick *et al.* 1982; Nilsson 2001). With regard to neurotransmitter activity, extracellular GABA (the major inhibitory neurotransmitter in the brain) levels rise in the brain of the Crucian carp (but less than in the turtle), while extracellular glutamate (the major excitatory neurotransmitter) remains low (Hylland & Nilsson 1999).

Thermobiosis

Thermobiosis is a form of cryptobiosis due to high or low temperatures. If the adaptation to cold temperatures involves a mechanism to prevent the formation of ice crystals in the organism, the term cryobiosis is used. For example, Antarctic notothenioids keep from freezing solid by secreting 'antifreeze proteins' that prevent their bodily fluids from turning into ice crystals. The extent to which Antarctic notothenioids have adapted to low, stable temperatures is revealed by their extreme stenothermy (Somero & DeVries 1967; Weinstein & Somero 1998), the presence of antifreeze glycoproteins in their blood (Ruud 1954; DeVries 1988), the loss of haemoglobin (in icefishes, family *Hannichthyidae*; Cocca *et al.* (1995), Ruud (1954)) and myoglobin (Sidell *et al.* 1997), and the use of a variety of other physiological adaptations (di-Prisco *et al.* 1991).

In the Southern Ocean, seawater temperatures rarely rise above about −2 °C, the freezing point of salt water, but the internal fluids of fish freeze at about −1 °C. The water is often filled with tiny ice crystals, which the fish ingest as they eat and which could potentially freeze them from the inside out. Antarctic notothenioid fish survive freezing waters by producing natural antifreeze proteins in the pancreas as
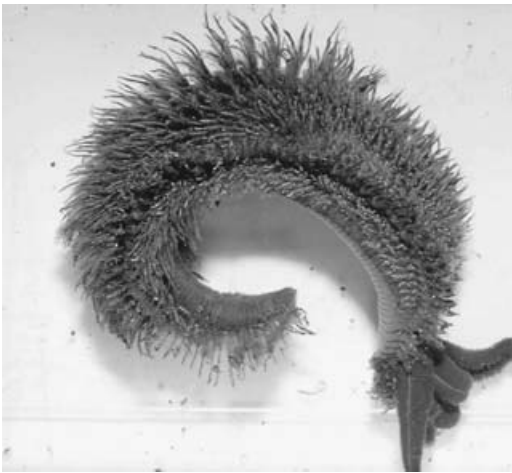
Fig. 5. Dorsal view of a specimen of *A. pompejana*. Filamentous bacteria are associated with cuticular secretions forming dense aggregates extending from the worm's integument (Haddad *et al.* 1995).

recently demonstrated by Cheng *et al.* (2006). The evolution of this adaptation was probably driven by the need to prevent the intestinal fluid from freezing, but has since allowed notothenioids to survive.

Adaptation to hot temperatures

Thermophilic ('heat loving') organisms have an affinity for temperatures above 40.5 °C. Deep-sea hydrothermal vent organisms are often cited as examples of adaptation to extreme environmental conditions (Dalhoff *et al.* 1991; Gaill *et al.* 1991; Desbruye'res *et al.* 1998). Many thermophiles are microbes from the kingdom Archaea; some, however, belong to the other kingdoms and include multicellular organisms. The multicellular organism most often cited with thermobiosis is the Pompeii worm (*Alvinella pompejana*). The annelid *A. pompejana* (Fig. 5) inhabits hot parts of hydrothermal ecosystems with documented occurrences at active vent chimneys from 21°N to 32°S along the East Pacific Rise (Desbruye'res & Laubier 1980; Desbruye'res *et al.* 1985, 1998). In this environment pressures commonly reach 260 bar and temperatures reach 350 °C. In addition, the hydrothermal fluids are acidic, anoxic and rich in metallic sulphides (Gaill 1993). The Pompeii worm is capable of withstanding temperatures as high as 105 °C (Chevaldonne *et al.* 1992), and is known as being the most eurythermal metazoan (Haddad *et al.* 1995). Experiments carried out by Brisa *et al.* (2005) demonstrated that *A. pompejana* influences mineralization processes at the interface between the smoker wall and the ambient oceanic water. The study also indicated that *A. pompejana* exerts a primary control on its environment by structuring the thermal and chemical gradients, creating a mosaic of micro-environments by the construction of protective tubes. The supply of water through the tube prevents exposure to the extreme temperature spikes and high sulphide concentrations. Circumventing the large erratic changes generally associated with hydrothermal venting, *A. pompejana*

