Sindre Andre Pedersen

Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor*

- a study on possible competition for the semi-essential amino acid cysteine

Thesis for the degree philosophiae doctor

Trondheim, January 2007

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
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II. **Pedersen S.A.**, Kristiansen E., Hansen B.H., Andersen R.A., Zachariassen K.E. 2006 Cold hardiness in relation to trace metal stress in the freeze-avoiding beetle *Tenebrio molitor*. *J Insect Physiol* 52: 846-853.

III. **Pedersen S.A.**, Kristiansen E., Andersen, R.A., Zachariassen, K.E. Isolation and preliminary characterization of a Cd-binding protein from *Tenebrio molitor* (Coleoptera). Preliminary accepted in *Comp Biochem Physiol C*.

IV. **Pedersen S.A.**, Kristiansen E., Andersen, R.A., Zachariassen, K.E. Cadmium exposure of the beetle *Tenebrio molitor* (L): Internal distribution of Cd and induction of a Cd-binding protein in the gut content. Manuscript.

1. Introduction

1.1. General

In their natural environment animals are confronted by both physical (e.g. extreme temperatures, desiccation) and chemical stressors (e.g. pollutants). Stress may be defined as a condition that is evoked in an organism by one or more environmental factors that bring the organism near to or over the edges of its ecological niche (van Straalen 2003). Various defence systems exist to cope with different forms of stress and restore homeostasis. Often, production of various proteins or enzymes are involved in these defence systems (Korsloot et al. 2004). Since an organism's resources may be considered to be limited, the ability to restore homeostasis depends on the severity of the different forms of stress it experiences. It has been proposed that pollutants present in the environment may alter the ability to respond to climatic stressors like e.g. low temperature, desiccation (Holmstrup 2002).

This work deals with the possible consequences of combined stress from metal exposure and low temperature in cold hardy insects. Many of these insects produce so called *antifreeze proteins* that protect them from lethal freezing. *Metallothioneins* are metal binding proteins that are considered to be important in detoxification when animals are exposed to metals. Metallothioneins and most forms of antifreeze proteins from insects are known to contain unusually high amounts cysteine. Cysteine is considered to be semi-essential, since it must be derived from the essential amino acid methionine (Choen 2004). Induction of one of these two types of proteins may potentially deplete the cysteine pool and thus reduce the capacity to produce the other type. Alternatively, the animals might have evolved other structures to avoid a potential competition for cysteine. The purpose of the present work was to explore these possible scenarios.

1.2. Metals

Metals are natural constituents of the environment and have played key roles in living organisms since life evolved some 3.5 billion years ago. Metals are essential in processes like photosynthesis, respiration, signalling, active transport and as electrolytes in biological systems (Butler 1998). They are required as co-factors in nearly one third of all known

enzymes (Voet et al. 1999). The list of metals identified as essential to animals so far includes: calcium, magnesium, potassium, sodium, iron, copper, manganese, zinc, cobalt, molybdenum, selenium and nickel (Anke 2004). Metals that are required in relatively high levels (gram(s) pr. kilogram dry mass of food; calcium, magnesium, potassium and sodium) are often referred to as *macro metals*, while those that are required in relatively low levels (mg(s) pr. kilogram dry mass of food; manganese, zinc, cobalt, molybdenum, selenium and nickel) are often called *trace metals* (Anke 2004).

All metals, essential or not, may cause damage if they are present in sufficiently high quantities (Bertrand 1912). Metal toxicity typically occurs when metals interfere with biochemical reactions where they are normally not involved (Hopkin 1989). This often involves replacement of other metals that act as co-factors or are normally associated with a particular protein or enzyme. To avoid such effects, organisms maintain the concentration of free metal ions in their cells within certain limits. In general, the metal concentrations where toxic effects occur are higher for the essential metals (e.g. zinc and copper) than for the non-essential metals (e.g. cadmium and mercury) (Burk and Levander 2006). Since the present work has focused on the effect of exposure to cadmium (Cd), copper (Cu) and zinc (Zn), the rest of this section will be to these metals.

1.2.1. Cadmium, copper and zinc in biological systems

Locally, metal pollution may arise from natural processes like weathering of metal-rich rock (e.g. Hågvar and Abrahamsen 1990), but in most cases human activities like mining, smelting, agriculture and combustion of fossil fuels are involved (for a review see Hopkin 1989).

Zink (Zn) is an essential metal for cell proliferation and differentiation. Zn is a structural constituent of many metabolic enzymes, transcription factors and cellular signalling proteins (for a review see Stefanidou et al. 2006), and increasing evidence suggest that it may also be directly involved in cellular signal transduction (Beyersmann 2002). The human daily dietary requirement for Zn is estimated to be 15 mg pr. day (Tapiero and Tew 2003) and the upper tolerable intake level is 25 mg pr. day (SCF 2003). The toxicity of Zn is generally considered to be low, meaning that ingestion of high doses of this metal seldom lead to adverse long-terms consequences in humans (Brown et al. 1964).

Copper (Cu) is an essential metal that may exist in a reduced (Cu^{+}) or an oxidised divalent state (Cu^{2+}). By serving as an acceptor and donor of electrons, Cu plays a role as a catalytic co-factor in a number of metalloenzymes including Cu/Zn superoxide dismutase (antioxidant defence) and cytochrome c oxidase (mitochondrial respiration). However, if not regulated properly by the cells, Cu may cause various kinds of damage due to its reactive nature. One such effect may be generation of reactive oxygen species, via the Fenton reaction, which in turn can damage DNA and other cellular structures (Halliwell and Gutteridge 1984). Cu may also inactivate enzymes by displacing other co-factors (Predki and Sarkar 1992). In humans the recommended dietary allowance is 0.9 mg Cu pr. day and the tolerable upper intake level has been set at 10 mg pr. day (DRI 2001).

Even though cadmium (Cd) is non-essential, this metal is easily taken up by cells via Ca^{2+} channels due to the similar ionic radii of the two metals (Beyersmann and Hechtenberg 1997). Cd is extremely toxic and mutagenic (McMurray and Tainer 2003). The tendency of Cd to form lipid soluble inorganic complexes means that this ion easily crosses the microvillus border of the digestive tract in animals (Gutknecht 1981). Inside the cells, Cd may cause damage by binding to cytosolic and nuclear material, with effects such as inhibition of Zn-containing enzymes, lipid peroxidation and disturbance of protein synthesis (Beyersmann and Hechtenberg 1997).

1.3. Uptake and detoxification of metals in terrestrial arthropods

With the exception of small soil-dwelling arthropods like collembolans, where uptake of metals from the pore water may also be important, diet is the main source of metals in terrestrial arthropods (Posthuma and Van Straalen 1993; Vijver et al. 2003). Generally, the accumulated metals become enriched in certain tissues instead of being evenly distributed throughout the body (Posthuma and Van Straalen 1993). For instance, in isopods like *Porcellio scaber* and *Oniscus asellus* most of the metals are found in association with the midgut gland (hepatopancreas) (Dallinger 1993). In most insect orders the midgut seems to be the main site for both absorption and accumulation of metals. Based on this, the midgut has been proposed to act as a barrier against transport of potentially toxic metals from the gut content to the haemocel (Balland-Dufrancais 2002). However, in some insect orders like Orthoptera and Dictyoptera, no midgut barrier seems to exist and the anterior hindgut (ileum) seems to be the most important structure for storage of metals (Ballan-Dufrancais 2002).

