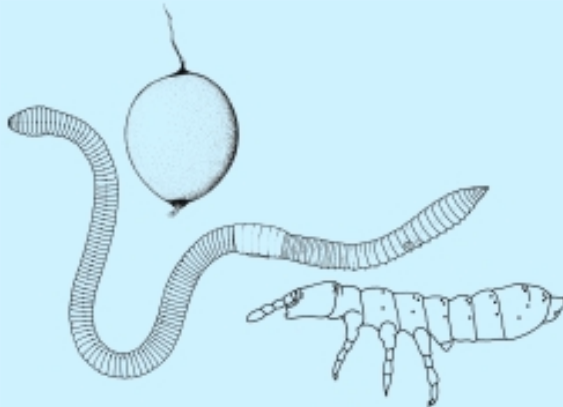**National Environmental Research Institute**
Ministry of the Environment · Denmark

Strategies for cold and drought tolerance in permeable soil invertebrates

Doctor's dissertation (DSc)

Martin Holmstrup

Denne afhandling er af Det naturvidenskabelige Fakultet ved Aarhus Universitet antaget til forsvar for den naturvidenskabelige doktorgrad

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Doctor's dissertation (DSc)
2002

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Data sheet

Abstracts:	The distribution and functioning of soil invertebrates is above all dictated by climatic factors such as temperature and water availability. In this doctor's dissertation including 23 original papers the problems of how soil animals are adapted to cold and drought are addressed. Using hygrophilic collembolans and earthworms as models, a cold hardiness strategy is described which is not based on the well-known ability to supercool, but on a dehydration mechanism that enables the animal to adjust the body fluid melting point to the temperature of the surroundings. Also the drought tolerance of Collembola is examined, and a mechanism is demonstrated by which these animals can adjust their osmotic pressure to remain active within the limits of water potentials tolerable to plants, and absorb water vapour from the soil atmosphere. Chemical pollution originating from the activities of humans should also be considered as an important environmental factor. Pollution in itself can have negative effects on the functioning of soil organisms. In the present work it is investigated if and how pollution can have an impact on the defence mechanisms against climatic stress such as drought or cold.

Contents

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Preface

This doctor's dissertation is an outline of 23 selected papers addressing stress tolerance in soil invertebrates. The dissertation was submitted in January 2002 to The University of Aarhus and accepted for defence December 4, 2002.

Dissertation papers

The dissertation includes the papers listed below and references are made to the numbers listed.

Page

1. Holmstrup, M, (1994) Physiology of cold hardiness in cocoons of five earthworm taxa (Lumbricidae: Oligochaeta). J. Comp. Physiol. B 164:222-228. 41-47

2. Holmstrup, M, & Westh, P, (1994) Dehydration of earthworm cocoons exposed to cold: a novel cold hardiness mechanism. J. Comp. Physiol. B 164:312-315. 48-51

3. Holmstrup, M, (1995) Polyol accumulation in earthworm cocoons induced by dehydration. Comp. Biochem. physiol. 111A:251-255. 52-56

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7. Holmstrup, M, & Petersen, B, F, (1997) Freeze-tolerance in the subarctic earthworm *Eisenia nordenskioeldi* (Eisen). CryoLetters 18:153-156. 83-86

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13. Bayley M, Petersen SO, Köhler K, & Holmstrup M, (2001) Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. J.Insect Physiol. 47:1197-1204. 117-124

14. Sjursen H, Bayley M, & Holmstrup M, (2001) Enhanced drought tolerance of a soil-dwelling springtail by pre-acclimation to mild desiccation stress. J. Insect Physiol. 47:1021-1027. 125-131

15. Holmstrup M, Bayley M, Sjursen H, & Ravn H, (2001) Dehydration tolerance and water vapour absorption in two species of soil-dwelling Collembola by accumulation of sugars and polyols. Functional Ecology 15:647-653. 132-138

16. Holmstrup M, & Sjursen H, (2001) Freeze induced glucose accumulation in the enchytraeid, *Fredericia ratzeli*, from Greenland. CryoLetters 22:273-276. 139-142

17. Holmstrup M, Ramløv H, & Bayley M, (2002). Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable arctic invertebrates. Proc. Natl. Acad. Sci. USA. 99:5716-5720.

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21. Friis K, Damgaard C, & Holmstrup M, (in press) Sublethal soil copper concentrations increase mortality during drought in the earthworm *Aporrectodea caliginosa*. Ecotoxicology.

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The following two papers have been added after the evaluation committee finalised the reviewing process.

22. Holmstrup M, Hedlund K & Boriss H, (2002) Drought acclimation and lipid composition in *Folsomia candida*: implications for cold shock, heat shock and acute desiccation stress. J. Insect Physiol. 48: 961–970.

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Introduction

Ecological significance of soil invertebrates

Soil invertebrates are important components of soil ecosystems worldwide, with the exception of deserts and land permanently covered by ice. This diverse group of animals covers a range of taxa, the most important being protozoans, nematodes, oligochaete worms (earthworms and enchytraeids), mites, springtails (Collembola), millipedes, centipedes, and a range of insects (mostly belonging to Diptera and Coleoptera) whose larval stages complete their development in the soil. Soil invertebrates perform important functions in soil related to the growth conditions of plants. Even though this was realised a long time ago (e.g. Darwin 1881), it is in the last 30-40 years that a large body of evidence of this has accumulated demonstrating the importance of soil fauna for soil structure improvement, nutrient cycling, and organic matter decomposition. Thus, engineering species such as earthworms can by their tunnelling through the soil increase soil porosity and average pore size (Edwards and Shipitalo 1998). These animals ingest considerable amounts of soil and dead plant material, thereby contributing to the mixing of organic matter and mineral soil. This improves aggregate stability and increases the surface of organic material so that it is more readily colonised and decomposed by soil bacteria and fungi (Parmelee *et al.* 1998; Lavelle *et al.* 1997). Soil mesofauna, for example represented by enchytraeids, mites and Collembola, are grazers of bacteria and fungi which are thought to stimulate microbial decomposition rates, even though they may reduce the standing crop of microbial biomass (Ineson *et al.* 1982; Seastedt 1984; Anderson 1988; Didden 1993; Hedlund and Öhrn 2000). At present there seems to be a consensus that the respiration of soil invertebrates amounts to little more than 10% of the total soil respiration (bacteria and fungi being the dominant biotic components), and often less than this. However, the indirect effects on soil respiration and decomposition, though difficult to quantify, may be substantial (Petersen & Luxton 1982; Verhoef & Brussard 1990; Beare *et al.* 1992; De Ruiter *et al.* 1993). A number of examples show that soil fauna increase nitrogen mineralisation with up to 25% (Persson 1983; Seastedt 1984; Verhoef and Brussard 1990).

In their comprehensive analysis of soil fauna populations from a variety of biomes Petersen and Luxton (1982) report average biomass estimates ranging between 2 and 8 g dry weight m^{-2} (total soil fauna). This places soil invertebrates as one of the dominant animal groups of terrestrial ecosystems, having higher biomass on an area basis than for example above ground herbivorous insects or vertebrates (Odum 1971 and references therein). Soil invertebrates represent, with their relatively high protein content, therefore a significant pool of nutrients such as nitrogen, which may ultimately become available for primary production. Soil invertebrates are also an important player in terrestrial food webs. They are an important food source for many predacious epigeic arthropods and vertebrates (Cooke *et al.* 1992; Whitehead *et al.* 1996; Leinaas & Ambrose 1999; Marcussen *et al.* 1999; Bilde *et al.* 2000; McNabb *et al.* 2001).

Stress tolerance

Environmental stress has played and still plays a significant role in the evolution and geographical distribution of species. According to Hoffmann and Parsons (1991) environmental stress may be defined as an "environmental factor causing a change in a biological system, which is potentially injurious". Broader or more narrow characterisations of stress have been introduced in the literature, but Hoffmann and Parsons' definition seems appropriate for the present purpose. Climatic stresses are environmental factors of paramount importance for biological systems. Numerous examples from terrestrial plants and animals show that in particular cold and drought are factors that may dictate the distribution of species (for a review see Hoffmann & Parsons 1991). Although it can be argued that biotic interactions such as competition or predator-prey relationships (e.g. Davis 1998ab) may be of significance, it appears that climate in general is a much more dominant factor (see for example discussion in Hodkinson 1999).

Seasonal departures away from optimal temperature and moisture conditions are foreseeable by organisms. One way to cope with seasonal environmental stress is to respond in a behavioural way and evade a stress, either by migration or by entering a dormant stage until favourable conditions are re-established. However, unfavourable climatic conditions do not necessarily occur in a predictable way. In fact, acyclic climatic stress events may occur both on an ecological and evolutionary time scale. In the extreme cases this may result in ecological crises where selection for tolerance mechanisms is intense. New types of environmental stress that should also be considered are the increasing levels of chemical pollution (e.g. pesticides and heavy metals) that have been inflicted on the biosphere at a global scale. Pollution in itself can have negative effects on the functioning of organisms, but in the present perspective it is also interesting

to study if and how pollution can have an impact on the defence mechanisms against climatic stress such as drought or cold. One classic example is the effects of acid rain and ozone where reduced cold and drought tolerance plays an important role in tree death (reviewed by McLaughlin & Percy 1999).

Many organisms have evolved sophisticated physiological and biochemical mechanisms to cope with different environmental stresses. Examples of such mechanisms include heat shock proteins to counteract thermal and other stresses (Feder & Hofmann 1999), detoxication systems (Walker *et al.* 1996), and the use of compatible solutes to counteract the deleterious effects of osmotic stress, dehydration or freezing (Yancey *et al.* 1982; Crowe *et al.* 1992; Storey 1997). Understanding the nature of physiological and biochemical adaptations of organisms to extreme environments is important for the understanding of the ecology and evolution of organisms. This field of science – ecological physiology or ecophysiology – has a relatively long history, and an impressive literature has built up.

The dissertation I present here is a synopsis of investigations on the physiology of cold and drought tolerance in soil invertebrates. Even though such topics have been intensely studied in the past, I have revisited some of these issues and tried to approach them with new ideas. These studies (with the aid of colleagues and students) have revealed new and exciting discoveries of invertebrate ecophysiology and phenomena that at the first glance may seem trivial.

This dissertation

The subject of this dissertation is cold and drought tolerance in a group of soil invertebrates that are characterised by having very limited cuticular resistance to desiccation. Using hygrophilic collembolans and earthworms as models, I will describe a cold hardiness strategy which is not based on the well-known ability to supercool, but rather on a dehydration mechanism that enables the animal to adjust the body fluid melting point (MP) to the temperature of the surroundings. I will also examine the drought tolerance (at summer temperatures) of Collembola, and demonstrate a mechanism by which these animals can adjust their osmotic pressure elegantly to remain active within the limits of water potentials tolerable to plants, and even absorb water vapour from the soil atmosphere. Drought tolerance in earthworms will also be treated. These issues form the core of my research and this dissertation, treating aspects in the boundary between physiology and studies of performance of individuals. Around this central research theme, there have been various excursions to further elucidate these matters. This has resulted in many interesting observations, but more questions have arisen than conclusive explanations of the central problem.

As will become apparent in the following sections, for these small hygrophilic animals, drought and cold cause, to a large degree, similar physiological problems associated with water balance. Special attention is therefore paid to the common features of these two stress types and the animal's responses. Connections to plant physiology will be drawn since these issues have been the concern of plant physiologists to a much greater degree than animal physiologists. Lastly, I will address the problems of combined stresses. One of my particular interests has been the combination of climatic stress and toxic stress, a field of research that may be of importance in the light of the global changes we are currently facing.

Cold hardiness strategies of ectothermic animals

Nature of freezing injuries

Cold hardy ectothermic animals are traditionally thought to have developed two different strategies for survival of sub-zero temperatures (reviews: Zachariassen 1985; Block 1990; Lee 1991; Ramløv 2000). The freeze avoiding species, which die if frozen, depend on supercooling of their body fluids, whereas a second strategy is deployed by the freeze tolerant species that are able to tolerate freezing of their extracellular body fluids. Intracellular freezing is generally considered to be lethal (Asahina 1969; Zachariassen 1985) although a few examples of tolerance of intracellular freezing do exist (Lee *et al.* 1993; Wharton & Ferns 1995). However, at the relatively high sub-zero temperatures organisms will encounter in nature, cells will usually supercool, and inoculation from extracellular ice is unlikely to occur because ice crystals are probably unable to penetrate the cell membrane at these temperatures (Mazur 1963; Asahina 1969). Moreover, when extracellular freezing occurs, the concentration of the non-frozen extracellular fluid fraction increases (since only pure water forms ice) resulting in an osmotic flux of water out of the cells. At normal cooling rates, water loss from cells due to extracellular freezing will be sufficient to allow the melting point of intracellular fluids to equilibrate with that of extracellular fluids and thus prevent freezing of cells (Mazur 1963).