tubes create more homogeneous chemical and thermal microniches and likely play a role in microbial diversity.

Collagens are among the most ubiquitous proteins found in the animal kingdom and can be found in sponges as well as in vertebrates (Scandurra *et al.* 2000). Collagens are extracellular proteins with triple-helical domains (vander-Rest & Garrone 1991). Among collagens, the most homogeneous subfamily is that of fibrillar collagens (Wharton & Brown 1991). Fibrillar collagens possess a long central triple-helical domain of 330 to 340 GNN triplets, flanked by an amino-propeptide (N-pro) and a carboxyl-propeptide (C-pro). Collagens are of great importance, not only in performing cohesion between cells, but also in cell differentiation and migration (Wharton & Brown 1991; vander-Rest & Garrone 1991). Whereas the interstitial collagen of coastal polychaete worms (e.g. *Arenicola marina*) is denatured at 28 °C, the collagen of *A. pompejana* remains stable at 45 °C and is thus the most thermostable fibrillar collagen known (Gaill *et al.* 1995). Its collagen is adapted to the hydrothermal vent environment by its stability at higher temperatures (Gaill *et al.* 1995) and high pressures (Auerbach *et al.* 1995), and by its associated enzymatic processes, which appear to be optimized under anoxic conditions (Kaule *et al.* 1998).

The molecular basis of this thermal behaviour includes the increase in proline content and in the number of stabilizing triplets, which correlate with the outstanding thermostability of the interstitial collagen of *A. pompejana*. Proline residues are of primary importance in performing cohesion between the chains of the triple helix of collagens (vander-Rest & Bruckner 1993; Prockop & Kivirikko 1995). Biochemical analysis has so far been unable to explain *Alvinella*'s collagen thermostability (Gaill *et al.* 1995). A molecular genetic approach has been used by Sicot *et al.* (2000), who have cloned and sequenced a large cDNA molecule coding the fibrillar collagen of *Alvinella*, including one-half of the helical domain and the entire C-propeptide domain. For comparison, the group also cloned part of the homologous cDNA from *Riftia* (Pohlschröder *et al.* 1997). Comparison of the corresponding helical domains of these two species, together with that of the sequenced domain of the coastal lugworm *Arenicola marina* (Sicot *et al.* 1997), showed that the increase in proline content and in the number of stabilizing triplets correlates with the outstanding thermostability of the interstitial collagen of *A. pompejana*. Phylogenetic analysis showed that the triple helical and the C-propeptide parts of the same collagen molecule evolve at different rates, in favour of an adaptive mechanism at the molecular level.

Thermobiosis is not limited to hydrothermal vent faunas, but also occurs in terrestrial species. For example, the desert snail *Sphicterochila boisseri* can survive in the desert at temperatures of up to 50 °C (Schmidt-Nielsen 1971) and the desert ant *Proformica longiseta* forages on sand surfaces when temperatures reach as high as 60 °C (Wehner *et al.* 1992).

Adaptation to cold temperatures

The biology of cold tolerance has a long history of scientific study, including pioneering works in the past centuries by

Boyle (1665), Dumeril (1849) and Reamur (1736). The research has diversified into many fields such as cold acclimation, plant and animal cold hardiness, and hibernation studies (Storey *et al.* 1981; Frank 1985; Bowler & Fuller 1987; Kamekura *et al.* 1992). This type of work is also relevant for cryomedical purposes to preserve mammalian organs (Storey *et al.* 1981).