1.3.1. Compartmentalization of metals

As metals by their nature cannot be metabolised, detoxification of metals that have entered the body must involve some form of sequestration. Two forms of metal sequestration are recognised; incorporation of metals in membrane enclosed vesicles (compartmentalization) and binding of metals to cytoplasmic proteins (Dallinger 1993; Vijver et al. 2004). Vesicles packed with different metals have been observed in all metal accumulating tissues in terrestrial invertebrates (Posthuma and Van Straalen 1993). Two main forms of vesicles can be distinguished; the spherocrystals which are small concretions where metals are organised in concentric layers on an organic peptoglycan stroma, and the mineralised lysosomes with concretions that lack the organised concentric layers (Ballan-Dufrancais 2002). Elimination of metal-rich vesicles through apocrine secretion has been observed in some metal exposed insect species (Ballan-Dufrancais 2002; Lauverjat et al. 1989). However, elimination does not seem to be an absolute requirement since no such process has been observed in the metal tolerant cockroach *Blatella germanica* (Ballan-Dufrancais 2002).

1.3.2. Metal binding proteins

Sequestration of metals by metal binding proteins is regarded as important in the defence against metal toxicity. The most studied type of metal binding proteins is the metallothioneins (MTs), a group of cytosolic low molecular weight proteins that are characterized by a high cysteine content (up to 33% of the residues). Since their discovery in horse kidney by Margoshes and Vallee (1957), MTs have been identified in vertebrates, invertebrates, plants, fungi and prokaryotes (Stephan et al. 1994). While being ubiquitous in vertebrate tissues (Hamer 1986), MTs have only been unequivocally identified (based on primary structure) in two terrestrial arthropods; *Drosophila* flies (Lastowski-Perry et al. 1985; Mokdad et al. 1987; Stephan et al. 1994) and the springtail *Orchesella cincta* (Hensbergen et al. 1999).

In some terrestrial arthropods no production of MTs has been observed in relation to metal exposure (Stone and Overnell 1985). In some of these species Cd-binding proteins without a high content of cysteine have been found to be induced. Induction of a Cd-binding glycoprotein with only ~ 2 % cysteine was found in the stonefly *Pteronarcys californica* by Clubb et al. (1975). Similarly, Martoja et al. (1983) found that Cd-exposure induced a Cd-binding glycoprotein with low cysteine content (2.1 %) in the locust *Locusta migratoria*. In

the isopod *Porcellio scaber* Dallinger et al. (1993) found a Cd-binding protein with low cysteine content (2.9 %) and an amino acid composition similar to that of the two glycoproteins. However, since all these proteins have been purified from extracts of whole animals, the internal localization is unknown and their functional role in detoxification of metals remains unresolved.

1.4. Cold hardiness in relation to metal exposure

Winter temperature is considered to be one of the most important determinants of the distribution of ectothermic species (Hoffman and Parson 1991). Among invertebrates that are exposed to sub-zero temperatures, two main strategies for cold hardiness are recognised (Zachariassen 1985; Duman 2001). Freezing is lethal to those species that have adopted the *freeze avoidance* strategy. These species rely on supercooling when they are exposed to temperatures below the equilibrium freezing point (melting point) of their body fluids. In the second strategy, *freeze tolerance*, internal ice formation is tolerated by limiting this to the extracellular fluids. A special case of freeze avoidance, often referred to as *protective dehydration*, is also recognised among some soil-dwelling ectotherms. In these species supercooling is avoided by allowing the melting point of the body fluids to fluctuate passively, according to the ambient temperature, by evaporative loss or gain of water from the surrounding environment (Holmstrup and Westh 1994).

Pollutants have been shown to reduce the tolerance to low temperatures in cold hardy invertebrates (Holmstrup et al. 1998; Holmstrup et al. 2000; Bindesbøl et al. 2005). So far, the effects of metals on cold hardiness have been studied in only a few invertebrates. Zachariassen and Lundheim (1995) observed a reduced catabolism of glycerol, accumulated during overwintering, when micro injections of Cd or Cu was administered to the freeze avoiding beetle *Rhagium inquisitor* undergoing warm acclimation. Glycerol is an important cryoprotectant in this species, providing a colligative reduction in the equilibrium freezing point (melting point) of the body fluids down to ~ - 6°C (Zachariassen 1973). The effect of metals exposure on glycerol production was not tested, but if enzymes involved in glycerol formation at low temperature were inhibited, the metals might reduce the capacity to induce cold-hardening (Zachariassen and Lundheim 1995). In the collembole *Folsomia candida*, Cu-exposure was found to reduce the tolerance to desiccation, possibly due to increased concentration of the toxicant in the body fluids (Holmstrup 1997). Since the cold tolerance of

hygrophilic collembola depend on protective dehydration, it is also likely that the Cu-exposure may reduce their cold tolerance (Holmstrup et al. 2000). Studies examining the survival of freeze tolerant earthworms and their cocoons, after Cu-exposure, have provided more direct evidence that metal exposure may reduce cold tolerance (Bindesbøl et al. 2005; Holmstrup et al. 1998).

Many freeze avoiding insects produce so called antifreeze proteins (AFPs) (Zachariassen 1985; Duman et al. 2004). These proteins are defined by their ability to separate the freezing and melting temperature of ice. Their effect may be observed by cooling a sample containing AFPs in the presence of a small ice crystal. Normally, when the temperature of such a sample is lowered, the ice crystal will gradually grow, so that the melting point of the unfrozen fraction is equal to the sample temperature. In the presence of AFPs however, no ice growth occurs when the temperature is lowered, down to a certain temperature, where a sudden explosive ice growth takes place. This phenomenon is referred to as *thermal hysteresis*, and the temperature where the ice growth occurs is called the *hysteresis freezing point*. The temperature interval between the stabilization temperature and the hysteresis freezing point is called the *hysteresis activity*, and refers to the strength of the thermal hysteresis. Thermal hysteresis is believed to be caused by adsorption of AFPs to the ice crystals (Raymond and DeVries 1977; Kristiansen and Zachariassen 2005). The function of AFPs in terrestrial arthropods appears to be to promote supercooling by preventing inoculative freezing across the body wall (Gehrken 1992; Olsen et al. 1998) and masking of ice nucleating structures within the body fluids (Olsen and Duman 1997a, b).