As stated, freezing of body fluids is lethal to freeze avoiding species, if ice formation goes to an equilibrium. Several so-called freeze avoiding species may survive some ice formation if warmed before the freezing process is complete and the body temperature reach equilibrium with the environment, a phenomenon that has been termed "partial freeze tolerance" (Todd & Block 1995; Sinclair 1999; Ansart *et al.* 2001). Even freeze-tolerant species may die from extracellular ice formation if the fraction of ice becomes too large, i. e. the temperature becomes too low. Injury due to freezing may be caused by temperature per se (Ramløv 2000), but may also be a consequence of freezing-induced dehydration of tissues (Zachariassen 1985). Thus, cells may be injured by high concentrations of salts and other deleterious molecules causing destruction of cell macromolecules upon rehydration (Lovelock 1953). Another explanation is that shrinking cells will reach a "critical minimum cell volume", at which the cell matrix is too packed to allow further shrinking. This may cause rupture and/or fusion of membranes and thus cell disintegration (Meryman 1971; Loomis 1991). Low temperature in itself can, however, also cause damages that

ultimately may become lethal. This may be due to phase transitions in membranes and denaturation of proteins that will lead to malfunctions such as altered permeability of membranes for ions, or inactivation of enzymes (for a recent review, see Ramløv 2000). Such "pre-freeze" mortality or cold shock injury is known from many insects from warmer regions, e.g. aphids and fruitflies (Knight & Bale 1986; Czaika & Lee 1990).

Cold hardy invertebrates have developed a number of physiological and biochemical mechanisms to tolerate freezing (freeze-tolerant species), or to avoid freezing (freeze avoiding species). These adaptations will be described in the following two sections.

Freeze-tolerant ectotherms

The strategy of freezing tolerance is found in many different organisms. It has been recorded in nematodes (Wharton and Block 1993), tardigrades (Ramløv & Westh 1992), molluscs (Aarseth 1982), oligochaetes (**7, 9, 16, 23**; Berman & Leirikh 1985), insects (Zachariassen 1985; Lee 1991), and in frogs and salamanders (Schmid 1982; Storey & Storey 1992). The freeze-tolerant organisms are generally able to establish a controlled, protective freezing of their extracellular fluids at high sub-zero temperatures, although freeze tolerant species with low supercooling points (SCP) have been recorded (Miller 1982; Ring 1982).

As the temperature of an aqueous solution is lowered, freezing typically does not occur at the equilibrium freezing point. Rather, the solution will for some time remain in a metastable supercooled liquid state. For freezing to occur, water molecules need to be aggregated in an ice-like pattern, an ice-nucleus, which may cause freezing of the remaining water when the nucleus grows to a sufficient size. Ice-nuclei can be ice crystals, food- or mineral particles, or ice-nucleating agents (INA) produced by the organism. It has been shown that most freeze-tolerant invertebrates during cold hardening accumulate extracellular INAs with potent ice-nucleating abilities (Zachariassen & Hammel 1976; Zachariassen 1982; Duman 2001). Another important way of freezing to occur is inoculative freezing (e.g. Lee 1991). As will be discussed later, inoculative freezing is of particular importance for many larger soil animals because they are often overwintering in close contact with ice. Either way, extracellular freezing induced at high sub-zero temperatures prevents extensive supercooling of body fluids and thus a very rapid formation of ice, which could be lethal.

Since freeze-tolerant animals undergoing freezing face the problems of dehydration of cells, they require physiological and/or biochemical mechanisms to alleviate dehydration damage. This protection is generally acquired by the accumulation of cryoprotectants such as sugars and polyols (SP) (e.g. glycerol, sorbitol, mannitol, trehalose, glucose) (Zachariassen 1985), but amino acids also may have cryoprotective ability (Loomis 1991; Ramløv 1999). These cryoprotectants may occur alone or in combinations. The most widespread cryoprotectant is clearly glycerol (Storey & Storey 1991). There seem to be at least two general mechanisms of protection by these low molecular weight substances. Firstly, their osmotic properties will decrease the speed of ice formation and lower the fraction of frozen water at equilibrium. In addition cryoprotectants will dilute the potentially toxic solutes (salts) by colligative means (Lovelock 1953). Glycerol readily penetrates the cell membrane and will therefore have the same effect inside and outside cells. Accumulation of glycerol (or other low molecular weight substances) will reduce the amount of ice in the extracellular compartments. The freeze-concentration of unfrozen extracellular fluids is therefore reduced, which will minimise water loss of cells and thus reduce the probability that the "critical minimum cell volume" is reached. A second cryoprotective mechanism seems to be that membrane and protein structures are stabilized during dehydration by e.g. glycerol, glucose and especially trehalose (Gekko & Timasheff 1981ab; Clegg 1986; Crowe & Crowe 1986; Crowe et al. 1992). This hypothesis termed "the water replacement hypothesis", states that polyhydroxyl compounds replace the structural water of cellular components as intracellular water levels are reduced, thereby preventing fusion of membranes and denaturation of proteins (Clegg 1986). Freeze-tolerant terrestrial arthropods seem generally to have a preparative accumulation of cryoprotectants. In contrast to this, semi-terrestrial animals (frogs, salamanders and oligochaetes) will not accumulate SPs until freezing of body fluids is taking place (Costanzo et al. 1993). This is likely to be an adaptive feature, because a preparative accumulation of cryoprotectants in significant concentrations would cause an osmotically driven uptake of water over the skin of these animals (**9**).

Not all water in an organism is freezable. A certain fraction of the body water is so-called osmotically inactive water (OIW) i.e. it does not act as a solvent for low-molecular-weight solutes in the body fluids. The OIW is likely to be the water that is perturbed by cellular (e.g. membranes) and macro-molecular (e.g. proteins) components in such a way that it is not readily freezeable, at least not under short-term (ecological) conditions. The role of OIW in freeze-tolerant invertebrates has been much debated (Lee 1991). However, there does not seem to be any clear relationship between the fraction of OIW and the degree of freeze-tolerance (Zachariassen et al. 1979; Zachariassen 1991).

Freeze avoiding ectotherms

The cold hardiness strategy of freeze avoiding species is generally reported to involve either prolonged supercooling (Sømme 1982; Block 1990) or escaping the most extreme sub-zero temperatures during winter (Danks 1991). Supercooling is promoted by removal of INAs and production of polyhydroxy cryoprotectants and antifreeze proteins. Low molecular weight cryoprotectants like glycerol and sorbitol are often accumulated in body fluids to multimolar concentrations by freeze avoiding species (Zachariassen 1985; Block 1990; Storey & Storey 1991).

Many cold hardy freeze avoiding insects and Collembola have so called thermal hysteresis factors (THF) in their body fluids (Zettel 1984; Duman 2001). THFs are peptides or glycopeptides that lower the freezing point of body fluids in a non-colligative manner, i. e. practically without lowering the MP (DeVries 1982). In this way, the freezing point can be depressed by several degrees Celsius. The presence of THF seems not to lower the SCP substantially, but the THF may stabilize the supercooled state over long periods (Zachariassen & Husby 1982; Duman et al. 1991).

Hibernating terrestrial invertebrates may be dehydrated during winter. As discussed by Zachariassen (1985), dehydration will increase the solute concentration in body fluids and thereby decrease the MP and SCP. However, a given dehydration will have only minor effects in animals with low initial solute concentrations, whereas animals with high solute concentrations will have their MP and SCP lowered appreciably. Dehydration has been shown to stimulate polyol production (e.g. glycerol in an oribatid mite; Young & Block 1980), thus making it difficult to distinguish between physical and biochemical (stimulating) effects of water loss.

Protective dehydration strategy – a different kind of cold tolerance mechanism

In the previous sections the two classic cold hardiness strategies, freeze avoidance and freeze-tolerance, were outlined. For permeable soil invertebrates, the physical conditions in soil during a frost have proven to be of major importance. I first realised this in a study of cold hardiness in earthworm cocoons (Holmstrup 1992). The cocoons of the earthworm *Dendrobaena octaedra* were subjected to -3°C in small vials where the air humidity was defined by ice. The cocoons (with a MP of -0.3°C) did not freeze under these conditions, and were thus supercooled from the beginning of the experiment. Unexpectedly, a drastic water loss of the cocoons were observed under these

conditions indicating that the ice had a desiccating effect, and that the cocoons did not offer any substantial resistance to desiccation.

The reason for the dehydration of the cocoons must be found in the physics of water and ice. Consider a closed system, held at subzero temperature, consisting of a volume of supercooled water surrounded by air and ice. In such a system, vapour pressure of the supercooled water is higher than the vapour pressure of the ice (Salt 1963; Weast 1989). This will cause a net transport of water (vapour) from the supercooled water to the ice, where water vapour is condensated forming ice. The force of this desiccating effect of ice can be calculated as the relative humidity (RH) from:

$$(1) \qquad RH(\%) = \frac{P}{P^*} = \frac{VPice}{VPwater} \times 100$$

where VP is the vapour pressure at a given temperature. Since the pressure difference between water and ice increases with decreasing temperature (at least down to -10°C), and there is an absolute pressure lowering of both water and ice with decreasing temperature, the RH will be steadily declining with the lowering of temperature (Fig 1).
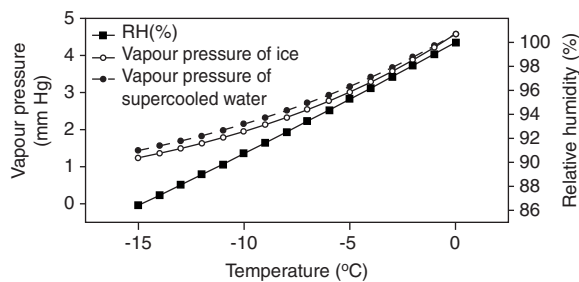
Figure 1. The vapour pressure of supercooled water is always greater than the vapour pressure of ice. The pressure difference between water and ice increases with decreasing temperature (at least down to -10°C). At the same time there is an absolute pressure lowering of both supercooled water and ice with decreasing temperature, which will cause the RH will be steadily declining with the lowering of temperature (Equation 1). Data from Weast (1989).

The pressure difference between ice and the supercooled water can more conveniently be expressed in bar from:

$$(2) \qquad \psi \, bar = -10.6T \log (100/RH)$$

where ψ (psi) is the water potential or "osmotic pressure of ice", and T is the absolute temperature (°K). These relationships make it possible to calculate the difference in water potential between a supercooled soil animal surrounded by ice in the soil. As it appears from Figure 2, these differences in water potential are substantial, even in situations where there is only a slight difference between the water potential of the animal body fluids and the ice (cor-

responding to a difference between the body fluid MP and temperature of the ice). For example, when considering the particular case with an animal body fluid MP of -2°C surrounded by ice at -3°C ("ambient MP") ambient temperature. By transforming MP to osmolality using the osmolal MP depression constant of -1.86°C Osmol^{-1}, we find using Van't Hoff's equation that the difference in osmotic pressure between the animals body fluids and the surrounding ice, is 12 bar (see Fig 2). Thus, even with a "MP inequality" of only 0.1°C there is a difference in osmotic pressure of about 1.2 bar.
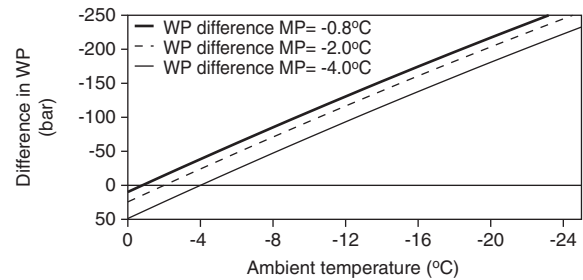
Figure 2. Examples of differences in water potential (WP) between the body fluids of a supercooled animal and the surrounding ice at varying ambient temperature. The more intense supercooling becomes, the larger becomes the WP difference. The water potential of body fluids at a given temperature has been calculated using Van't Hoff's equation: y = Osm×R×T, where Osm is the osmolality of body fluids, R is the gas constant, and T is absolute temperature (°K). The melting point of body fluids (MP) is calculated by application of the osmolal melting point depression constant.