A particular research emphasis for low-temperature adaptations is the study of insects. Insects from temperate, polar and high-altitude environments must adapt behaviourally, physiologically and biochemically to survive potentially lethal subzero temperatures (Danks 1978; Kukal *et al.* 1988; Block 1990) they encounter in their habitat, and a large literature has developed regarding this important component of the life histories of insects (Lee & Denlinger 1991; Sømme 1999). Behavioural adaptations also include subzero avoidance by migration (Lee Jr 1989) or by seeking sheltered microhabitats (Danks 1978, 1991). There are two basic strategies to adapt to cold temperatures. Freeze-tolerant species survive the formation of internal ice, while freeze-avoiding insects die upon freezing, and survive subzero temperatures by keeping their body fluids liquid (Sømme 1999). Despite variations in mechanisms and patterns of cold tolerance within each of these categories (Bale 1996; Sinclair & Sjursen 2001), it is accepted that freeze tolerance and freeze avoidance represent alternative strategies for survival in cold weather (Convey 2000). Therefore, insects encounter subzero temperatures by the use of either one of these strategies (Storey *et al.* 1981; Sinclair & Sjursen 2001), although several species switch between them (Horwath & Duman 1984).

Beetle larvae *D. canadensis* can overwinter as either freeze-avoiding or freeze-tolerant insects; however, most individuals in the population are either one or the other at any given time (Horwath & Duman 1984; Olsen & Duman 1997). In the case of freeze avoidance, *D. Canadensis* maintains a metastable supercooled state. The larval supercooling points decrease from a summer maximum of -2 to $-7\,°C$ to a winter minimum of $-20\,°C$ or lower via the removal of highly active hemolymph and gut fluid ice nucleators along with the inhibition of residual nucleators by antifreeze protein (AFP; (Olsen & Duman 1997)). *Dendroides canadensis* (Pyrochroidae) overwinter as non-diapausing larvae (Horwath & Duman 1983) beneath the bark of decaying deciduous trees. Their hemolymph contains polyols and AFPs that increase seasonally in activity and concentration (Kukal *et al.* 1988; Olsen & Duman 1997) cued by seasonal photo- and thermo-periodic cycles (Horwath & Duman 1983). Their hemolymph thermal hysteresis activity increases from 0.5 °C in summer to 5 °C or greater during midwinter (Kukal *et al.* 1988; Olsen & Duman 1997). Immunofluorescent staining of transverse sections of cold-hardy *D. canadensis* larvae indicated that AFPs are found in the subcuticular epidermal layer (Kukal *et al.* 1988); however, the role of these epidermal AFPs in inoculative freezing resistance is unknown. Olsen & Duman (1997) found that seasonal modifications in epidermal and hemolymph AFPs in *D. canadensis* contributed most strongly to the inhibition of inoculative freezing. Winter

integument patches (cuticle with epidermis) were more resistant to inoculative freezing than were summer integument patches. The results suggested that some integumentary ice nucleators were removed in cold-hardy larvae and that AFP promoted supercooling by inhibiting the activity of these nucleators.

Cryobiosis (*as a special case of thermobiosis*)

Over vast areas of the globe, temperatures drop during the winter season far below the freezing point of water. The formation of ice crystals within cells is uniformly damaging throughout the animal and plant kingdom, and organisms that inhabit seasonally cold areas of the Earth must have well-developed mechanisms for enduring or avoiding long-term exposure to temperatures below freezing (Storey *et al.* 1981). Cryobiosis takes place in reaction to decreased temperature when freezing occurs. In cryobiosis, the organism freezes all of the water within its cells and becomes dormant until more hospitable conditions reappear. It has been indicated through various studies that the longer an organism remains in cryobiosis, the longer it takes for the organism to come out of cryobiosis (Keilin 1959). The organism has to use its own energy sources to come out of cryobiosis and the longer the organism remains in cryobiosis the less energy it has at its disposal. The common organisms of choice to study cryobiosis are tardigrades, which are known to survive freezing to near absolute zero. Cryobiosis, anhydrobiosis and osmobiosis all involve restructuring and/or removal of water from cells, and therefore their metabolic depressant effect may share a common mechanism (Keilin 1959).