1.5. The model organism: *Tenebrio molitor* Linnaeus (Yellow mealworm)

The beetle *T. molitor* belongs to the family Tenebrionidae of the suborder Polyphaga. It is one of the most important pests on stored grain products. Even though the species has a cosmopolitan distribution today, it is considered to be indigenous to Europe (Metcalf and Flint 1951), where rotholes of deciduous trees are assumed to be their primary habitat (Palm 1959). The species is univoltine and produces 200 - 300 eggs that hatch into yellow coloured larvae. Over a period of 3 months or more the larvae grow up to 30 mm in length and progress through several instars varying from 8 to more than 20 (Cotton and St. George 1929). They overwinter as quiescent larvae, which in the spring move to the surface, moult to the prepupal stage and develop into pupae in a few days. The pupal stage last approximately 3 weeks

before pupae develop into black beetles in the summer. The length of the life cycle depends on food availability and the environmental conditions. The shortest period from egg to adult stage is about 120 days. Under optimal laboratory conditions (25°C, 60-70 % RH, 16:8 L:D cycle) the time for completion of its life cycle is about 6 months (Cotton and St. George 1929). *T. molitor* is popular as a model species for beetles because it is relatively easy to maintain cultures of them under laboratory conditions.

T. molitor is a freeze avoiding species, as freezing in any stage of development is lethal (Johnston and Lee 1990). Upon acclimation to winter conditions (low temperature, short day and low humidity) the supercooling point of the larvae is depressed from ~ - 7°C to ~ - 15°C (Patterson and Duman 1978). Accompanying the winter acclimation, the titre of AFPs in the hemolymph increases, causing an increased thermal hysteresis that is believed to be involved in the depression of the supercooling point of the larvae (Graham et al. 2000; Horwath et al. 1996; Patterson and Duman 1978). The first primary sequence of AFPs from *T. molitor* was determined by Graham et al. (1997), and further work by Liou et al. (1999) have revealed that the AFPs in this species constitute a family of isoforms that are rich in cysteine (21 - 25% of the residues).

2. Aims and hypothesises of the present study

The main objective of the present work was to investigate if metal exposure might interfere with the mechanisms for cold-hardening in insects or if strategies to avoid such problems might have evolved. In order to achieve this objective, the following second-order objectives were addressed.

1. The physiological mechanism behind cold hardiness in insects was reviewed. Emphasis was placed on how cryoprotective mechanisms may affect the ability of animals to deal with metal exposure, and how defence mechanisms against metal exposure could interfere with cryoprotective mechanisms (**Paper I**).

2. To see if accumulation of high levels of MTs might reduce the capacity to produce AFPs, protein level and gene expression of AFPs was measured in larvae of the freeze avoiding beetle *T. molitor*, chronically exposed to different levels of Cd, Cu or Zn, and acclimated to summer or winter conditions (**Paper II**).

3. To determine whether high levels of MTs are accumulated in *T. molitor* in response to Cd-exposure, or if alternative strategies for dealing with metals might have evolved, molecular association of Cd in extracts of Cd-exposed larvae was investigated (**Paper III**). This was complimented by further investigation of the internal distribution pattern of Cd and Cd-binding protein(s) in *T. molitor* larvae during a 16 day period of exposure to Cd (**Paper IV**).

3. Summary of the individual papers

3.1. Paper I: Inorganic ions in cold hardiness

This paper reviews the role of inorganic ions in context of the two classical cold-hardiness strategies; freeze avoidance and freeze tolerance. The aim of the work was to give an overview of the different challenges posed by the two cold hardening strategies on ionoregulatory mechanisms, and how metals may interfere with the cryoprotective mechanisms of the two strategies. Since the objective of the present work concerns possible effects of metals on freeze avoiding organisms, the summary will focus on the parts of the paper that address this issue.

A possible scenario is presented where mechanisms against trace metal injury may affect the cryoprotective mechanism in freeze avoiding organisms, where production of AFPs is a part of the arsenal. Metal binding metallothioneins (MTs) and many AFPs may contain up to ~ 30% cysteine. Since cysteine occurs in relatively low levels in insects, the consumption of large amounts of free cysteine to produce MT could reduce the capacity of insects to produce AFPs. It was proposed that cold-hardy insects exposed to metals might develop AFPs without high cysteine content or to switch over to the freeze tolerance strategy.

3.2. Paper II: Cold hardiness in relation to trace metal stress in the freeze-avoiding beetle *Tenebrio molitor*

The aim of the study was to see if metal exposure could reduce the production of AFPs in a freeze avoiding insect. A metal-induced reduction in the capacity to produce AFPs would be consistent with the hypothesis from **Paper I**, that a high accumulation of cysteine rich MTs could reduce cysteine pool and thus reduce the capacity to produce cysteine rich AFPs. Gene expression was analysed as well as protein levels of AFPs since a metal-induced reduction in AFPs could potentially come about by reduced gene expression of the proteins rather than a reduced capacity to produce them.

Mealworm larvae (*T. molitor*) were dietary exposed either Cd (0.005, 0.05 or 0.5 mg g^{-1} d.w. wheat bran), Cu (0.1, 1, or 10 mg g^{-1} d.w. wheat bran), (0.5, 5 or 50 mg g^{-1} d.w. wheat bran),

or uncontaminated wheat bran (controls). The larvae were chronically exposed in the laboratory by keeping them under summer conditions (25°C and 18:6 h, L:D) for 3 months, at which stage they had developed into medium sized larvae. Chronically metal exposed larvae that were winter-acclimated were obtained by adding a one-month period at 4°C and short day conditions (18:6 h, L:D).

In the summer-acclimated larvae all levels of the different metals caused a reduction in the level of AFPs compared to the control group ($p < 0.05$). All groups, except those feed the lowest dose of Cd (0.005 mg Cd g^{-1} d.w.), also showed a significant reduction (0.5 mg Cd g^{-1} d.w., or 5 mg Zn g^{-1} d.w., or 0.1 mg Cu g^{-1} d.w., $p < 0.05$) or a statistical tendency towards reduced gene expression of isoform AFP YL-3 (0.05 mg Cd g^{-1} d.w., or 0.5 mg Zn g^{-1} d.w., $p < 0.1$). Among the winter-acclimated groups no differences between the levels of AFPs were apparent compared to the control group. Also, no difference in gene expression of AFP YL-3 was found between the metal exposed groups and the control group in these larvae.

3.3. Paper III: Isolation and preliminary characterization of a Cd-binding protein from *Tenebrio molitor*

The aim of the study was to isolate and characterise the Cd-binding protein(s) that were induced in response to Cd-exposure in *T. molitor* larvae. Special emphasis was put on revealing if accumulation of high levels of MTs was involved in the detoxification of Cd. The isolation procedure was therefore adapted to allow purification of low molecular, heat stable proteins like MTs.

Larvae exposed to Cd, and non-exposed controls, under laboratory conditions (25°C, L/D: 18/6 h) were obtained by feeding them Cd-contaminated wheat bran (0.5 mg Cd g^{-1} d.w.) and simple wheat bran with no metal supplement (controls), respectively.

Cd-binding capacity in extracts of larvae, measured by a Cd-saturation technique, was found to be approximately doubled in Cd-exposed larvae, compared to the controls ($p< 0.05$).
Ion exchange chromatography of the extracts revealed that most of the increase in Cd-binding capacity was due to the induction of a Cd-binding ligand which eluted as a distinct peak. Further analysis of the fractions corresponding to the Cd-peak with size-exclusion

chromatography revealed that a Cd-binding ligand with an apparent molecular mass of ~ 7500 Da was responsible for the Cd-binding.