As shown above, the driving force for dehydration in frozen soil is indeed substantial (if the MP is very different from the ambient temperature the pressure difference is enormous, see Fig 2). The second prerequisite for frost-induced dehydration is a high integumental permeability for water. Hygrophilic soil invertebrates are characterised by a high permeability for water through their skin or cuticle (Ghilarov 1983), and in fact they have practically no resistance to desiccation (**1**, **2**, **4**; Carley 1978; Vannier 1987; Harrison 1991). In small permeable soil invertebrates the dehydration process may therefore occur rapidly, as
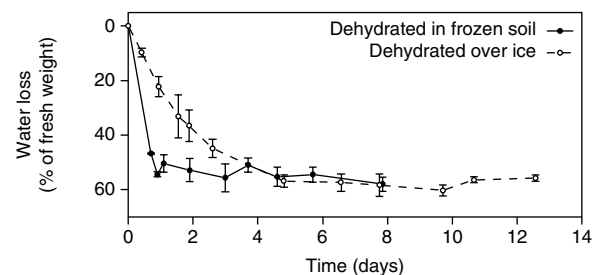
Figure 3. Supercooled earthworm cocoons (*Dendrobaena octaedra*) lose water when surrounded by ice (here at -3°C). Dehydration rates are much faster when the cocoon is buried in frozen soil as compared to cocoons exposed in air, probably due to the shorter distance between cocoon and ice. Data from (**5**).

in the example with cocoons of *D. octaedra* (Fig 3). Factors that determine the rate of dehydration are distance between the ice and the organism (**5**), and difference between MP of the organism and temperature of the ice (Fig 2). The dehydration will continue until equilibrium between water potentials of the organism and the ice is attained, i.e. until the body fluid MP (due to its solutes) equals the temperature of the ice. This relationship also holds true when the pressure gradient is reversed (soil ice temperature > animal MP).

The example in Figure 4 shows that *D. octaedra* cocoons in a way function as small osmometers, and that the dehydration process and vapour pressure equilibration takes place at all temperatures.

This mechanism has also been studied in an Arctic collembolan, *Onychiurus arcticus* (**8**; Worland *et al.* 1998). These studies showed that *O. arcticus*, too, become severely dehydrated when exposed to sub-zero temperatures. Thus, as was the case with earthworm cocoons, the water content of this collembolan could equilibrate to a level dictated by the temperature (i.e. the water potential) of its surrounding environment (Fig 5).
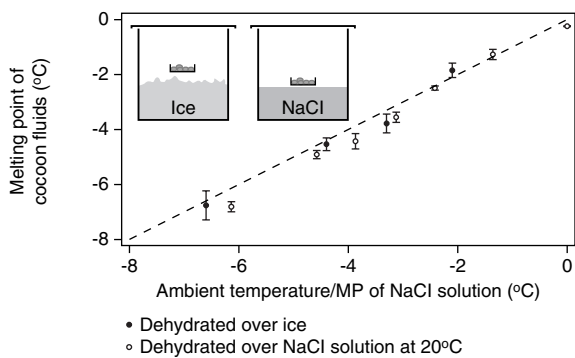
Figure 4. *Dendrobaena octaedra* cocoons equilibrate their osmotic pressure to ambient temperature in a frozen environment (filled circles), and to the relative humidity of air at 20°C (open circles). For comparison, relative humidity at 20°C has been transformed to melting point. Data from (**2**) and (**4**).
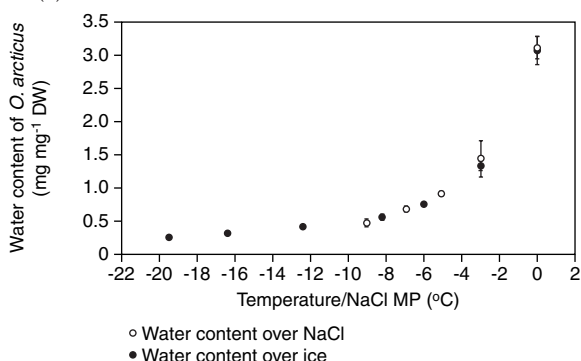
Figure 5. Equilibrium water content of the collembolan *Onychiurus arcticus* surrounded by ice at subzero temperatures (filled circles), and at various relative humidities at +0.5°C (here transformed to melting points: open circles). It is evident that water content at equilibrium is controlled by water potential, irrespective at which temperature. Data from (**8**).

For both of these species it is clear that these animals must be able to tolerate extensive dehydration, which is indeed the case. Both species can tolerate the loss of practically all OAW (**4**, **8**), using accumulation of SPs as dehydration protectants (**3**; Worland *et al.* 1998) in a manner bearing similarities to anhydrobiotic organisms (Crowe *et al.* 1992). An outline of the protective dehydration mechanism is shown in Figure 6.

Frost exposure →

Full hydration	Dehydration	Vapour equilibration
Risk of freezing by inoculation and nucleation.	Lowered MP and SCP (reduced contact with surrounding ice?) leads to reduced risk of freezing. Accumulation of sugars and sugar alcohols in animal begins.	Freezing cannot occur. Sugar and sugar alcohols reduce water loss in animal and protects against deleterious effects of dehydration.

Figure 6. An outline of the "protective dehydration mechanism" (see text for details).

However, in the examples shown above temporal changes in body fluid MPs were measured after a sudden transfer to constant sub-zero temperatures. Under such conditions freeze avoiding organisms are forced to rely on supercooling until equilibrium has been reached. In the field, overwintering soil animals will be subjected to the combined hazards of fluctuating freezing temperatures, the danger of body water freeze-inoculation via contact with ice crystals in the environment, and in arctic and sub-arctic regions exposure to low sub-zero temperatures for the entire winter. For these animals therefore, supercooling is unlikely to be a sustainable winter survival strategy (**5**, **16**). On the other hand, for the dehydration mechanism to be effective, the important question is: can these organisms lower their body fluid MP quickly enough and hence avoid the need for supercooling, to meet the cooling rates that they will encounter in their habitat? To address this question, experiments were conducted in which *O. arcticus* and *D. octaedra* cocoons were exposed to a temperature regime based on real logged temperature data from the winter habitat of *O. arcticus* on Svalbard, where snow cover is typically lacking. Thus, this represents a "worst case" temperature decline for the early winter of arctic soils (**17**). Results from short-term (14 d) and long-term (42 d) experiments suggest that supercooling of the body fluids of these two organisms during overwintering is limited to a few days, and that supercooling during this period is restricted to a few °C (**17**; Fig 7).
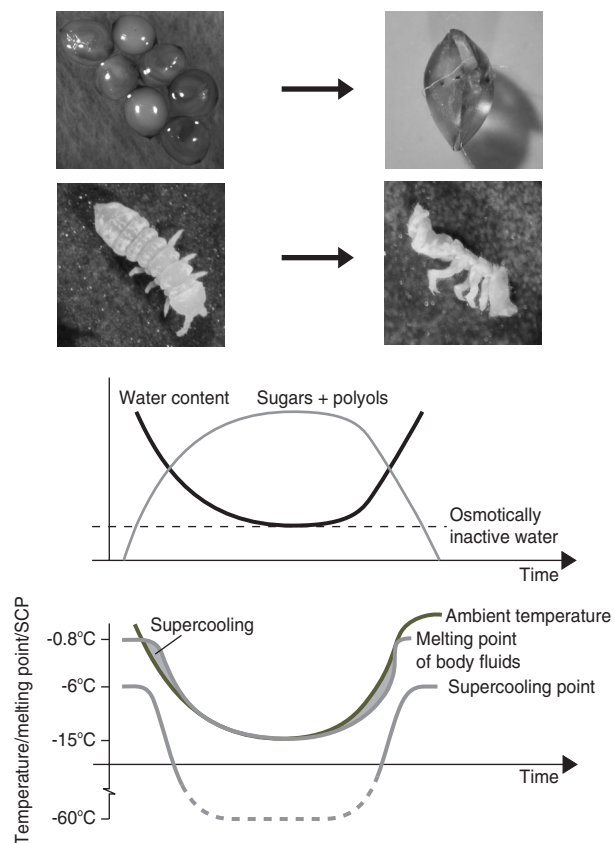
Figure 7. A general model of protective dehydration. Decreasing soil temperature cause dehydration of the organism, which in turn induce accumulation of sugars and polyols, SPs (upper panel). At low temperature (-15 to -20°C) practically all osmotically active water is lost. Dehydration and SP accumulation bring about a lowering of melting point (MP), largely at the same rate as soil temperature decrease. Supercooling is therefore restricted to only a few degrees and only during a short initial period at relatively high subzero temperatures (lower panel). A temperature rise causes the animal to take up water, and increase the body fluid MP. Fully hydrated animals have supercooling points at about -6°C. Dehydration result in a depression of the supercooling point. When dehydration approach the level of osmotically inactive water freezing cannot occur at environmental temperatures. The photos show fully hydrated cocoons of *Dendrobaena octaedra* and collembolan *Onychiurus arcticus* (photos at left), and animals dehydrated at -6°C for 7 days (photos at right). The model is based on results from (**2**, **3**, **8**, **17**) and Worland *et al.* (1998).

Only two options for cold tolerance in permeable soil invertebrates: Freeze or dehydrate

Having established that supercooling is of minor importance in permeable invertebrates from arctic and subarctic regions, it may be stated that permeable soil invertebrates are left with only two sustainable strategies available for survival of sub-zero temperatures: freeze-tolerance or protective dehydration. One major problem for soil animals is inoculative freezing from external ice in the soil. It is unlikely that larger

animals living or hibernating in the soil, such as large enchytraeids, earthworms, frogs or turtles, will be able to avoid contact with ice crystals in frozen soil. There are numerous examples in the literature of inoculative freezing taking place and being of importance in cold tolerance (**16**; Salt 1963; Danks 1971; Hoshikawa *et al.* 1988; Packard *et al.* 1993; Frisbie & Lee 1997; Costanzo *et al.* 1997; Costanzo *et al.* 1999). Because of the high risk of inoculative freezing in frozen soils, natural selection should be expected to favour freeze-tolerance in larger soil animals. Inoculative freezing is also of significance in nematodes, many of which are known to be freeze-tolerant, because these organisms are associated with the thin films of water covering soil particles (Wharton & Block 1993). Other small soil animals associated with the air-filled pores of the soil, e.g. collembolans, can in some cases also be vulnerable to inoculative freezing (Sømme & Conradi-Larsen 1977). However, the smaller soil arthropods may be able to avoid inoculative freezing (see Sømme 1982) by behavioural means, probably because they are small in comparison to ice crystals in the soil. When soils freeze, ice first forms in the larger pores. Water in very fine pores may still be warmer than its freezing point due to capillary forces. Because of differences in chemical potential, water from the finer pores moves to ice in larger pores and freezes on the interface. This process is termed "freezing-induced redistribution", and is the mechanism by which ice lenses in arctic soils are formed (Miller 1980). Since ice almost exclusively forms as a pure water phase, the ice formed when soils freeze may be found segregated in quite large ice crystals. This is easily observed if one looks at frozen soil in a dissection microscope. It is therefore reasonable to speculate that small animals in many cases simply can avoid ice crystals in the soil. Consistent with these considerations, freeze-tolerance is not known in any soil arthropod (Collembola or mites). However, as discussed in the previous sections, if the organism is very permeable for water, the avoidance of freezing will inevitably lead to dehydration.

Which traits are in favour of protective dehydration?

The "protective dehydration strategy" has so far only been studied in few species. However, as shown here, its effectiveness as an over-wintering strategy has been demonstrated in two organisms as phylogenetically distant as an earthworm and a collembolan, whose only relevant common feature in this context is their high water permeability. This fact strongly suggests that the frost-induced dehydration is a widespread winter survival strategy in soil invertebrates. Adoption of the "protective dehydration strategy" by invertebrates requires that water loss rates are sufficiently high to ensure vapour pressure equilibration

within relatively short time. Otherwise, the probability of freezing of body fluids by inoculation may be high. The particular importance of water loss rates is probably the reason why the described cold hardiness mechanism has not been associated with insects. These are often highly adapted to the terrestrial environment by being very drought resistant, i.e. having efficient water conserving morphological and physiological mechanisms (e.g. impermeable cuticle). However, many other soil invertebrate species possess the characteristics necessary for the effective use of this strategy, namely small size and high permeability for water (Table 1). Thus, frost induced dehydration has been shown in nematodes (Forge and McGuidwin 1992), enchytraeids (Sømme and Birkemoe 1997), enchytraeid cocoons (Bauer *et al.* 2001), and in chironomid larvae (Scholander *et al.* 1953; Danks 1971). These are all organisms possessing the traits that will favour the protective dehydration mechanism. Other soil invertebrates with similar characteristics, such as tardigrades and rotifers, could therefore also be candidates, but this remains to be investigated.