Tardigrades as an example of thermobiosis and cryobiosis

Tardigrades are metameric invertebrates and are found worldwide in various habitats such as marine, freshwater and terrestrial ecosystems. The species inhabiting terrestrial habitats are active only when surrounded by at least a film of water. When deprived of their free water, they survive by entering the cryobiotic state. This capability allows tardigrades to colonize extreme environments such as deserts, high mountains and polar regions (Bertolani *et al.* 2004). Together with nematodes and bdelloid rotifers, tardigrades are known to enter cryptobiosis at any stage of their life cycle, from egg to adult (Bertolani *et al.* 2004). Tardigrades overcome adverse conditions also by forming cysts, for example, by reducing their metabolism even less than in the cryptobiotic state (Pigòn & Węglarska 1953), without losing large quantities of water. This capability characterizes several limnic tardigrades, but also some soil and moss-dwelling tardigrades.

Tardigrades resemble closely Onychophora, Euarthropoda and Annelida. However, tardigrades are animals that have small body sizes. The traditional concept is that tardigrades had large body sizes in the ancestor of this common taxon (Articulata; Schmidt-Rhaesa (2001)). If so, the small size and the absence of organs such as a dorsal heart, segmental coelomic cavities and metanephridia must be interpreted as specialized adaptations to their environment (Fig. 6; Schmidt-Rhaesa (2001)). When exposed to cryptobiotic

Fig. 6. Image of a tardigrade using a scanning electron microscope (photo: H. Greven).

stresses, tardigrades contract into a so-called tun, and the permeability of the cuticle declines (Wright 1989; Drinkwater & Crowe 1991). This reduces the rate of transpiration and gives the animal time for synthesis of tissue protectants such as trehalose (Drinkwater & Crowe 1991; Westh & Ramlùv 1991). These protectants replace the bound water in membranes and allow structural cell integrity to be maintained in completely dry or frozen organisms (Drinkwater & Crowe 1991; Clegg 2001).

In order to investigate cryobiotic effects, Bertolani *et al.* (2004) conducted a laboratory study with four species of eutardigrades belonging to two families and living on different substrates. Two species belonged to the family Hypsibiidae, *Ramazzottius oberhaeuseri* (lichen-dwelling) and *Hypsibius dujardini* (fresh water-dwelling); the other two species belonged to the family Macrobiotidae, *Dactylobiotus parthenogeneticus* (fresh water-dwelling) and *Macrobiotus richtersi* (leaf litter-dwelling). Active animals were frozen at different temperatures (-9, -20 and -80 °C), kept frozen for 6 days, placed at -9 °C overnight and then gradually thawed to 12 °C. Survival was evaluated 24 hours after the beginning of thawing. The four species considered showed very different patterns of cryobiotic survival with the terrestrial species *R. oberhaeuseri* and *M. richtersi* showing the highest survival rates. Interestingly, the survival rates of *R. oberhaeuseri* showed very little dependence on the variation of the experimental temperatures.

Many other organisms exist that use cryobiotic adaptation mechanisms (Table 2). These adaptation mechanisms allow these organisms to persist in environments that most other species are excluded from.

Hibernation

Hibernation is not a cryptobiotic state, because metabolism is maintained through the process but, owing to its importance to more complex organisms in particular, it will be discussed here. Hibernation is a state of inactivity in an animal lasting several days or weeks that allows animals to conserve energy during the long cold winter season when food is scarce or just difficult to find. The ability to hibernate is the most dramatic example of phenotypic plasticity displayed by mammals.