Reverse phase chromatography and mass spectrometry of the fractions corresponding to the Cd-binding ligand showed the presence of a single component with a molecular mass of 7134.5 Da. Amino acid analysis of the ligand revealed a protein without cysteine residues. The mass spectra obtained by mass spectrometry of a tryptic digest of the protein showed no similarity to other described proteins. Sequence information from four peptide-fragments, obtained by *de novo* sequencing also showed no presence of cysteine.

3.4. Paper IV: Cadmium exposure of the beetle *Tenebrio molitor* (L): Internal distribution of Cd and induction of a low cysteine Cd-binding protein in the gut content

The aim of the study was to see if the Cd-binding protein purified from *T. molitor* (**Paper III**) might be involved in detoxification of Cd. This was done by examining the internal location and time dependency for induction of Cd-binding proteins during exposure to Cd.

Briefly, mealworm larvae (*T. molitor*) were dietary exposed under laboratory conditions (25°C, L/D: 18/6h) with wheat bran contaminated with 0.5 mg Cd g^{-1} d.w. over a 16-day period. During the course of the exposure period the molecular association of Cd in extracts prepared from whole alimentary tract extracts, gut wall tissue and gut content of larvae, were analysed using size exclusion and ion exchange chromatography. Also, the internal distribution of Cd in the larvae was analysed during the course of the exposure period.

Chromatography revealed the accumulation of a Cd-binding protein in the gut content, consistent with that of a Cd-binding protein of 7134 Da previously isolated in **Paper III**. The protein could be detected after 4 and 8 days, using ion exchange and size exclusion chromatography, respectively. No increase in the capacity to bind Cd was apparent in gut wall tissue extracts of Cd-exposed larvae. Accumulation of Cd in the gut wall tissue stabilized after 8 days of exposure, at a rather low level. There was a statistical trend ($P = 0.07$) towards Cd being incorporated in the gut content in a manner that was disproportionally high compared to the amount of Cd in the gut wall tissue, as the exposure period progressed.

4. Methodological considerations

4.1. Choice of study organism

The choice of the yellow mealworm *T. molitor* as the study organism had several reasons. Firstly, the AFPs from *T. molitor* contain the highest amount of cysteine known for this type of proteins. This made it an ideal candidate to test whether metal exposure might exhaust the cysteine pool and thus reduce the capacity to produce AFPs. Secondly, the mRNA sequence of the *T. molitor* AFPs are available. This made it possible to study the gene expression of AFPs. This was considered to be important since gene expression data together with protein levels would allow for discrimination between effects on AFP protein and effects on gene expression of AFPs. Thirdly, literature on the effect of environmental conditions (light, temperature, humidity) on AFP levels and gene expression of AFPs was available. Finally, in contrast to the other insects where the primary structure of AFPs is available, *T. molitor* is easy to maintain under laboratory conditions and has a relatively short generation time.

4.2. Choice of metals and exposure conditions

The choice of Zn, Cu and Cd (in **Paper II**) was based on the fact that these metals were known to both induce and bind to MTs (Coyle et al. 2002). The metal was administered via the food since ingestion is the most likely route for metal uptake in terrestrial arthropods like *T. molitor*, with water impermeable skin and wax coating (Vijver et al. 2003). Chronic exposure regimes, starting from when the larvae hatched, were chosen since the levels of AFPs have been reported to increase with larval development in *T. molitor* (Graham et al. 2000). The metal concentrations used were quite high compared to levels the larvae may normally experience in their diet. However, this was to ensure that the larvae received exposure levels sufficiently high to determine if a reduction in the capacity to produce AFPs could be imposed.

In the studies involving molecular associations of metals with metal binding proteins (**Paper III** and **IV**), the highest level of Cd (0.5 mg Cd g^{-1} d.w.) was chosen to expose the larvae. This decision was based on a preliminary screening of the effect of different levels of Zn, Cu or Cd-exposure on the Cd-binding capacity in the larvae.

4.3. Measurement of AFP protein levels

Direct determination of the molar antifreeze concentration could not be performed since no polyclonal antibodies that cross reacts with many of the AFP isoforms is available. Instead an indirect method was adopted (Graham et al. 2000), where the level of AFPs was estimated from the hysteresis activity. The hysteresis activity was measured on a Clifton nanolitre osmometer. Briefly, a hemolymph sample, suspended in paraffin oil, was frozen and carefully heated again until only a small ice crystal ("seed crystal") was left in the sample. After the crystal had been stabilized at this temperature (for 1 min.), the temperature was lowered again

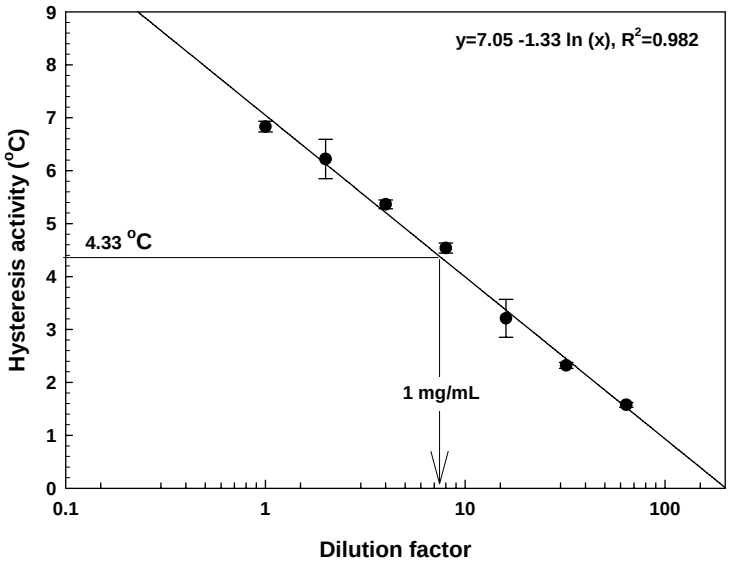

Fig. 1. Semi-logarithmic plot of hysteresis activity in pooled hemolymph from *T. molitor* larvae as a function of dilution in 0.9 % NaCl. Values are means ± SD of measurements of each dilution (n = 4). The corresponding dilution factor of the summer-acclimated control group was found by interpolation from the mean hysteresis activity (4.33°C).

(0.1 °C min^{-1}), until a rapid ice growth could be observed (hysteresis freezing point). The hysteresis activity was calculated by subtracting the stabilization temperature from the hysteresis freezing point. The hysteresis activity is not linearly related to the concentration of AFPs (Kristiansen et al. 2005; Zachariassen et al. 2002). Thus, to compare the AFP levels among the groups, the hysteresis activities were transformed to relative dilution factors (rDF)

according to the method described by Graham et al. (2000) as shown below. This involved making a standard curve based on the hysteresis activity at different dilutions (in 0.9% NaCl) of a standard hemolymph sample from *T. molitor* (pooled from control animals) (see Fig. 1).