The "protective dehydration mechanism" bears some resemblance to freeze-tolerance in which extracellular ice formation induces dehydration of cells (Zachariassen 1985). Parallel to this, organisms using the dehydration strategy are protected by the dehydrating effects of environmental ice. As a response to dehydration both groups of organisms accumulate cellular SPs, which may prevent deleterious effects of the extensive water loss coupled with water potential equilibration (Table 1).

For those organisms that use the dehydration strategy it is characteristic that no preparative accumulation of SPs occurs (as opposed to freeze avoiding and freeze-tolerant insects) before the moment where ice is present in the environment. Due to the elevated osmotic pressure this would result in an increased influx of water into the body at above-zero temperatures, requiring increased energy consumption to avoid salt losses in connection with excretion, and causing problems with the organism's volume control. This is also true for permeable, freeze-tolerant earthworms (and probably also freeze-tolerant enchytraeids). Deferring cryoprotectant synthesis to the moment of tissue freezing obviates the energetic expense of mobilising cryoprotectant, and the potential for loss of energy-rich compounds from the body, that would be unnecessary so long as body temperatures remain above freezing. Also for freeze-tolerant oligochaetes, this system eliminates the osmoregulatory burdens discussed before.

Cold hardiness strategy	Adaptive traits
"Classic" Freeze avoidance • Extensive and long-term supercooling • Conservation of body water	• Anticipatory depression of MP by accumulation of SPs • Thermal hysteresis factors • Removal of INAs • Integumental impermeability
Freeze-tolerance • Supercooling usually avoided • Conservation of body water • Cellular dehydration • Osmotic potential of cells in equilibrium with extracellular ice	• INAs promoted • Inoculative freezing • Control of ice content and ice formation velocity by accumulation of SPs (anticipatory or freeze-induced)
Dehydration strategy • Supercooling not taking place • Extensive dehydration of whole organism • Osmotic potential of cells and extracellular fluids in equilibrium with environmental ice	• Dehydration tolerance • High integumental permeability • Small size (large surface: volume ratio) • Dehydration-induced accumulation of SPs

Table 1. Characteristics of three major cold hardiness strategies in terrestrial ectotherms. Important adaptive traits for each strategy are listed. See text for a discussion.

Dehydration tolerance and water vapour absorption in soil-dwelling Collembola

One of the most important environmental factors determining the performance of soil-dwelling Collembola, and soil fauna in general, is the availability of water in the soil (Rapoport & Tschapek 1967). This has long been realised, and the ecophysiology of desiccation tolerance and water balance in Collembola has been rigorously studied.

In essence, there are three survival strategies open to soil dwelling Collembola during drought. Firstly, they may have evolved the ability to tolerate extensive loss of body water. The most extreme case of this is termed anhydrobiosis (Keilin 1959), a strategy utilised by some species of Mediterranean dry areas (Greenslade 1981; Belgnaoui & Barra 1988; Poinsot-Balaguer & Barra 1991). Secondly, they may have evolved resistance to desiccation by reducing the water permeability of their integument. This strategy has been adopted by many surface-dwelling species, whereas euedaphic species have little or no control of evaporation across the integument (Vannier 1983).

Finally, Collembola may migrate to moist microsites during drought periods, either vertically or horizontally (Verhoef & van Selm 1983; Hopkin 1997 and references therein). This last strategy may enable Collembola to evade transient drought spells but longer lasting or extreme drought is likely to incur physiological stress.

Evapo-transpiration by plants is a much more important pathway of soil water removal than evaporation from bare soil surface (Gardner 1960; Herbst *et al.* 1996; Hillel 1998; Kappen *et al.* 2000). Soil water loss through plants will therefore tend to dry out the soil where roots are most abundant. The root zone of annual plants is primarily occupying the upper 30 cm of the soil profile (Gardner 1964; Andersen 1986; Kappen *et al.* 2000). This is the same soil stratum in which the majority of soil dwelling Collembola are found (Usher 1970; Hågvar 1983; Lagerlöf & Andrén 1991; Filser & Fromm 1995; Detsis 2000). Moreover, a number of soil invertebrate species are associated with the rhizosphere because this is where they often seek their food (Coleman *et al.* 1988). Migration to deeper and moister soil layers is a possibility for collembolans under moderate drought periods. However, because of the reduction of soil pore size with increasing depth (Schaeffer & Schachtschabel 1984) there is probably a limit to the depth these animals can migrate. It is therefore likely that occasional extreme drought situations will leave soil Collembola with no place to migrate to. Under such circumstances, a species would run the risk of local extinction, if physiologically based tolerance mechanisms were not available.
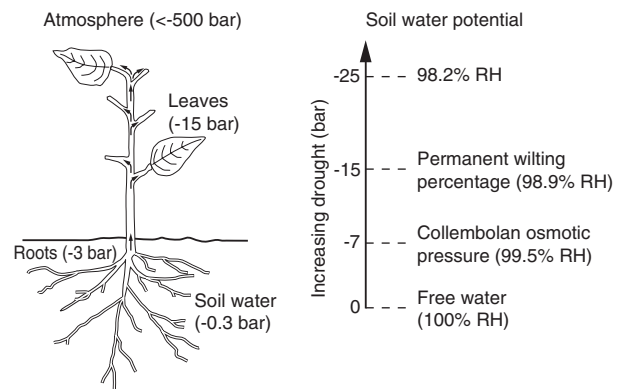
Figure 8. Plants are the reason why soil is often desiccated in the whole root zone during drought. In a moist soil Collembola constantly take up water because of the osmotic gradient, and exude excess water with other excretion products. If soil water potential becomes more negative than the osmotic pressure of the animal, water will be lost to the environment.

Plants are typically able to absorb soil water until the soil water potential reaches a value known as the permanent wilting percentage. At this stage, suction pressure of the soil is -15 bar, equivalent to a relative humidity of the soil air of approximately 98.9% RH (Hillel 1998). This should be compared with the osmolality of collembolan body fluids, which is typically 300 mOsm, equivalent to a RH of 99.5%, or a water potential of approximately -8 bar. Soil-dwelling Collembola are therefore likely to meet water potentials during summer drought that will cause a loss of body water (Fig 8). Although a seemingly mild desiccation level, e.g. 98.9% RH as mentioned above, will cause a dramatic water loss in soil-dwelling species (**6**), most previous studies on desiccation tolerance of Collembola have been made using very low humidities. There are a number of studies, predominantly on epigeic Collembola, where transpiration rates and survival have been measured at low humidities, typically using dry air. This type of experimental situation is not appropriate for the study of soil-dwelling Collembola because even extreme drought will seldom produce soil air humidities lower than 96% RH (Hillel 1998). If any physiological mechanisms involved in the drought tolerance of soil-dwelling Collembola are to be studied, it is necessary that the experimental desiccation levels be adjusted to simulate the conditions that the animals are likely to meet in the field.

In this section of the thesis it will be shown that common soil dwelling Collembola possess the ability to actively reduce water loss in the range of ecologically realistic soil humidities (e.g. 98.2% RH), and even re-absorb water vapour from the atmosphere.

Simulating ecologically relevant drought conditions in the laboratory

It is extremely difficult to administer a precise drought stress to soil organisms in the soil itself under laboratory conditions. Instead, a system using aqueous NaCl solutions to define the relative humidity in a small chamber was developed (Fig 9).
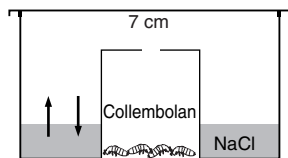
Figure 9. The system used to control relative humidity with aqueous NaCl solutions. Arrows symbolise the equilibration of air humidity with the salt solution.

The animals were placed in open-top plastic sample vials (4 cm height, 2 cm diameter) in the centre of a 200 ml plastic cup containing 25 ml NaCl solution, and sealed with a tightly fitting plastic lid. This system closely emulates the method used for calibrating instruments used to measure soil pore air humidity (Wescor 1986). The air in this small closed system rapidly equilibrates with the salt solution (following Raoult's law) and very precise RH-levels (± 0.1% RH) can be established by adjusting the NaCl concentration (Lang 1967; Weast 1989). In general, the water potential can be calculated from:

(3) $$\psi = \frac{RT}{V_1} \ln \frac{P^o}{P}$$

where R is the gas constant, T is absolute temperature, V_1 is molar volume of water, P^0 the vapour pressure of pure water at that temperature, and P the vapour pressure of the solution in question.

The RH in this system can be affected by temperature in two ways. Firstly, temperature has a weak effect on the water potential of salt solutions. A given concentration of NaCl will therefore give slightly different RH at 0°C and 25°C after equilibration has been reached. However, Lang (1967) reported that water potential varied less than 1 bar between 15 and 25°C, meaning that this effect of temperature is without any practical importance. Secondly, and more important, is the fact that water vapour pressure is highly dependent on temperature (Weast 1989). Small sudden changes in temperature (even 0.1°C) can therefore cause instant changes in RH. However, in such a small system (Fig 9) the water vapour pressure of the air will quickly adjust with the salt solution, with the result that the steady state RH is restored (Fig 10). In practise the experiments were carried out at as constant temperature as possible (± 0.1°C or better). In effect, the average RH in the system could therefore be kept at a precisely defined level but with transient excursions away from the steady state level (see Fig 10).
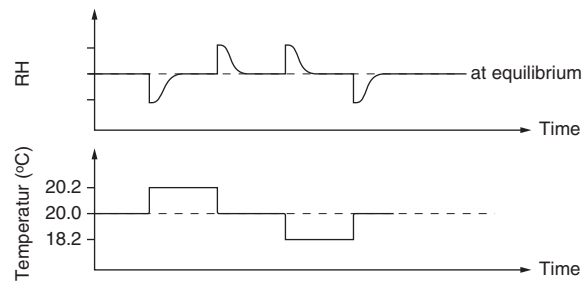
Figure 10. The relative humidity (RH) is precisely defined by NaCl concentration at constant temperatures but sudden changes in temperature can cause a change in RH. In a small system (small volume of air, large surface of salt solution in relation to air volume) vapour pressure equilibrium will quickly be re-established. Thus, a temperature rise will cause the RH to decrease, and vice versa. In practise, temperature driven movements away from equilibrium RH due to temperature are probably moderate compared to the present example because temperature shifts in the experiments are less sudden than shown here.

In most of the experiments discussed in the following, 5-15 animals were placed in the inner vial of the drought chamber, and exposed for 8 days at 20 ± 0.1°C. For analysis of temporal developments, animals were sampled destructively. When survival was the issue, the animals were allowed to recover at 100% RH for 2 days before assessment. A similar system has been used for studies of earthworm cocoons.

A case study: passive absorption of water vapour in *Folsomia candida*

The desiccation tolerance of *F. candida* can be studied by exposing animals to a series of decreasing relative humidities (Fig 11). Using this procedure it appears that *F. candida* tolerates desiccation down to 98% relative humidity without any substantial mortality, and has a lower tolerance limit at approximately 96% RH. This result is surprisingly robust as judged from numerous experiments in the laboratory. In several studies the physiological responses of *F. candida* were followed at a sub-lethal but stressful desiccation level to describe the mechanism by which this species is able to cope with desiccation stress.
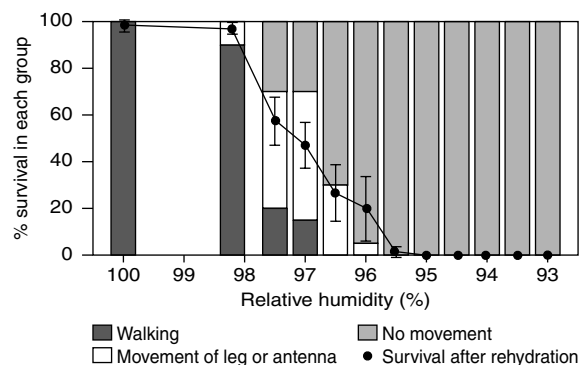
Figure 11. Survival of adult *Folsomia candida* after 7-day exposure to 98.2% RH. Data from (**14**).
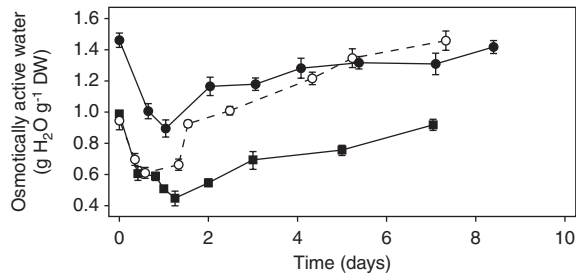
Figure 12. Three independent experiments showing water vapour absorption in *Folsomia candida* at 98.2% RH. Error bars show SEM. Data from M. Holmstrup (unpublished study) and (**10**).