Each year mammalian hibernators undergo a complex suite of morphological, physiological and behavioural changes in response to seasonal periods of high-energy demand coupled with reduced energy availability in the environment. The adaptive value of the hibernating phenotype is realized by eliminating the need to maintain a constant, high body temperature by entering torpor.

During hibernation, the body temperatures of mammalian hibernators drop to below 10 °C or even 5 °C, and the metabolic rate is reduced to only a few percent of the euthermic level. Heart rate and breathing rate also fall. However, unlike the lower vertebrate and invertebrate hibernators, mammalian hibernators maintain the thermoregulatory control with a lowered set point during hibernation and retain the capacity to arouse from hypothermia by physiological means. Mammalian hibernation accompanies various physiological changes (Wang 1989). Several histological and morphological studies reveal that polyglandular involution takes place prior to the onset of hibernation and that endocrine functions are generally depressed during the first half of the hibernation season (Wang 1982). Also, the lymphoid organs undergo an involution during Fall, and the immune system is depressed during hibernation (Shivatcheva 1989). A number of molecular and cellular changes that occur during the preparation, entrance and torpor stages of hibernation have been defined (MacDonald & Storey 1999; O'Hara *et al.* 1999). The mRNA translation process is conventionally divided into three distinct phases termed (peptide-chain) initiation, elongation and termination (Proud 1992). The global rates of protein synthesis have been determined to be profoundly reduced in hibernating animals. The decreased rate of protein synthesis is due to inhibition of both initiation and elongation (Frerichs *et al.* 1998).

The mechanism that contributes to the regulated suppression of metabolism and thermogenesis during hibernation is reversible phosphorylation of enzymes and proteins that limits rates of flux through metabolic pathways (Storey *et al.* 1981). The acute regulation of mRNA translation depends on changes in the activity of initiation factors or elongation factors, which is also commonly mediated by alteration in their states of phosphorylation (Hovland *et al.* 1999). A substantial decrease in the rate of protein synthesis initiation in hibernators is contributed by phosphorylation of eIF2α (Frerichs *et al.* 1998); however, the mechanism(s) involved in the regulation of elongation in hibernators is not known (Chen *et al.* 2001). mRNA translocation on the ribosome involves elongation factor-2 (eEF-2). eEF-2 is phosphorylated and inhibited by a specific kinase (eEF-2 kinase), with phosphorylation reducing the ability of the factor to promote translocation, possibly by decreasing its affinity for pretranslocation ribosomes (Nairn & Palfrey 1987; Ryazanov & Shestakova 1988; Redpath & Proud 1989; Carlberg *et al.* 1990). Increased levels of phosphorylation of eEF-2 occur in response to cellular stimulation by mitogens, growth factors and neurotransmitters that influence intracellular Ca^{2+} levels (Redpath *et al.* 1996; Ryazanov & Shestakova 1988). Results provided by Chen *et al.* (2001) suggest that the level of eEF-2

Table 2. *Some example organisms using cryobiosis as a survival mechanism*

Organism	Survival ability
Tardigrades	These organisms are resistant to temperatures near absolute zero and those well above 100 °C, and are also resistant to very high hydrostatic pressures and ionic radiation (Rahm 1921; Becquerel 1950)
Ice Worm (*Mesenchytraeus solifugus*)	This worm completes its life cycle at about 0 °C exclusively on glacier ice/snow in Alaska (Farrell *et al.* 2004)
Periwinkle (*Littorina littorea*)	This plant survives at temperatures down to -22 °C with 76% of its body water converted to ice (Wharton 2002)
Woolly bear caterpillars (*Gynaephora groenlandica*)	The larvae of this caterpillar are freeze tolerant to at least -70 °C (Kukal *et al.* 1988)
Fruit fly (*Chymomyza costata*)	These fly larvae survive temperatures below -100 °C. Frozen larvae are able to survive at -196 °C in liquid nitrogen for at least 1 hour (Shimada 1995; Moon *et al.* 1996)
Arctic fly (*Rhabdophaga strobiloides*)	*Rhabdophaga* larvae can supercool to -62 °C indicating that much of their body water is physiologically bound and therefore unfreezable (Storey *et al.* 1981)
Nematodes (*Panagrolaimus davidi*)	These organisms can survive freezing in water to temperatures as low as -80 °C (Wharton & Brown 1991)
Skunk cabbage (*Symplocarpus foetidus*)	The flowers of this plant maintain a temperature of about 20 °C even though the air temperature drops as low as -14 °C (Knutson 1974)