The equation for the line in Fig. 1 was transformed so that the dilution factor (DF) for a sample could be calculated from the measured hysteresis activity (TH):

Equation 1 $$DF = e^{\left(\frac{TH - 7.05}{-1.33}\right)}$$

The relative dilution factor (rDF) was found by relating the dilution factor for each sample to the dilution factor (7.73) corresponding to the mean hysteresis activity (4.33°C) of the summer-acclimated control group:

Equation 2 $$rDF = \frac{7.73}{DF}$$

The thermal hysteresis activity corresponding to a given rDF may be obtained by using the equation:

Equation 3 $$\text{Hysteresis activity } (^{\circ}C) = 4.33 + 1.33 \ln(rDF)$$

When measuring hysteresis activity on the Clifton nanolitre osmometer it is important to standardize parameters like time for stabilization of the ice crystal (1 min.), rate for lowering the temperature (~ 0.1 °C min^{-1}), sample size (~ 20 nL) and size of the "seed crystal". To reduce the "noise" from these sources of variation each sample was measured repeatedly, until three values of hysteresis activity were obtained that differed maximally ± 0.5%. The mean of the three measured values was taken as the hysteresis activity of the sample.

Since the procedure for estimating the levels of AFPs is based on measurements of hysteresis activity, it is potentially sensitive to other factors that could influence the hysteresis activity of AFPs. Low molecular mass substances (e.g. glycerol), endogenous proteins, ice nucleators and some AFP forms have been reported to enhance the activity of AFPs *in vitro* (Wu and

Duman 1991; Olsen and Duman 1997a; Li et al. 1998; Wang and Duman 2005). The generally low hemolymph osmolality observed in the present work (see Table 1, **Paper II**) does not indicate high levels of polyols in the larvae. This is also supported in a study by Patterson and Duman (1978), where no significant levels of glycerol were found in *T. molitor*. Influence of low molecular solutes on the antifreeze activity in this study may therefore be considered unlikely. According to Graham et al. (1997) the high hysteresis activity observed *in vitro* for purified AFPs from *T. molitor* (> 5°C) also suggests that activators are not very important in this species. Even so, it cannot be excluded that these parameters might have influenced the hysteresis activity measured in **Paper II**.

4.4. Measurement of AFP mRNA levels

The measurement of gene expression of AFP mRNA in **Paper II** deserves a brief discussion. The real-time PCR technique that was used in this work involves amplification of double stranded cDNA which is selectively marked by a dye which turns fluorescent upon binding to double stranded DNA. The fluorescence level at a given PCR cycle reflects the amount of double stranded DNA present in the PCR reaction. Since a fixed amount of first-strand cDNA, derived from total mRNA is used in all reactions to be compared, the amount of double stranded DNA again reflects the level of target mRNA in the different samples.

As different AFP isoforms could potentially be differently regulated, an analysis of the gene expression of AFPs should ideally include as many of the different isoforms as possible. For this reason, several primer pairs for different AFP isoforms were designed and tested under different conditions. However, the desired specificity could only be obtained for AFP isoform YL-3 (gene bank accession no. AF160496). Specificity, in the sense that only the target cDNA is amplified, is an absolute premise for real time PCR, since the fluorescent dye will bind to any double stranded DNA amplified during the PCR. However, AFP YL-3 represents one of the main AFP isoforms in *T. molitor*, and other investigators have found the different isoforms to be regulated in a coordinated fashion (Graham et al. 2000). This suggests that YL-3 is representative for the gene expression of AFPs in *T. molitor*.

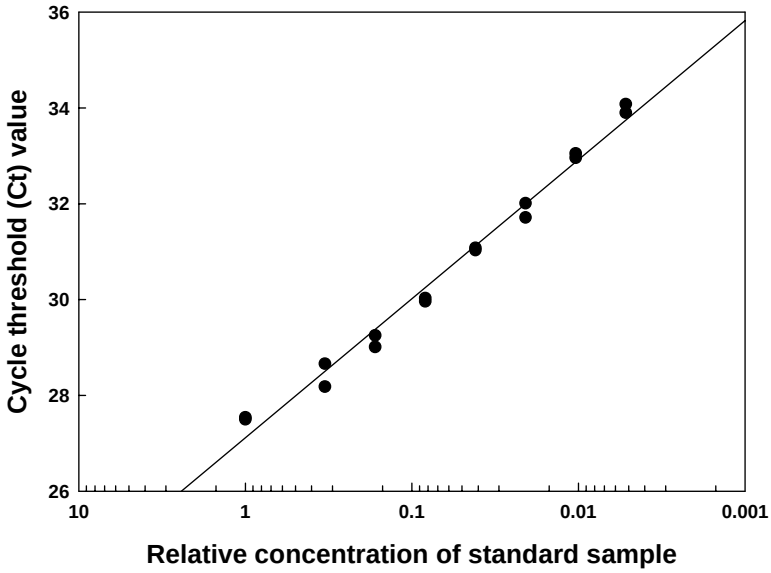

Fig. 2. Example of a standard curve for reading off the AFP mRNA level of a sample from the cycle threshold value. Duplicate measurements of each standard sample dilutions were used in all standard curves.

A standard sample (pooled mRNA from winter-acclimated control animals) was used to make a dilution series. Aliquots of these dilutions were included in each real-time PCR run to serve as standards from which concentration of the target sequence in the samples could be obtained (see Fig. 2). All concentrations were normalised to the mean transcript expression in the summer control group.

It cannot be excluded that inhibiting agents, possibly present in the standard sample, may have been diluted. This might have tended to increase the slope of the standard curve, thus underestimating the actual difference between samples. In retrospect it would have been preferable to use dilutions of plasmids cloned with the AFP YL-3 transcript as standards, to reduce the risk of this effect.

In real-time PCR the gene expression of target genes is often normalized against so called household genes. In *T. molitor* the sequences of two such genes were available at the time of the study: α-Tubulin and 18s rRNA. However, the kit used to make first-strand cDNA only targets mRNA templates with a poly-A tail, thus eliminating ribosomal RNA, like 18s, which lacks this tail. The other household gene considered, α-tubulin, having been shown to be

highly sensitive to metal exposure in insects (Mattingly et al. 2001), was also found to be unsuited for the study.

4.5. Purification of Cd-binding ligands

4.5.1. Marking Cd-binding ligands with ^{109}Cd

Addition of radioactive ^{109}Cd as a radiotracer to extracts prior to chromatographic steps is a method that is often employed in purification of proteins with a high affinity for Cd. Initially, this technique was attempted on whole animal extracts of *T. molitor* (**Paper III**). However, high variability in the elution profiles of Cd and "ghost peaks" (peaks that appear only some times) made it difficult to interpret the results. Treating the extracts with a synthetic cation exchanger (Chelex-100), after pre-incubation with ^{109}Cd solved the problems described above. The cation exchanger prevented "spillover" to ligands with low ^{109}Cd affinity by binding excess ^{109}Cd, not bound to high affinity ligands.

The method for administration of the radiotracer ^{109}Cd prior to chromatography (used in **Paper III**) was changed in Paper IV. By exposing the larvae directly to ^{109}Cd via the food (**Paper IV**), the chelex-step used in Paper III could be omitted (except for gut tissue) thereby avoiding the sample loss associated with this step (~33%). This decision was motivated by the modest size of the dissected tissues that were available for preparation of extracts, making it important to reduce the sample loss to a minimum. Prior to adopting the new method for administering Cd, it was verified that the two methods produced similar elution profiles for Cd (see Fig. 1B, **Paper III** and Fig. 2A, **Paper IV**).