Figure 13. Individual dry weight of adult *Folsomia candida* remains constant during a 7-day exposure to 98.2% RH (upper panel), indicating that the subsequent increase in water content (statistically significant at 192 hours, P<0.05, n=5) is due to vapour absorption, and not due to metabolic water production or decrease in dry weight. In controls (lower panel) no significant change in dry weight or water content were observed. Data from Holmstrup *et al.* (**22**).

Figure 12 shows results from three different experiments where the water content of animals exposed to 98.2% relative humidity was followed. Initially, the pressure difference between ambient air and the animals' osmotic pressure is approximately 17 bar (Fig 8), and because the integument of the animal is so permeable, water will quickly leave the body to produce a drastic water loss within the first 24 hours (Fig 12). However, after 48 hours an increase in the water content begins to take place. Other experiments show that the dry weight of the animals remains constant during the 8-day experiment, so the increase in water content is not due to metabolic water production or decreases in dry weight (Fig 13). Moreover, there is no free water available to the animals at this relative humidity making drinking impossible. The increase in water content must therefore be due to absorption of water vapour from the air in the desiccation chambers.

Investigations of the osmotic pressure of the animal's body fluids reveal that this initially is approximately -8 bar. Within the first 24 hours the osmotic pressure reach the level of the environment at approximately –25 bar. This coincides with the lowest water contents observed (Fig 14). At this time, the water loss has caused the concentration of the original and induced solutes to equal the water potential of the environment, which will cancel out the pressure difference and therefore stop further water loss. After this, a further increase of body fluid osmotic pressure is observed, until the normal pressure difference is re-established. There is therefore again a pressure gradient that will cause a net influx of water to the animal. When the animals are returned to saturated conditions, a quick decline of the osmotic pressure occurs, and the normal osmotic status is regained (Fig 14). Thus, water loss in itself increases the body fluid osmotic pressure to a level equal to the ambient water potential. But for animals to become hyperosmotic again, they need to up-regulate osmotic pressure by some active process. Chemical analyses have revealed that glucose and myo-inositol account for a large proportion of the measured increase in osmotic pressure. When the animals are placed in saturated conditions, these compounds are rapidly removed from the body fluids (Fig 14).

Figure 14. A general model of water vapour absorption in permeable Collembola. Exposure to 98.2% RH (approximately -25 bar) cause a quick dehydration of the organism (upper panel), which in turn induce accumulation of sugars and polyols, SPs (lower panel). During the first 24-48 hours of exposure the osmotic pressure (OP) of the animals is up-regulated and the normal inward gradient in OP is restored (centre panel). This is the basis for vapour absorption and the increasing water content. When the animals are returned to saturated conditions (100% RH) SPs are quickly removed from the body fluids, and OP returns to a normal level. At the time with lowest water contents the animals may appear inactive and darker in colour (e.g. in *Folsomia candida*). Based on data from (**10**) and (**15**). Photos by Heidi Sjursen.

A number of terrestrial arthropods have the capability to balance their water budget by actively absorbing water from highly unsaturated atmospheres. These truly terrestrial arthropods achieve this by a combination of a cuticle with very low permeability and by locally creating extremely low water activity in specialised organs/tissues (reviewed in Hadley 1994). In common with the majority of soil arthropods *F. candida* has a highly permeable integument making localised active water absorption inappropriate. This animal is therefore forced to maintain all its body fluids hyperosmotic to its surroundings to allow net water uptake from the atmosphere by passive diffusion along the water potential gradient. As stated, *F. candida* will tolerate acute desiccation with a lower tolerable limit at about 96% RH. However, if acclimated at a mild desiccation level, it will endure much harsher desiccation (Fig 15) and tolerate lower body water contents (Fig 16) (**14**). Thus, after a 7-day acclimation period at 98.2% RH *F. candida* was able to maintain a hyperosmotic status to the surroundings at 95.5% RH, suggesting that it can absorb water vapour down to this limit. At these low humidities trehalose levels increased whereas myo-inositol levels declined. This may indicate a change in survival strategy where *F. candida* at mild desiccation levels seek to preserve water by colligative means (remain hyperosmotic), but at more severe desiccation levels switches to an anhydrobiotic-like strategy to protect cellular structures from the damage caused by lack of water (**14**).
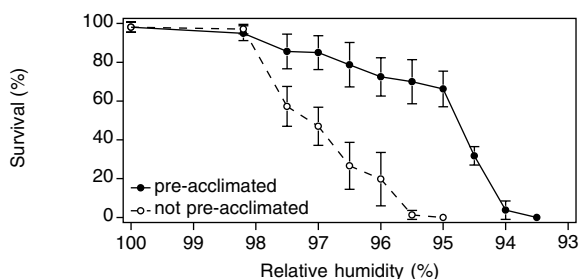
Figure 15. Pre-acclimation of *Folsomia candida* to a mild desiccation stress (98.2% RH) improve the tolerance to a subsequent severe drought stress. Error bars indicate standard deviations (n = 5). Data from (**14**).
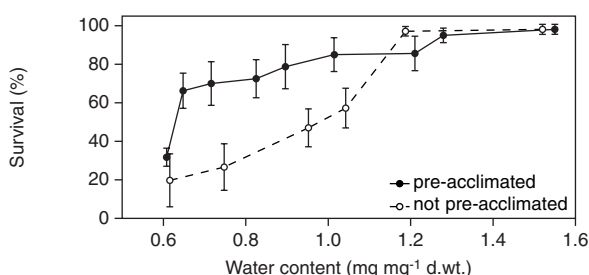
Figure 16. Pre-acclimated *Folsomia candida* (7-day exposure to 98.2% RH; solid circles) are less sensitive to low water contents than animals directly exposed to a severe desiccation stress (open circles). Error bars indicate standard deviation (n = 5). Data from (**14**).

How widespread is the vapour absorption mechanism?

The question of how widespread this mechanism is in soil arthropods is only beginning to be uncovered. As suggested earlier, the existence of the mechanism only becomes evident when experiments are designed appropriately. There has been a tendency to apply methods in Collembola research that were originally designed for studies of drought resistant insects. The number of studies at ecologically relevant relative humidities is at present therefore limited. However, two other species, *F. fimetaria* and *Protaphorura armata*, have been studied in detail (**15**). These species are similar to *F. candida* with respect to general morphology and both possess exactly the same drought tolerance mechanism, suggesting that this strategy is indeed widespread in hygrophilic soil Collembola. It may be hypothesised that two crucial features, namely small size and high permeability, are necessary for the presence of this strategy in a species. Collembola as a group embraces a whole spectrum of species ranging from very small and permeable species (Vannier's type I species, hygrophilic species), through mesophilic species of moderate permeability, to more insect-like epedaphic species, which may be large and relatively impermeable (Vannier's type III species, xerophilic). Therefore, along this "spectrum" within the Collembola it is possible to survey the presence of this strategy in order to determine by which groups the strategy is utilised. A survey was therefore designed consisting of three steps: (1) Determination of the desiccation level at which mortality began to occur (i.e. the "critical desiccation level"), (2) investigation of the water loss and osmotic pressure at the critical desiccation level, and (3) a semi-quantitative screening for SP accumulation at the critical desiccation level. This approach was used because it was anticipated that if a particular species utilizes the mechanism, it would be evident at this desiccation level where physiological compensation is possible (Fig 17), as was the case with the three hygrophilic species mentioned before (**10**, **15**).
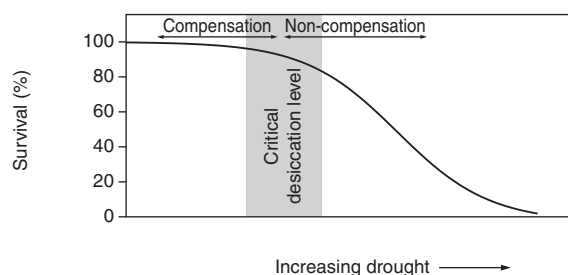
Figure 17. If a species possess the ability to synthesise sugars and polyols for regulation of osmotic pressure, accumulation is likely to be triggered at the critical desiccation level. This is the level where mortality becomes evident.

In this survey covering nine species of Collembola (Kærsgaard *et al.* in prep) several desiccation related physiological parameters were investigated. The critical desiccation level, given as water potential, was identified. Thereafter water contents and osmotic pressure of the body fluids were determined making it possible to detect whether an active up-regulation of osmotic pressure had taken place during the 7-day desiccation exposure. The results show that several of the mesophilic (hemi-edaphic) species did actively up-regulate their osmotic pressure since the measured osmotic pressure was significantly higher than what was expected from the observed water loss (Table 2). In many of the cases non-quantitative chemical analyses of tissues showed that SPs were present in drought stressed animals but not in control animals. However, only in two species, *H. nitidus* and *I. anglicana*, did the animals become hyperosmotic to the ambient water potential. These two species may therefore possess the ability to absorb water vapour as seen in the three hygrophilic species. In *S. curviseta* and *H. assimilis* active up-regulation of the osmotic pressure was demonstrated, but no re-establishment of a normal pressure gradient was observed (Table 2). These spe-

cies could therefore not absorb water vapour to compensate a water loss, but they are possibly able to slow down the dehydration process, or even stop it. In the two larger species, *P. flavescens* and *O. cincta*, which probably have the most epigeic life-style, no significant active up-regulation of the osmotic pressure was evident. On the contrary, the measured osmotic pressure was in good agreement with the anticipated osmotic pressure calculated from water loss estimates (Table 2). Based on the survey of these nine species, it may be suggested that active up-regulation of osmotic pressure of body fluids by synthesis of solutes is common in hygrophilic species, and probably also in many mesophilic species, whereas this mechanism is not likely to be found in epigeic (xerophilic) species. In general terms, the more pronounced the adaptations to truly terrestrial conditions become in a species, the more rare becomes the utilisation of active up-regulation of solutes such as SPs.

Other examples support the suggestion that SP-accumulation induced by dehydration is not confined to a few arthropod species. For example, when specimens of *O. arcticus* were slowly dehydrated at sub-zero temperatures a similar dehydration-induced

Species	LWP50 (bar)	Tested WP (bar)	Original OP (bar)	Original WC (mg mg^{-1} dry weight)	WC after 7 d desiccation stress (mg mg^{-1} dry weight)	OP after 7d due to water loss (bar)	Determined OP after 7 d (bar)	OP (bar) originating from active up-regulation	SPs
			A	B	C	D = (B/C)×A	E	F = E-D	
Hygrophilic/euedaphic species									
F. candida	-30	-25	-7	1.5	1.4	-8	-32	-24	Glucose, myo-inositol
F. fimetaria	-33	-25	-8	1.8	1.8	-9	-28	-19	Myo-inositol two unidentified SPs
P. armata	-40	-25	-9	1.9	1.4*	-12*	-34*	-22	Trehalose
Mesophilic/hemiedaphic species									
H. assimilis	-34	-25	-9	3.1	2.2	-13	-26	-13	None detected
H. nitidus	-25	-25	-9	2.7	1.9	-13	-30	-17	Myo-inositol glucose
I. anglicana	-33	-25	-10	5.0	2.6	-19	-29	-10	Glucose
S. curviseta	-49	-25	-9	1.8	1.7	-9	-15	-6	Glucose unidentified
		-38	-9	1.8	1.4	-11	-32	-21	
P. flavescens	-16	-17	-6	2.6	1.9	-8	-11	-3	Glucose
Xerophilic/epigeic species									
O. cincta	-66	-54	-6	4.5	2.7	-15	-14	≈0	None detected

*After 12 days

LWP$_{50}$: the water potential causing 50 % mortality in a 7-d test; WC: water content; OP: osmotic pressure of body fluids; SP: sugars and polyols

Table 2. Results of a survey of desiccation related physiological parameters in nine species of Collembola. The water potential causing 50% mortality (LWP$_{50}$) was estimated as described in (**20**). From the dose-response curve, a critical desiccation level was chosen, at which further physiological experiments were made. The body fluid osmotic pressure (OP) and water content (WC) was determined for control animals as well as for animals exposed to the relevant critical desiccation level for a 7-day period, using the methodology described in (**10**). By comparing the OP resulting from water loss alone with determined OP after the desiccation period, it was possible to judge if active up-regulation of OP had taken place. The contribution from active up-regulation is calculated by subtraction of these two OP values. SPs in desiccation treated animals were identified using methods described in (**15**).

accumulation of trehalose took place (Worland *et al.* 1998). Accumulation of glycerol in the Antarctic mite, *Alaskozetes antarcticus*, was also shown to be induced be dehydration (Young & Block 1980). Finally, Verhoef & Prast (1989) reported that *O. cincta*, subjected to slow dehydration at 96% RH increased the osmotic contribution of the organic solutes slightly more than expected from the observed water loss. However, this is largely consistent with the results shown in Table 2.