phosphorylation is increased in hibernators through both an increase in eEF-2 kinase activity and a decrease in PP2A activity. However, the precise physiological role of eEF-2 phosphorylation has not been elucidated.

Changes in gene expression

Hibernation's unique characteristics as a phenotype probably constrain the types of molecular changes that are employed. Seasonal changes prepare the animal for hibernation; one example is the storage of enormous reserves of energy in the form of white adipose tissue (WAT) in late summer and early autumn. These seasonal changes may be regulated less by changing the amounts of gene product via altered transcription, translation or stability, and more by employing rapidly reversible molecular switches such as phosphorylation to control protein activity (Storey *et al.* 1981). Because the hibernating mammal arouses periodically from torpor throughout the hibernation season, the ability to function at euthermic temperatures must be maintained continuously. At the molecular level, this indicates that the steady-state levels of mRNA and protein expressed from the majority of genes will remain relatively constant between the active and hibernating seasons. A relatively small number of genes that perform crucial functions for hibernation will be up-regulated during winter, whereas other gene products that are not essential during hibernation may be down-regulated. Several mRNAs are differentially expressed during hibernation. An example of a seasonally up-regulated gene at the mRNA level in hibernators is -2-macroglobulin, which encodes a broad-spectrum protease inhibitor that is produced in the liver and then secreted into the plasma (O'Hara *et al.* 1999). Both its mRNA and protein were shown to increase during winter in ground squirrels. This protein plays a pivotal role in controlling blood clotting. It is well known that clotting times of blood taken from hibernators are significantly increased (O'Hara *et al.* 1999). Because improving microcirculation in hypothermic rats (Lee *et al.* 1996) and preventing or reversing blood clotting in cardiac arrest patients (Bottiger & Martin 2001) enhances survival, reduced clotting is likely to be a

significant adaptation for hibernation. In the liver, the genes encoding thyroxine-binding globulin, apolipoprotein A1 and cathepsin H are also up-regulated at the mRNA level during the hibernation season (Epperson & Martin 2002). The mRNAs for the immediate-early genes, c-*fos*, c*jun* and junB are differentially expressed in the brain of ground squirrels across the hibernation cycle (O'Hara *et al.* 1999). This probably reflects the established link between neuronal activation and immediate-early gene expression (Christman *et al.* 1985). The switch from carbohydrate to fatty acid metabolism is regulated by differential gene expression at multiple levels, including altering the steady-state levels of key proteins and the mRNAs encoding them. The general pattern is to suppress carbohydrate metabolism and increase fatty acid metabolism. Elevated levels of pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) mRNA and protein in hibernating ground squirrels suppress glycolytic activity (Christman *et al.* 1985; Buck *et al.* 2002). Pancreatic triacylglycerol lipase (PTL), which hydrolyses ester linkages in dietary triacylglycerols and is normally expressed exclusively in the pancreas, is also expressed at high levels in the heart of hibernating ground squirrels, at both the mRNA and protein levels (Andrews *et al.* 1998). Glyceraldehyde-3-phosphate dehydrogenase is down-regulated at the mRNA, protein and activity levels in skeletal muscle of hibernating jerboas (Soukri *et al.* 1996), as is acetyl CoA carboxylase in the hearts of hibernating ground squirrels (Belke *et al.* 1998). Other reports of up-regulated mRNAs in hibernating ground squirrels include: uncoupling protein 2 (UCP2) in WAT and UCP3 in skeletal muscle (Boyer *et al.* 1998); heart-type fatty acid binding protein (FABP) in brown adipose tissue (BAT), skeletal muscle and heart (Hittel & Storey 2001); adipose type FABP in BAT and heart; cytochrome-*c* oxidase subunit 1 and ATP synthase 6/8 in kidney, BAT and heart; NADH-ubiquinone oxidoreductase subunit 2 in heart, skeletal muscle and liver; and ventricular myosin light chain 1 in heart and skeletal muscle (Fahlman *et al.* 2000). Down-regulated mRNAs during hibernation have also been reported, including: those for prostaglandin D2 synthase in the brain of