4.5.2 Chromatography

Several precautions were taken to increase the yield of the Cd-binding protein in **Paper III**. Steps to reduce breakdown of the protein included addition of the protease inhibitor phenylmethylsulphonylfluoride (PMSF). Also, since many proteins may become more labile to degradation during freeze-thaw cycles after purification steps, the protein was concentrated by centrifugal concentration at 0°C rather than freeze drying. To increase the processing capacity for sample extracts, ion-exchange chromatography was used prior to size exclusion in the purification protocol. This modification allowed sample volume application to be

increased from 5 mL to 40 mL. However, despite these efforts, the yield of the Cd-binding protein in **Paper III** was still low and posed limitations to how much the protein could be characterized. In light of the finding in **Paper IV**, that the protein seems to be accumulated in the gut content, the yield of the protein could perhaps have been increased if only gut content had been used as starting point for the purification protocol rather than extracts prepared from whole larvae.

5. Discussion

To the author's knowledge, this is the first work where the effect of metal exposure on production of AFPs has been studied. Again, to the author's knowledge, this is the first time metal binding proteins have been studied in a beetle, even though this insect order contributes to about one third of all known living species.

5.1. Effect of metal exposure on production of AFPs

In response to winter acclimation the metal exposed larvae were able to attain the same level of AFPs as the control group (**Paper II**). Thus, the results in **Paper II** did not support the hypothesis in **Paper I**, that metal exposure could reduce the capacity to produce cysteine-rich AFPs, by tying up free cysteines in form of cysteine rich-MTs.

The results from **Paper II** also showed that metal exposure may reduce the production of AFPs in larvae during summer conditions. The reduced levels of AFPs observed in the metal exposed larvae seemed to come from reduced gene expression of AFP mRNA. Graham et al. (2000) found that gene expression and the level of AFPs increase with development in *T. molitor* larvae, reared under summer conditions. In fact, presence of AFPs (by measurement of hysteresis activity) under summer conditions has been observed in about half of the insect species known to produce AFPs (Duman et al. 1982). Even though there seem to be no obvious reason why animals should have AFPs in their body fluids during summer conditions, the phenomenon has been proposed to serve as a protection against a sudden drops in temperature in the autumn or the spring (Patterson and Duman 1978). Thus, even though the metal exposed larvae was able to compensate by producing more AFPs, when exposed to winter conditions (**Paper II**), they might still be more vulnerable to lethal freezing during the time period involved before the concentration of AFPs is restored to normal winter levels. But since the exposure levels of metals in **Paper II** were generally high, its ecological relevance is uncertain.

5.2. Induction of metal binding proteins

The hypothesis that metal exposure might reduce the capacity to produce AFPs **(Paper I)**, was based the assumption that accumulation of high levels of MTs plays an important role in detoxification of metals in freeze avoiding insects, thereby potentially binding up a high proportion of the animals' free cysteine pool. MTs are often assumed to be ubiquitous proteins that are found in all animals (Hamer 1986). MTs, however, have only been indisputably identified, in form of protein or DNA sequence, in two types of insects; the springtail *Orchesella cincta* (Hensbergen et al. 1999) and from *Drosophila* flies (Lastowski-Perry et al. 1985; Mokdad et al. 1987). It was therefore considered important to determine if the model organism, *T. molitor*, really accumulated high levels of MTs in response to metal exposure.

The gut wall tissue has been shown to be the most important organ for induction of MTs in both *D. melanogaster* and *O. cincta* (Durliat et al. 1995; Hensbergen et al. 2000). However, no sign of increased binding capacity for Cd, suggesting no accumulation of MTs, was found upon inspection of gut wall tissue extracts of exposed *T. molitor* larvae (**Paper IV**). Two possible scenarios might explain this observation; MTs may not be produced, or they might be rapidly taken up in lysosomes. The latter scenario have been taken as an explanation to why the MT isoform involved in Cd resistance in *Drosophila* flies has never been identified at the protein level (Silar et al. 1990; Maroni et al. 1995; Lauverjat et al. 1989). Irrespective of this, it appears that the assumption for the hypothesis formulated in **Paper I**, and later tested in **Paper II**, that freeze avoiding insects produce and accumulate high levels of MTs to detoxify metals does not necessarily apply. This may explain why no reduction in the capacity to produce AFPs could be observed during cold acclimation in metal exposed larvae (**Paper II)**. If metal exposure do not cause accumulation of high levels of MTs, this would also mean that little of the cysteine "pool" should be expected to be committed in form of MTs, therefore posing no limitation on the production of cysteine rich AFPs.

5.3. Cd-binding proteins of the low cysteine type

Combined, **Paper III** and **IV** revealed that a 7134 Da, non-MT, cadmium binding protein is induced in the gut contents of Cd-exposed *T. molitor* larvae. The apparent absence of cysteine residues in the inducible Cd-binding protein (**Paper III**) suggests that it belongs to a group of

little studied proteins classified by Stone and Overnell (1985) as “Cd-binding proteins without a high content of cysteine”. Among terrestrial arthropods, proteins that fit into this category have been found in the stone fly *Pteronarcys californica* (Clubb et al. 1975), the locust *Locusta migratoria* (Martoja et al. 1983) and the wood lice *Porcellio scaber* (Dallinger 1993). Besides a low content of cysteine, the proteins share a low apparent molecular mass (5-20 kDa), a high proportion of acidic amino acids and a substantial content of aromatic amino acids and histidine.

In terrestrial arthropods, proteins have often been interpreted to be MTs, or referred to as “MT-like proteins”, based on two criteria; affinity for metals and apparent molecular weight in the same range as MTs (see Aoki et al. 1984a, b; Bouquegneau et al. 1985; Godocikova et al. 1993; Polek 1991; Polek et al. 1993; Yamamura et al. 1983; Znidarsic et al. 2005). Since Cd-binding proteins of the low cysteine type also fit both of these criteria, it is possible that proteins may have been wrongly classified as MTs in some of the studies listed above. This means that proteins of this type could be more widespread among terrestrial arthropods than the present literature suggests.

The appearance of the Cd-binding protein in the gut content, in the same time frame that incorporation of Cd in the gut wall tissue appeared to stabilize (**Paper IV**), suggests that the protein might be involved in lowering the concentration of free Cd in the gut fluid, thereby reducing the uptake of Cd in the gut wall tissue.

5.3.1. A contribution from the gut microflora?

The internal location of Cd-binding proteins of the low cysteine type has not, to the authors’ knowledge, been investigated previously in terrestrial arthropods. It was therefore especially interesting to find that the Cd-binding protein isolated from *T. molitor* (in **Paper III**) seems to be induced in the gut content (**Paper IV**). Given the location of the protein, it cannot be excluded that it is produced by the micro organisms that inhabit the gut (i.e. bacteria or fungi). If the protein turns out to be a contribution from the gut microflora, that alleviates the toxic effect of Cd-exposure, it would be a new example of beneficial effects of microsymbionts in insects. An interesting observation in this respect was made by Suzuki et al. (1984) on germ-free silkworms (*Bombyx mori*) exposed to Cd under aseptic conditions. Only two high molecular weight Cd-binding agents, which could not be cleaved by reduction, were found in

the extracts of the alimentary tract of the larvae. The combination of germ free animals and the absence of Cd-binding proteins in the low molecular range (i.e. MTs or Cd-binding proteins of the low cysteine type) in the study by Suzuki et al. (1984) support the notion that the Cd-binding protein isolated form *T. molitor* is indeed a contribution from the gut flora. However, a similar experiment using germ-free *T. molitor* larvae is required before more substantial conclusions regarding the source of the Cd-binding proteins can be drawn.