Other physiological responses during acclimation

As argued in the previous sections the accumulation of SP is a major adaptive mechanism in drought tolerance. However, there are a number of other physiological mechanisms that should also be taken into consideration. Two of the most obvious candidates, the induction of heat shock proteins and alterations in the membrane phospholipid composition are known to be of significance in the tolerance to departures away from optimal temperature and moisture conditions. Expression of heat shock proteins such as Hsp70 is triggered by a whole variety of stresses (Feder & Hofmann 1999). But until now only a few reports of their induction in animals by desiccation stress exist in the literature (Tamariello *et al.* 1999). Drought acclimation induced the expression of Hsp70 in *F. candida* (**13**). Reported functions of Hsp70 proteins are numerous and apparently diverse but all include the basic process of binding and subsequent release of partially unfolded proteins in an ATP-dependant cycle which end result is either the reestablishment of their normal 3D structure or their degradation (Forreiter & Nover, 1998). This function fits well with the model for their induction proposed by Ananthan *et al.* (1986) who showed that the common factor in the diversity of stresses causing transcription of the Hsp70 gene was the presence of partially denatured proteins within the cell. This generally accepted theory may explain the observations of Hsp70 expression in drought stressed *F. candida*, because the severe water loss occurring would increase the concentration of ions drastically and possibly cause protein denaturation. Other reports exist of a desiccation induced 28 kDa desiccation stress protein isolated from the meal worm *Tenebrio molitor* (Graham *et al.*, 1996), as do numerous reports of desiccation stress proteins from plants, the so-called dehydrins (Close 1996, 1997). A large body of evidence suggests that dehydrins are expressed and become associated with membrane-bound proteins in drought-stressed plants and algae. At present, the hypothesis is that dehydrins are "surfactants" capable of inhibiting the coagulation of a range of macromolecules, thereby preserving structural integrity of cell membranes during desiccation. It would be interesting to screen for these other dehydration-induced molecules in drought tolerant Collembola and earthworms, but such attempts have not yet been made.

Membrane integrity and function is an essential pre-requisite for all living organisms. The reasons for this are many and will not be treated here in detail (for reviews, see Hazel & Williams 1990; Hazel 1995). However, in the context of desiccation tolerance, two main roles of membranes seem to be especially important, namely the function as a physical barrier to solute diffusion, and the mediation of transmembrane movement of specific solutes and water via membrane-bound proteins. The phase behaviour of membrane phospholipid fatty acids (PLFA) is extremely sensitive to changes in temperature. Such phase alterations induce membrane organisational changes posing serious challenges to physiological function in ectotherms, with the functional optimum occurring in a relatively narrow range of viscosities (Hazel 1995). There is considerable evidence that organisms grown at different temperatures tailor the PFLA composition of their membranes to maintain optimal fluidity of their membranes (**11**; Hazel 1995). This homeoviscous adaptation typically results in an increased degree of fatty acid unsaturation with falling temperature, but thermal compensation of membrane function can also occur in the absence of homeoviscous adaptation and vice versa (Hazel, 1995).

In a study of *F. candida*'s responses to drought it was shown that 8-day acclimation at 98.2% RH caused a statistically significant increase in the mole percent of mono-unsaturated fatty acids and 18:2ω6c, while the other polyunsaturated fatty acids showed only minor increases (**13**). Compared to typical effects of cold acclimation seen in other poikilotherms the changes were, however, not very pronounced, and there was a net status quo in the overall degree of unsaturation. Nevertheless, a follow-up study has largely confirmed this trend (**22**) suggesting that drought does indeed induce membrane phospholipid changes in *F. candida* that resemble typical changes following cold acclimation (Hazel 1995). It should be noted that homeoviscous adaptation is not simply a matter of increasing the unsaturation of membrane phospholipids. The number and position of cis-cis double bonds in long-chain fatty acids has a pronounced influence on the properties of membrane lipid bilayers by changing the gel to liquid phase transition temperature, T_m (Hazel, 1995). In general, T_m decreases with increasing number of double bonds and with increasing distance to the ends of the molecule. Also, the effect of the first double bond introduced is stronger than the effect of the next, and so on. Hence, the observed shift towards a higher proportion of mono-unsaturated fatty acids with a mid-chain double bond in the reported study can be expected to correspond to a relatively strong decrease of T_m in the drought acclimated animals and contribute to the observed cross tolerance to cold shock (**13**).

Hazel & Williams (1990) rigorously treat membrane adaptations to changes in water activity, which seems relevant to the observations made for *F. candida* under drought stress (and organisms that undergo freezing-induced dehydration). Water activity is important because the interactions between water and phospholipids are the fundamental feature of membrane organisation in a bilayer structure. Changes in water activity may result in two types of adaptation, (1) a restructuring of membrane lipid composition in response to moderate levels of dehydration, and (2) synthesis of SPs that are known to have specific membrane stabilising effects (Crowe *et al.* 1992). With reference to plant studies, Hazel & Williams (1990) suggest that moderate water stress in some cases have been shown to result in membrane alterations resembling the changes of cold acclimation. However, very little literature exists concerning the effects of desiccation in membrane composition, and the picture is far from clear. In plant and bacteria studies, desiccation stress alone have been shown to induce a reduction in the level of membrane PFLA unsaturation, resulting in reduced membrane fluidity and water permeability (Liljenberg and Kates 1985; Kieft *et al.* 1994). This is opposed to other studies suggesting that only minor, or no changes in phospholipid composition occur in plants as a response to dehydration (e.g. McKersie & Stinson 1980; Stewart & Bewley 1982; Singh *et al.* 1984; Repellin *et al.* 1997).

Probably more important, several studies have shown that in sufficient concentrations, a variety of carbohydrates can stabilise membrane structure (Crowe & Crowe 1986; Newman *et al.* 1993). Sugars and polyols, at molarities similar to those expected in the animals in the study referred to here (**13**), have also been shown to exert a direct influence on membrane fluidity. It would therefore appear that both the alterations in PLFA's measured in this study and the high concentrations of SP's reported previously, might contribute to the animals' tolerance to drought and to their cross-tolerance to cold shock (**13**).

It has been proposed that increased membrane fluidity in itself increase the water diffusion across cellular membranes. However, the recent discovery of a class of water-transport proteins named aquaporins has changed this view. Aquaporins are membrane-bound proteins that facilitate water transport in bulk flow across the cell membrane without the passage of most other molecules (see reviews in Maurel 1997; Borgnia *et al.* 1999). Even though water molecules may passively diffuse through the cell membrane this transport is orders of magnitude less than the water transport mediated by aquaporins. As a response to drought aquaporin genes may be up- or down-regulated, or posttranslational modifications may alter the permeability characteristics of aquaporins so that cells or tissues may adapt to the changed water activities (Maurel 1997; Borgnia *et al.* 1999). It has been propo-

sed that observed reductions in cell membrane fluidity of drought stressed wheat plants may regulate overall membrane permeability by changing the environment of aquaporins (Carvajal *et al.* 1996). Another aspect of drought exposure where aquaporins may be of importance is the case of cell volume regulation. For instance, the re-hydration of desiccated collembolans or other soil invertebrates may impose a considerable hypo-osmotic shock during which cells will quickly imbibe water because of a high internal concentration of SP. At present nothing is known of aquaporin function or regulation in soil invertebrates, except that these proteins must have the same significance as found in plants, bacteria and animals. There can be no doubt that research into this would provide a significant contribution to the molecular basis for understanding water relations of drought tolerant soil invertebrates.

Drought tolerance in earthworms

In the previous sections it has been argued that the hygrophilic Collembola in many respects have characteristics similar to freshwater animals. Bearing this in mind, earthworms are even more adapted to a life in water. Most earthworm species and their cocoons may be kept in water for long periods, e.g. *Allolobophora chlorotica* that may stay alive submerged for months provided the water is sufficiently oxygenated (Roots 1956). It is commonly thought that earthworms will drown when submerged, but this is not so because oxygen and carbon dioxide are readily exchanged across the skin-water interface. With regard to their excretory and respiratory physiology, earthworms (and enchytraeids) have many similarities with freshwater invertebrates (Ramsay 1949; Oglesby 1969; Schmidt-Nielsen 1990). Another feature relating them to freshwater invertebrates is the fact that the MP of their body fluids is high (about -0.4°C) compared to arthropods where the MP during summer is about -0.8°C (Zachariassen 1985). Earthworms have only very moderate morphological or physiological means for reducing water transport through the cuticle (Carley 1978). Because of the very low osmotic pressure of the body fluids, it is expected that earthworms would be even more sensitive to desiccation than hygrophilic Collembola. Many studies have shown that activity can be maintained only if free water is available in the soil (Lee 1985). If soils become too dry, earthworms may move to deeper soil layers or micro-sites where conditions are more favourable (Gerard 1967; Rundgren 1975). The earthworm *Aporrectodea caliginosa* and several other species enter diapause during summers if the water potential becomes too low (**12**; Gerard 1967; Nordström 1975). During this process the worm excavates a spherical cell in the soil, lined with mucus and egested gut contents. By coiling itself into a ball in the soil cell,

water loss is minimised during drought. However, earthworms can generally tolerate extensive water loss (Grant 1955; El-Duweini 1968). For example, *A. caliginosa* is able to survive loss of up to 80% of its normal water content for several days. Whereas this dehydration would cause a protective accumulation of SPs in hygrophilic Collembola, this it not the case in *A. caliginosa* (**21**). Earthworms must therefore be able to tolerate extremely high concentrations of inorganic ions as Chloride, Potassium and Sodium.

In experiments with *A. caliginosa* exposed to decreasing soil matric potentials in the range –2 to –300 kPa (i.e. > -3 bar or 99.75% RH) it was shown that physiological and behavioural responses in earthworms are triggered at much weaker water potentials than in Collembola (**12**, **21**). Estivation ("diapausing") in this species was induced at water potentials > -100 kPa, which in itself would not cause the animals to dehydrate (Fig 18). Life history parameters like reproduction and growth was also affected or brought to a complete halt at these seemingly mild water potentials (**12**). It appears therefore that the strategy of earthworms for survival of drought in the soil is based on an "early warning" and precautionary formation of an estivation chamber, before the drought conditions are actually becoming harmful. The costs of this strategy are that growth and reproduction is completely shut down, also in periods with potentially favourable environmental conditions. It should be noted that temperature is also an important factor for induction of diapause (Edwards and Bohlen 1996). Earthworm cocoons are predominantly to be found in the upper soil layers (Gerard 1967; Bouché 1972) where drought is most intense. Opposed to worms, that may prepare themselves to drought, the cocoons depend on tolerance to drought and dehydration. Field investigations show that earthworm cocoons have a greater tolerance to environmental stress than hatched individuals and that populations under extreme conditions survive mainly as cocoons (Bouché 1972, 1977; Huhta 1980). Indeed, laboratory studies have shown that earthworm cocoons are extremely desiccation tolerant, some species being able to tolerate loss of practically all osmotically active water (**4**). This ability is likely to be a result of accumulation of SP in the embryo (**3**) as known from many anhydrobiotic invertebrates (Crowe *et al.* 1992). Even though earthworm cocoons will not survive loss of all water their drought tolerance resembles the strategy known as anhydrobiosis.