Fig. 7. Arctic Ground Squirrel (*Spermophilus parryii*), an ideal object to study hibernation in mammals. During hibernation ground squirrels typically spend 80–90% of the hibernation season at body temperatures below 5 °C (Wang 1978), although due to periodical interruption body temperatures reach above 35 °C. The metabolic rate of hibernating mammals is less than 6% of corresponding normal thermic values, which saves energy considerably (Geiser & Ruf 1995; Song *et al*. 1997; Kortner & Geiser 2000) (image from http://www.nhptv.org/natureworks/ arcticgroundsquirrel.htm with permission of Nature Works, New Hampshire Public Television).

ground squirrels (O'Hara *et al*. 1999); glyceraldehyde-3-phosphate dehydrogenase in the muscle of jerboas (Soukri *et al*. 1996); and liver-expressed HP-22, -25, -27 and -55, which form a protein complex in chipmunk plasma and which are also decreased in hibernation (Takamatsu *et al*. 1993, 1997). Recent work on the plasma proteins HP-25 (Kojima *et al*. 2000, 2001), HP-20 (Ono *et al*. 2001) and HP-27 (Ono *et al*. 2003) has focused on the liver-specific regulation of their expression at the transcriptional level.

At the protein level, myoglobin appears to increase in skeletal muscle as animals prepare for and undergo hibernation. This increase may facilitate shivering thermogenesis during torpor and arousal (Postnikova *et al*. 1999). Immunoreactivity for the gap junction protein connexin43 is elevated in cardiac myocytes of hibernating hamsters and reverses to euthermic control values within 2 hours of arousal to euthermy (Saitongdee *et al*. 2000). It was suggested that an increase in gap junctions might prevent ventricular fibrillation at the low body temperature values of torpor. However, because connexin43 immunoreactivity is elevated in cold-acclimated control hamsters that did not hibernate (Saitongdee *et al*. 2000), this change is not strictly associated with the hibernating phenotype.

Histamine (HA) is a multifunctional messenger that acts as a neurotransmitter or neuromodulator in the brain (Schwartz *et al*. 1991; Wada *et al*. 1991). In mammals, histaminergic perikarya are located in the tuberomammillary nucleus of the posterior hypothalamus, projecting fibres to almost all parts of the brain (Airaksinen & Panula 1988; Inagaki *et al*. 1988;

Panula *et al*. 1989). HA has been implicated in the regulation of the sleep–wake cycle, body temperature, energy metabolism, reproductive behaviour and neuroendocrine secretion (Schwartz *et al*. 1991). Hibernation is a physiological state and is under strict neuronal control involving the hypothalamus, septum, hippocampus and brainstem reticular formation (Beckman & Stanton 1982; O'Hara *et al*. 1999).

The fact that at least one species of mammalian hibernator, the arctic ground squirrel, maintains subzero body temperature values during deep torpor raises the possibility that specialized antifreeze proteins similar to those found in Antarctic fishes might be expressed during hibernation that would prevent freezing of body fluids at body temperatures below 0 °C (Fig. 7). To date, however there is no evidence for antifreeze activity such as thermal hysteresis in blood from supercooled arctic ground squirrels (Barnes 1989). Other mammals that are specially adapted to very cold temperatures are the Arctic fox, which can sleep on open snow at temperatures as low as −80 °C for up to 1 hour without decreasing its body temperature (Wharton 2002), and the Antarctic springtail (*Gomphiocephalus hodgsoni*), which can live at temperatures below −39 °C in the Antarctic environment (Sinclair & Sjursen 2001).