5.3.2. Avoiding competition for cysteine?

In contrast to vertebrates where MTs seem to be ubiquitous, some terrestrial arthropod species have MTs, while others seem to have metal binding proteins of the low cysteine type (Dallinger 1993). Dallinger (1993) commented upon the apparent paradox that Zn both induces and binds to MTs in vertebrates (Dalton et al. 1996; Palmiter 1994), but seems to be a poor inducer (Silar et al. 1990; Silar and Wegnez 1990) and exhibit only weak binding to MTs (Domenech et al. 2003) in *Drosophila* flies. Dallinger (1993) proposed that this difference could reflect that MTs in terrestrial invertebrates are more specialized towards detoxification of metals instead of being involved in both maintenance of Zn-homeostasis and detoxification of metals, like in the vertebrates. The more specialized role towards detoxification of metals might have increased the possibility for other proteins to have replaced MTs.

One question Dallinger (1993) raises is what factor(s) might have favoured development of non-metallothionein proteins for detoxification of metals in some species. In the present work it is speculated that among freeze avoiding insects which produce AFPs, a shortage for cysteine could be one such factor. However, the recent discovery that some terrestrial arthropods have AFPs with only 2 cysteine residues (Kristiansen et al. 2005; Graham and Davies 2005) shows that this may not apply to all AFP producing species. Among the species where Cd-binding proteins with low cysteine content have been reported, the author could only find confirmation that *P. scaber* produces no AFPs (shown by absence of hysteresis activity; Lavy et al. 1997). Presence of AFPs in *L. migratoria* also seems highly unlikely, since it is a tropical species. Thus, AFP does not appear to be a common denominator for terrestrial arthropods where Cd-binding proteins of the low cysteine type have been observed. On the other hand, in the insects where production of MTs has been confirmed (Stephan et al. 1994; Hensbergen et al. 1999), no production of AFPs has been observed (Nicodemus et al.

2006, Lavy et al. 1997). However, it should also be considered that cysteine rich proteins, other than AFPs, might favour evolvement of metal binding proteins of the low cysteine type.

6. Conclusions

The main findings in the present work can be summarized as follows:

Exposure to metals (Cd, Cu, or Zn) may reduce the normal developmental production of AFPs in summer-acclimated larvae of the freeze avoiding species *T. molitor*. The effect seems to be caused by a reduced AFP mRNA expression rather than a reduction in the capacity to produce AFPs, as a consequence of cysteine shortage. This indicates that metal exposure may have a negative effect on the cold hardiness in freeze avoiding insects that produce AFPs.

T. molitor does not seem to accumulate high levels of MTs in response to dietary exposure to Cd. Instead, the main Cd-binding ligand induced upon exposure is a 7134 Da protein without any cysteine residues. Since the Cd-binding protein is induced in the gut content it may be involved in reducing the uptake of ingested Cd to the hemolymph side (haemocel). It is uncertain whether the protein is an excreted product from the animal itself or a contribution from the gut microflora.

The apparent absence of MT accumulation in *T. molitor* could be an adaptation to avoid a competition for cysteine with AFPs, another type of cysteine rich protein. However, this is probably not an important issue in all AFP-producing species, since recent studies have shown that some freeze avoiding insects produce AFPs with low cysteine content. Also, no accumulation of MTs has been found in several species without any AFPs. Thus, cysteine shortage may not be the only factor that determines the production and accumulation of cysteine rich MTs in metal exposed terrestrial arthropods.

7. Future investigations

The present work may be regarded to have contributed to increase the understanding of how metals may reduce cold hardiness in freeze avoiding insects, and how non essential metals like Cd are detoxified. Several approaches seem appropriate in further studies on the effect of metals on production of AFPs and metal binding proteins:

1. The metal levels used in the present study were rather high compared to the levels that insects like *T. molitor* might become exposed to during natural conditions. The effect of lower metal levels on the production of AFPs should therefore be tested. Studies of possible interactions between metals, using full factorial designed experiments, should also be included since synergistic effects may occur.

2. The high degree of similarity between the various AFP-isoforms in *T. molitor* makes it challenging to work with these proteins. Different levels of salts, polyols, activator proteins and interactions between different AFP-isoforms have been shown to influence the hysteresis activity of antifreeze proteins. Thus, estimations of AFP-levels by measuring the level of hysteresis activity will always be sensitive to confounding factors as those listed above. However, new analytical approaches like proteomics offer tools where the level of the different isoforms may be studied simultaneously during metal exposure.

3. It would be interesting to compare the induction of metal binding proteins in species that produce cysteine rich AFPs and species that produce low cysteine AFPs. If species with cysteine rich AFPs do not accumulate MTs while species with low cysteine AFPs do, it would be a strong indication that cysteine shortage may be an important determining factor in these species.

4. The discovery that a Cd-binding protein is induced in the gut content does not exclude that this protein might be a produced by the microflora. This could be tested by Cd-exposure of germ free larvae (reared from sterilized eggs) under sterile conditions. If no Cd-binding protein is induced in the gut content of these larvae it would be a strong indication that it is a product of the gut microflora.

5. If the protein were found to be produced by microorganism(s), it would be possible to grow these in large quantities in the laboratory. This would increase the possibility of obtaining sufficient quantities of the protein to allow elucidation of its primary structure and to study its metal affinity and binding capacity.

6. If MTs are produced by *T. molitor* it is likely that a high turnover rate of these proteins makes it difficult to both detect and purify them. Considering that the primary sequence of insect MTs are relatively conserved, it should be possible to recognise MTs from mRNA sequences. This could be done by constructing a cDNA subtraction library, based on mRNA from control and Cd-exposed larvae, and sequencing those mRNA forms that are induced in response to Cd-exposure.

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Inorganic ions in cold-hardiness☆

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Abstract

Cold exposure and freezing may affect ion distribution in several ways and reduce physiologically important ionic gradients. Both freeze-avoiding and freeze-tolerant organisms have developed mechanisms to handle this stress. Supercooled insects seem to be able to maintain their ionic gradients even at temperatures far below zero. When freeze-tolerant insects freeze, ions diffuse down their concentration gradients across the cell membranes and reach electrochemical equilibrium. They quickly reverse this transmembrane diffusion when they are thawed.

Trace metals may affect mechanisms for cold-hardening in different ways and reduce cold-hardiness. Freezing may give rise to toxic concentrations of metal ions, and freeze-tolerant organisms probably need to inactivate toxic trace metals. Ice nucleating agents may be important in this context.