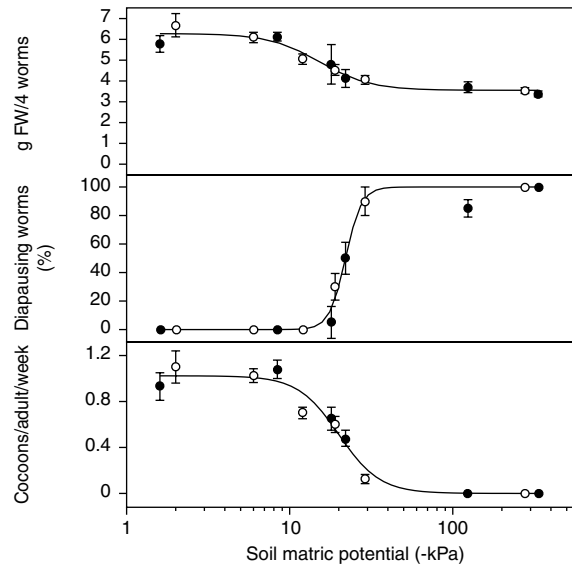**Figure 18.** Influence of soil water potential on fresh weight, induction of diapause, and cocoon production in adult earthworms of the species *Aporrectodea caliginosa*. Note that effects appear at much weaker water potentials than seen in Collembola (-100 kPa equal -1 bar). Values are shown as mean ± SEM. Data shown for two different soil types (open and filled circles). Data from (**12**).

Poikilohydry – a common mechanism for survival of cold and drought

Poikilohydric organisms can withstand the equilibration of their internal water content with the water potential of the environment. Many lower plant species (e.g. algae, lichens and bryophytes) dehydrate rapidly in accord with the ambient humidity (Bewley *et al.* 1993; Alpert 2000). Some higher plant species (e.g. ferns and grasses) exhibit a modified form of poikilohydry, in that they have morphological and physiological features to slow down the dehydration process. Whereas the first group seems to display a constitutive tolerance to dehydration, the moderately poikilohydrous plants are dependent on physiological and biochemical defence mechanisms (Bewley *et al.* 1993). In many ways the hygrophilic soil invertebrates dealt with here are similar to this latter group of plants. Firstly, soil is a buffered environment, and dehydration processes will therefore be relatively slow under the vast majority of circumstances. Secondly, most Collembola and earthworms will only tolerate loss of osmotically active water, but not the osmotically inactive water as it is the case in entirely anhydrobiotic species. As has been described in the previous sections, physiological acclimation responses are also necessary for desiccation tolerance in Collembola and earthworms.

The early colonisation of land by aquatic organisms have necessitated the evolution of desiccation tolerance mechanisms, which is perhaps the most fundamental feature of terrestrial organisms (e.g. Ghilarov 1958; Hadley 1994). Several authors have therefore proposed that many of the adaptations promoting cold tolerance were in fact originally develo-ped to tolerate desiccation (e.g. Block 1996; Ring & Danks 1994, 1998). One of the most important arguments in favour of this is that accumulation of SPs seems to be central in tolerance to both drought and cold. Thus, SPs may slow down dehydration rates in freeze avoiding species during winters, and have the same effect under summer drought (Ring & Danks 1994, 1998). SPs will reduce cellular dehydration in freeze tolerant organisms during winters, and reduce the equilibrium dehydration level in summer drought exposed organisms (**8**, **10**). The specific protection of membranes and proteins by SPs are useful both during winter dehydration of freeze avoiding and freeze tolerant species (or exposure to low temperatures *per se* (Ramløv 2000)), and during summer dehydration, with anhydrobiotic organisms as the most extreme and convincing example (Crowe *et al.* 1992). In hygrophilic soil invertebrates this seems to make a particular strong case because both cold and drought result in dehydration – and poikilohydry – which in turn triggers a substantial and necessary accumulation of SPs.

These suggestions are bolstered by the fact that acclimation of *F. candida* to mild desiccation stress at 20°C indeed improved cold shock tolerance considerably (**13**). Similar cross tolerance are also known from several plant studies (Chen *et al.* 1975, 1977; Siminovitch & Cloutier 1982; Anisko & Lindstrom 1995, 1996), suggesting that this is possibly a widespread phenomenon, not restricted to a few taxonomic groups.

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Interactions between climatic and toxic stresses

Annual and seasonal fluctuations in population size of soil invertebrates are natural phenomena. The amplitude and duration of these fluctuations depend first of all on variations in soil humidity and temperature. Soil animals will therefore occasionally encounter environmental "bottlenecks" such as severe drought in summer or extreme and sudden frosts in autumn or spring when no insulating snow cover is present (Fig 19). Such events may drastically reduce populations or even cause local extinction of species (Hoffmann & Parsons 1991). Environmental pollution may also cause significant reductions in soil invertebrate populations (e.g. Bengtsson *et al.* 1983; Spurgeon & Hopkin 1999). However, in the environment organisms will meet several stress factors at the same time. In this connection it is hypothesised that the presence of pollutants in the environment may alter climatic tolerance limits and hence an organisms potential geographic distribution. The corollary to this is that the exposure of an organism to natural stress may increase the toxicity of pollutants in its environment. The idea of studying the interactions between dominant environmental stress factors and environmental contaminants seems therefore to have obvious ecological significance.

Toxic compounds may interfere with several physiological mechanisms important for tolerance to cold and drought. One significant effect is the destabilisa-
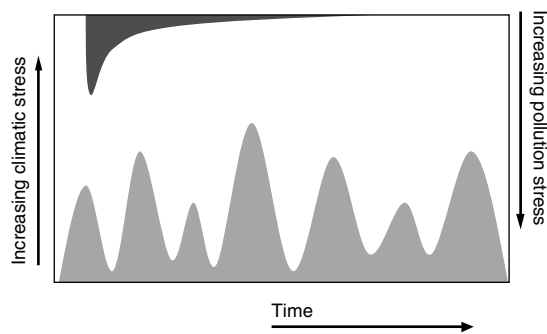
tion of membranes. It has been shown that membranes of muscle cells of the blue mussel *Mytilus edulis* become more permeable for Na^+ and K^+ when the mussels were exposed to an oil dispersant (Aarset & Zachariassen 1983). Likewise, it has been shown that copper and several other contaminants impair the retention of a dye ("neutral red") by lysosomes from coelomocytes of the earthworm, *Lumbricus rubellus* (Weeks & Svendsen 1996). Non-ionic organic pollutants may be absorbed by cell membranes due to their lipophilic characteristics. Many of these compounds are thought to have toxic effects due to their presence in high enough concentrations in membrane lipids and interactions with membrane proteins, and not due to a specific interaction with a receptor (Moriarty 1983; Chaisuksant *et al.* 1999).

Membrane permeability and stability may be crucial factors when soil invertebrates are exposed to freezing temperatures or desiccation because both situations will cause substantial dehydration of the animals (see previous sections). Dehydrated animals may be particularly vulnerable during thaw or post-drought rainfall because in these situations they may experience a substantial hypoosmotic shock with the associated risk of cell swelling and lysis (**5**). Contaminants may therefore increase the detrimental effects of this hypo-osmotic shock. No matter what the particular mechanism may be, several studies have recently shown that significant

Figure 19. A simple presentation of the "tolerable amount of stress" an organism may encounter in its habitat. The lower curve (light grey area) symbolises a seasonally changing climatic stress level. This could be low winter temperature alternating with summer drought, both of which reduce the ability of the organism to endure additional stress. The upper curve (dark grey area) depicts toxic stress of an introduced pollutant. The toxic stress is reduced with degradation of the toxicant. Under certain conditions, the simultaneous occurrence of both stress types may cause an environmental bottleneck, where the added stress effects can cause a major reduction in population levels. This will be even more likely if synergistic effects are present. Modified from (**19**).

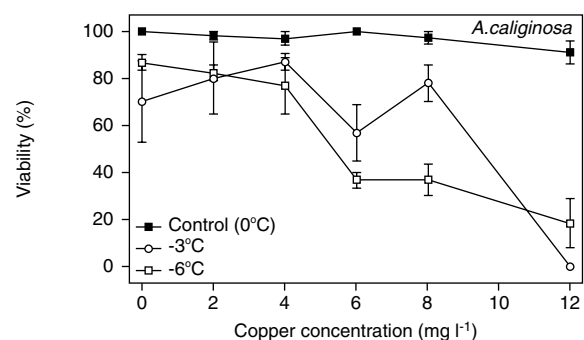**Figure 20.** Combinations of copper exposure and cold stress can result in synergistic effects. Earthworm cocoons (*Aporrectodea caliginosa*) were first exposed to aqueous $CuCl_2$ solutions for 14 days, and then cooled in soil at -3 or -6°C for 14 days. The copper concentrations used had no detrimental effects on viability if cocoons were not exposed in frozen soil after copper exposure. Data from (**18**).

interactions between drought and toxic stress do exist. Using Collembola and earthworms as models, it has been shown that copper (**6**, **18**, **21**), detergents (**6**, **20**) and polycyclic aromatic compounds (PACs) (Sjursen *et al.* 2001) may have significant impacts on the drought tolerance of these organisms.

For example, the viability of *A. caliginosa* cocoons is not influenced by copper ions at the concentrations used in the experiment reported in Figure 20. However, when a subsequent cold stress is added significant synergistic effects appear due to the combination of the two stress types. Based on results from a full factorial design experiment using the collembolan *F. candida* subjected to nonylphenol (NP) and a subsequent drought stress it was possible to model the influence of any concentration of NP on the drought tolerance (**20**). The results show, under these experimental conditions, that a soil concentration of 100 mg NP kg^{-1} would increase the RH causing 50% mortality from about 98% RH to 99% RH, and thus have a drastic effect on the drought tolerance of this species (Fig 21). Further investigations revealed that NP had a detrimental effect on at least some of the physiological mechanisms underlying the animal's drought tolerance (permeability of the integument and SP accumulation), suggesting that the observed synergistic effects are likely to occur in other species and as a result of other toxic compounds.

Figure 21. The estimated effect of an 8-day exposure to nonylphenol on the drought tolerance (8-day exposure) of *Folsomia candida*, expressed as the relative humidity causing 50% mortality (LRH$_{50}$). Data from (**20**).

So far, relatively few laboratory studies have been published on this topic. However, the potential for synergistic effects may be present under field conditions, not only for earthworms and Collembola but also for other soil animals in polluted areas. This is probably most important in extreme climatic zones (e.g. the subarctic) where the frequency and intensity of ecological crises are highest. The ecological implications of this could be that pollution in such regions plays a much more dramatic role for the local extinction or geographical boundaries of species than in more benign climates. Along this line it should be noted that ecotoxicological risk assessments in many cases are based on laboratory studies where the test

organisms have optimal temperature and moisture conditions. The involvement of combination effects in ecotoxicological tests could therefore improve risk assessment of soil polluting compounds and play a role in the extrapolation from laboratory experiments to the field.
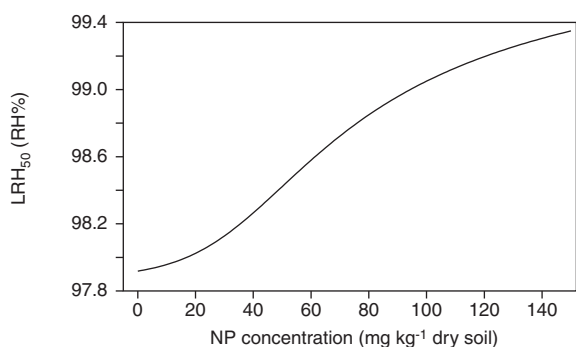
Conclusions

The findings of the work presented in this dissertation include the discovery of a new and alternative over-wintering strategy in freeze avoiding animals where supercooling is of little significance. The small and permeable soil invertebrates that have evolved this strategy dehydrate in frozen soil and equilibrate the vapour pressure of their body fluids to match the vapour pressure of ice at any temperature. During this process the animals are severely dehydrated. This dehydration induces a synthesis of sugars and polyols, which protect cells and tissues against damaging effects of dehydration. The principal studies on which this theory is built are reported in papers (**1**, **2**, **3**, **4**, **5**, **8**, and **17**).

In papers (**7**, **9**, **11** and **23**), I have described freeze tolerance in adult earthworms from the Arctic. This is the first physiological study of freeze tolerance in oligochaete worms. The freeze tolerant *Eisenia nordenskioldi* will immediately produce a high concentration of glucose once ice formation is initiated at a high subzero temperature. The species tolerate freezing down to -30°C. It is interesting to note that the physiology of this earthworm is similar to that of freeze tolerant frogs and salamanders. In paper (**16**) preliminary results suggest that a freeze tolerant Arctic enchytraeid, *Fredericia ratzeli*, also possess this physiological mechanism. I propose that freezing-induced cryoprotectant synthesis is probably a common feature of all freeze tolerant semi-terrestrial (or semi-aquatic) ectotherms, because it is most advantageous to defer mobilisation of cryoprotectants until the moment where they are needed.