Diapause

Diapause is a common feature of the life cycle of many animal species (Lees 1955; Yamashita & Hasegawa 1985; Marcus 1996). The significance of diapause includes the acquisition of mechanisms that led to resistance to environmental stress or as a timing device to synchronize favourable environmental states with an appropriate stage of development or both (Sandell 1990; Mcnamara 1994). Diapause is a dynamic state of low metabolic activity that is neurohormonally mediated. Reduced morphogenesis, increased resistance to environmental extremes and altered or reduced behavioural activity is associated with diapause. It occurs during a genetically determined stage of metamorphosis, and its full expression develops in a species-specific manner, usually in response to a number of environmental stimuli that precede unfavourable conditions. Upon beginning of diapause, metabolic activity is suppressed even if conditions favourable for development prevail.

Discussion

Most scientists associate micro-organisms with the word extremophile, particularly Archaea. However, there are many multicellular and macroscopic organisms that are well adapted to extreme environmental conditions. Here we have reviewed some of the processes used by animals and plants to adapt to extreme environmental stresses such as temperature extremes, lack of oxygen and water, and high salt conditions. We barely understand the responses to these processes, the associated biochemical changes, genetic switches and what leads to the induction of the cryptobiotic state or hibernation. Perhaps the largest strides have been made in the research

about hibernation. Most of the hibernation research has been geared towards mammals, which shows our own bias as humans towards our own heritage and also the economic potential for biomedicine for the freezing and preservation of organs and organ tissue. Even here, however, we only have an incomplete picture about what induces hibernation and the associated biochemical changes and genetic switches to maintain it during torpor.

This research has great significance for astrobiology as it does put in question the common assumption that increased complexity has necessarily to result in an increased sensitivity to environmental changes and extremes. As some of the multicellular extremophiles discussed here clearly underline, this does not necessarily have to be the case. Although microbes are the ultimate masters of adaptation owing to techniques such as gene swapping, transposition, transformation, etc, increases in complexity may in certain cases actually provide increased opportunity for adaptation. These opportunities have been utilized in a broad spectrum of organisms ranging from plants to animals and from rather simplistic organisms such as worms to highly complex organisms such as mammals. While the exploration of life is probably limited to microbial forms on our neighbouring planets and moons, understanding the adaptation mechanisms would enhance our ability to make planets such as Mars suitable for the habitation of terrestrial life forms (if we ultimately decide to pursue terra-forming concepts). The multicellular extremophiles on Earth also represent the best analogue for the study of possible forms and adaptation mechanisms employed by multicellular extraterrestrial organisms. This may even be relevant for intelligent life as many of the larger mammals (e.g. the omnivorous northern bears such as the brown bear, black bear and Grizzly bear) have the capability to hibernate during the cold winter periods and show incredible adaptation capabilities to their environment. In essence, even the human species *Homo sapiens* has adapted quite well to an extreme range of environments, which includes the temporary habitability of space. *Homo sapiens* achieved that feat by using a strategy not employed by any other species on our planet: manual dexterity and intelligence culminating in technology.

Conclusions

Adaptation to extreme environmental stresses not only occurs in microbes, but it also occurs in many multicellular and macroscopic organisms. Increases in complexity within an organism do not necessarily imply an increased sensitivity to environmental stresses, but, in fact, can lead under certain circumstances to new opportunities and adaptation strategies. Examples are organisms such as certain species of fish that can survive without oxygen for months without giving up brain activity and resurrection plants that can survive near-complete desiccation. These organisms are clearly relevant as an analogue for possible complex extraterrestrial life as well as for terraforming planets or moons in our solar neighbourhood.

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