Traditionally, hygrophilic Collembola are thought to survive desiccation by migrating to moister micro-habitats during summer drought. The studies found in papers (**10**) and (**15**), however, show that three common species are able to survive water potentials in the soil where plants would normally wilt. These Collembola adjust their osmotic pressure by producing sugars and polyols to re-establish the normal hyper-osmotic status in relation to their environment. The result is that the inward bound vapour gradient enables the animals to increase their water content by passive absorption. This is the first description in arthropods of a type of water vapour absorption, which in essence is passive, but mediated by accumulation of sugars and polyols. Effects of pre-acclimation to a mild drought stress are described in papers (**13**) and (**14**). Here it was shown that pre-acclimation will enhance the tolerance of subsequent severe drought, and increase the tolerance to cold shock in the species *Folsomia candida*. The latter support the notion that dehydration tolerance and cold tolerance in these organisms are highly linked. In contrast to hygrophilic Collembola and earthworm cocoons, postembryonic earthworms do not accumulate sugars and polyols in response to drought. However, earthworms become inactive and enter estivation at much milder drought conditions than seen in Collembola. These issues are described in detail for the species *Aporrectodea caliginosa* in papers (**12**) and (**21**).

In four papers (**6**, **18**, **19** and **20**) I have treated the interactions that may occur between pollution and climatic stress. It was hypothesised that pollution can reduce an organism's tolerance to drought and cold. Experiments that were carefully designed to investigate this showed that synergistic effects were evident and, depending on the level of pollution, could be substantial for a number of common environmental pollutants.

In conclusion, the series of papers forming this dissertation are aiming at understanding the physiological adaptations to cold and drought evolved by permeable soil invertebrates. Cold and drought are the most important environmental stress types determining the distribution and functioning of soil invertebrates. The new theories presented here will be valuable in future studies of soil biology.

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Dansk resumé

Jordbundsdyr er utrolig vigtige brikker i jordøkosystemets nedbrydning af dødt plantemateriale, og dermed frigivelsen af plantetilgængelige næringsstoffer. Jordbundsdyrene findeler det døde plantemateriale, fordøjer en del af det, men øger også materialets overflade således at bakterier og mikrosvampe har en bedre mulighed for at omdanne det videre. Desuden græsser jordbundsdyrene på jordens svampe og bakterier, og sørger derfor for en høj vækstrate af disse vigtige nedbrydere af dødt organisk stof. Kendskab til jordbundsdyrs tilpasninger til ugunstige klimatiske forhold er en forudsætning for at forstå deres økologi og beregne effekten af varige klimaændringer på jordbunden.

I denne afhandling er der fokuseret på dyrenes tilpasninger til ekstreme situationer som tørke og kulde, fordi disse forhold er afgørende for populationsdynamik, spredning og kolonisering af nye områder. Som modeller for dette arbejde er brugt springhaler (collemboler) og regnorme, som udgør to af de væsentligste brikker i jordbundssystemet. Formålet var at forstå de økofysiologiske mekanismer som dyrene har udviklet for at kunne tolerere stress.

Jordbundens talrige arter af springhaler, regnorme, enchytraeider, rundorme osv. er karakteriseret ved at deres hud er meget gennemtrængelig for vand, og at de derfor vil dehydreres hurtigt hvis de flyttes ud af jordens fugtige atmosfære og udsættes for udtørring i luften. Jordens vandforhold er derfor suverænt den vigtigste parameter for jordbundsfaunaens aktivitet og trivsel. Faktisk har disse dyr fysiologisk set større ligheder med ferskvandsdyr end man lige skulle tro, selvom de oftest betragtes som terrestriske livsformer. Den russiske videnskabsmand, Ghilarov, indså dette allerede i 1949, hvor han foreslog at jordbunden har fungeret som en evolutionær mellemstation i udviklingen af akvatiske arter til "ægte" terrestriske dyr. Selvom jorden for en stor del af tiden har en poreluft mættet med vanddamp, kan der fra tid til anden opstå udtørringstress i jorden. Under tørke vil planterne optage vand fra jorden samtidig med at der sker fordampning fra jordoverfladen. På et tidspunkt vil planterne ikke være i stand til at optage mere vand fra jorden fordi vandpotentialet i jorden bliver ligeså højt som det osmotiske potentiale i planten. Her er "den permanente visnegrænse" nået, hvilket tilsvarer et vandpotentiale (eller sugetryk om man vil) af størrelsesordenen -15 bar. Springhalernes normale osmotiske tryk er ca. -8 bar, og der er derfor en trykgradient mellem dyret og jorden, der bevirker en nettotransport af vand ud af dyret. Ved -15 bar er den relative fugtighed ca. 98.9%,

hvilket ikke umiddelbart lyder som et særlig kraftigt tørkestress, men fordi jordbundsdyr er så "utætte", vil de miste vand meget hurtigt ved denne fugtighed. Nu skulle man tro at jordbundsdyr ikke tolerer tørke i det hele taget, men faktisk har de meget effektive fysiologiske mekanismer der sætter dem istand til at klare selv kraftig tørke i jorden. Mange jordbundsdyr kan gå i en slags dvale i et mere eller mindre dehydreret stadie, når tørken optræder, men hos de ægte jordlevende (euedafiske) springhaler findes en mekanisme, der sætter dem istand til at optage vanddamp fra jordens poreluft, selv ved et meget kraftigt tørkestress.

Når springhalen *Folsomia candida* udsættes for et kraftigt tørkestress i jorden, f.eks. -25 bar (98.2% relativ fugtighed), vil dyret meget hurtigt dehydreres og blive forholdsvis inaktivt. Men i løbet af få timer begynder dyret at akkumulere sukkerstoffer og sukkeralkoholer hvorved det er i stand til at øge sit osmotiske tryk så meget, at dyret igen bliver hyperosmotisk i forhold til den atmosfære hvori det befinder sig. På den måde kan *F. candida* gendanne den normale gradient i vanddamptryk i forhold til omgivelserne, således at dyret begynder at optage vand i form af vanddamp fra atmosfæren. Der er ikke tale om aktivt (energikrævende) optag af vanddamp fra atmosfæren, sådan som det er kendt fra visse insekter, f.eks. melorme, kakerlakker, lopper m.fl., men derimod passivt vandoptag, sandsynligvis over hele den tynde og gennemtrængelige kutikula. Under disse forhold kan *F. candida* fuldstændig genoprette sit oprindelige vandindhold i løbet af 5-7 dage, og i løbet af denne periode bliver dyrene aktive igen, og får deres normale udseende tilbage. Selvom springhalerne ikke er særlig tørketolerante i sammenligning med "ægte" terrestriske leddyr, har de altså en bemærkelsesværdig tilpasning til netop de tørkeforhold, de kan komme til at opleve i jorden. De er således meget elegant tilpasset de vandpotentialer som planter er istand til at tolerere.

Vekselvarme dyr, der i tempererede og arktiske områder jævnligt udsættes for temperaturer under smeltepunktet af deres kropsvæsker, har udviklet forskellige fysiologiske og biokemiske tilpasninger til enten at undgå isdannelse i vævene, fryseundvigelse, eller til at overleve, at store dele af ekstracellulærvæsken fryser til is, frysetolerance. Disse to overordnede strategier drejer sig i vid udstrækning om at kontrollere, eller undgå, isdannelse. Tilpasningerne består bl.a. i (**1**) syntese af makromolekyler som "antifryseproteiner", først beskrevet fra antarktiske fisk og i melormen *Tenebrio molitor*, og "isnukleatorer",

som virker som vækstpunkter for is i frysetolerante dyr, samt (**2**) akkumulering af lavmolekylære stoffer som sukkerarter, sukkeralkoholer og frie aminosyrer under ét kaldet "cryoprotektiver". De fryseundvigende arter overvintrer ofte i en underafkølet tilstand, hvor de er i stand til at holde deres kropsvæske flydende i lange perioder, selvom temperaturen er lavere end kropsvæskens smeltepunktet. Underafkøling er ikke en stabil tilstand fysisk set, og der er derfor en tids- og temperaturbetinget risiko for at der sker spontan isdannelse, som altså er dødeligt. De frysetolerante arter kan inducere og tolerere en isdannelse i deres ekstracellulære kropsvæske på et tidligt tidspunkt (ved en "høj temperatur"), og på den måde kontrollere isens vækst under udfrysningen.

De jordbundsdyr der behandles i denne afhandling har en helt anden mekanisme til overlevelse af frost i jorden, end den vi kender fra f.eks. terrestriske insekter. Som i eksemplet med tørketolerance er det et vigtigt træk hos jordbundsdyrene, nemlig den meget vandgennemtrængelige kutikula, der er afgørende for mekanismen. Godt nok kan jordlevende springhaler ikke overleve isadnnelse i vævene, men de baserer ikke deres overvintring på underafkøling. I stedet mister de vand, når jordvandet fryser til is. Dette skyldes at vanddamptrykket over is er mindre end damptrykket over underafkølet vand ved den samme temperatur. Smeltepunktet af dyrenes kropsvæske er defineret af damptrykket af is, når der er en ligevægt mellem is og opløsning i sådan et system. Det vil omvendt sige, at sålænge smeltepunktet af dyrenes kropsvæske er højere end temperaturen af den omgivende is i jorden, vil der være en (ikke ringe) trykforskel mellem dyret og isen, som bevirker en nettotransport af vand ud af dyret. Induceret af dehydreringen sker der en kraftig akkumulering af sukkerstoffer og sukkeralkoholer i organismen. Herved sænkes dyrets smeltepunkt, ikke mindst som følge af opkoncentrering af opløste stoffer under vandtabet. I afhandlingen er det vist for den arktiske springhale, *Onychiurus arcticus*, og kokoner (ægkapsler) af regnormen *Dendrobaena octaedra*, at sænkningen af organismens smeltepunkt sker lige så hurtigt som temperaturen falder i deres miljø. På den måde er dyrene stort set aldrig underafkølede, og de er derfor beskyttede mod intern isdannelse, som ellers ville være dødeligt. Denne strategi er derfor betinget af at organismen kan tolerere et kraftigt vandtab. Men dette er også tilfældet, for både den arktiske springhale og regnormekokoner tåler at miste praktisk taget alt det osmotisk aktive vand, altså alt det vand der ikke er bundet til cellestrukturer og proteiner. Dette svarer til et vandtab på op til 85% af det totale oprindelige vandindhold.

Hos jordbundsdyr er der derfor fælles træk i responsen til tørke og kulde, som evolutionært set kan påstås at udgøre et fælles selektionstryk. Begge former for stress resulterer i udtørring som dyrene tolererer ved bl.a. at danne sukkerstoffer og sukkeralkoholer. Opdagelsen af disse mekanismer kan på længere sigt måske også forklare springhalernes vide udbredelse i arktiske områder, fordi evnen til at overleve så ekstrem udtørring åbner mulighed for spredning over store afstande med vinden.

Af andre betydningsfulde stressfaktorer skal også nævnes forurening, som i sig selv kan have effekter på jordbundsdyrene. I forbindelse med klimastress er det interessant at undersøge betydningen af toksiske stoffer for dyrenes evne til at tåle naturligt stress som tørke og kulde. Når kombinationer af disse faktorer undersøges, viser det sig, at der opstår synergistiske effekter. Dette kan betyde at forurening generelt har en større effekt under forhold hvor jordbundsdyr tillige er stresset af tørke eller kulde.

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The distribution and functioning of soil invertebrates is above all dictated by climatic factors such as temperature and water availability. In this doctor's dissertation including 23 original papers the problems of how soil animals are adapted to cold and drought are addressed. Using hygrophilic collembolans and earthworms as models, a cold hardiness strategy is described which is not based on the well-known ability to supercool, but on a dehydration mechanism that enables the animal to adjust the body fluid melting point to the temperature of the surroundings. Also the drought tolerance of Collembola is examined, and a mechanism is demonstrated by which these animals can adjust their osmotic pressure to remain active within the limits of water potentials tolerable to plants, and absorb water vapour from the soil atmosphere. Chemical pollution originating from the activities of humans should also be considered as an important environmental factor. Pollution in itself can have negative effects on the functioning of soil organisms. In the present work it is investigated if and how pollution can have an impact on the defence mechanisms against climatic stress such as drought or cold.

Strategies for cold and drought tolerance in permeable soil invertebrates