Keywords: Inorganic ions; Sodium; Calcium; Trace metals; Cold-hardiness; Glycerol; Antifreeze proteins; Ice nucleators

Inorganic ions are essential to life in that they have a variety of important functions in living organisms. They are important osmolytes in intracellular as well as extracellular fluids and co-factors for enzymes and other functional proteins. They are also important by providing electrochemical energy for transmembrane solute transport and action potentials in excitable cells [5]. Furthermore, they are signal substances in the control of cellular processes. The quantitatively most important ions are sodium, potassium, and chloride. The intracellular and extracellular concentrations of these ions differ substantially, and their concentrations are usually precisely regulated. Particularly cations such as K^+, Na^+, and Ca^{2+} have high concentration gradients across the cell membranes, and the regulation involves various active transport mechanisms, some of which require metabolic energy in the form of ATP [5].

In addition to the quantitatively dominating alkaline metals, all organisms contain trace metals. The levels of these metals are always far lower than those of alkaline metals, but the trace metals are still of great importance to organisms. Trace metals such as Fe, Cu, Zn, Mn, and Ni (the essential trace metals) are important as co-factors for functional proteins. Other trace metals such as

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Al, Cd, and Pb (the non-essential trace metals) have no known beneficial function, but they may have toxic effect by inhibiting enzymatic processes [1], perhaps including mechanisms for cryoprotection [31]. In freeze-tolerant organisms the freezing is likely to cause an increase in the concentrations of free trace metals, which thus may reach toxic levels.

Exposure of organisms to subzero temperatures may disturb the function of the regulatory mechanisms of the alkali metals in different ways. The nature of the disturbance is related to the mechanisms by which the organisms seek to survive cold exposure. There are two main strategies for survival. Many insects seek to avoid freezing by maintaining their body fluids in a liquid state even at temperatures far below the equilibrium freezing point, i.e., they stay supercooled. In these insects the disturbance is mainly due to the low temperature itself [18]. Other organisms have developed tolerance to freezing, provided the ice formation is restricted to the extracellular fluid [10]. The extracellular freezing is initiated at a high subzero temperature. The ice formation may cause a great increase in solute concentrations and in transmembrane concentration gradients [11], causing a re-distribution of alkali metals across the cell membranes [11]. Furthermore, the concentrations of alkali metals as well as trace metal may increase to toxic levels.

There is also evidence indicating that toxic trace metals in different ways may interfere with cryoprotective mechanisms [31], thus, reducing the ability of organisms to survive at low temperatures.

The present article reviews the physiological functions of metal ions and the regulatory mechanisms involved. It also reviews the mechanisms of cold-hardening and outlines how cold exposure and cryoprotective mechanisms may affect ionoregulatory mechanisms. Finally, it describes how the metals may interfere with cold-hardiness.

Physiological functions and regulation of alkali metal ions

The physiologically most important alkali metals are sodium and calcium, but also potassium and magnesium have important physiological functions.

In most animals sodium is the dominating extracellular cation, and the extracellular sodium concentration is about 10-fold higher than that of the intracellular fluid [19]. The high concentration gradient is the result of the active extrusion of sodium from the cells by the so-called sodium pump or sodium/potassium ATPase (Fig. 1), which usually pumps three sodium ions out of the cells and two potassium into the cells under the consumption of one molecule ATP [5]. The transmembrane permeability for sodium is relatively low, and sodium will accumulate in the extracellular fluid until the high extracellular concentration makes the passive sodium influx equal to the active extrusion. Together with the negative

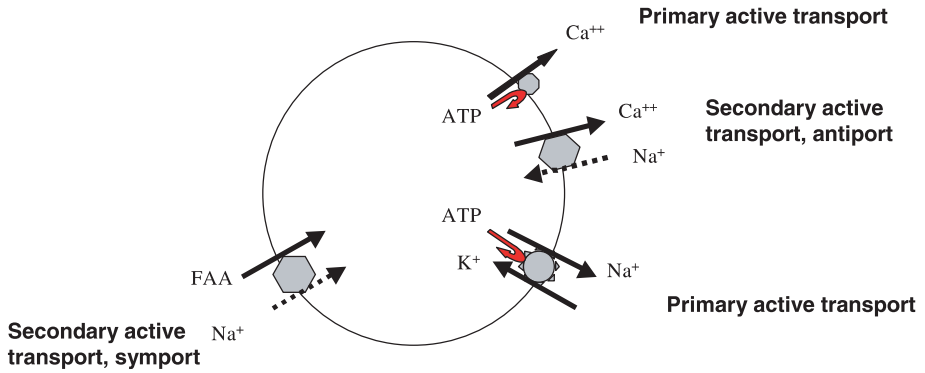

Fig. 1. Some of the known transport systems involving inorganic ions across cell membranes of animal cells.

electric charge of the intracellular compartments the high concentration quotient gives sodium a high transmembrane electrochemical potential difference. The energy contained in this electrochemical potential difference is the energy source of a number of important cellular processes, such as cellular uptake of free amino acids (FAAs), ATP-independent extrusion of calcium from cells (Fig. 1) and action potentials in neurons and muscle cells [5].

Many herbivorous insects differ from other animals by having a far lower extracellular sodium concentration [2,23] and a lower electrochemical potential difference [2]. The extracellular sodium concentration of these insects may be as low as 20 mM, and they may have potassium or magnesium as the dominating extracellular cation [2,23]. As pointed out by Zachariassen [27], the reduced sodium gradient is accompanied by a reduced capacity for cellular accumulation of FAAs, leading to high extracellular FAA concentrations.

Calcium is important as an activator of enzymes and other functional proteins. The activation is based on a passive influx of a minute amount of calcium into the cytoplasm from the extracellular fluid and/or the endoplasmatic reticulum, leading to an increase in free calcium concentration in the cells and thus to binding of calcium to the enzymes it activates [17]. Calcium is potentially highly toxic to cells, and most cellular calcium is bound to intracellular structures, making cellular levels of free calcium extremely low, approximately 10^{-7} M [5]. The low concentration of free calcium makes the calcium based regulation systems highly responsive, because it takes only a tiny influx of calcium to cause a substantial increase in the intracellular level of free calcium. The passive influx of calcium into cells is continuously counteracted by a membrane bound calcium ATPase, which pumps calcium out of the cells under the consumption of ATP. However, when cellular ATP levels are low, the activity of the ATP dependent pump will also be low. In this situation the sodium energy gradient functions as an energy back-up, in that a sodium dependent antiport extrusion of calcium takes over and secures that the cellular level of calcium remains low (Fig. 1) [5].

The high transmembrane concentration gradient of sodium leaves the intracellular fluid with a large sodium deficit [5], which is compensated for by a high intracellular concentration of potassium. Hence, potassium has an important physiological function by being an intracellular cationic osmolyte.

Magnesium is important as a co-factor for various enzymes.

Also various trace metals function as co-factors for enzymes and other organic molecules [22]. They bind to specific sites on organic molecules, and the binding of the metal co-factor is required for the normal function of the organic molecule. Among these so-called essential trace metals are iron, copper, zinc, manganese, and nickel.

Other trace metals such as aluminium, cadmium, mercury, and lead have no known physiological function. However, due to their chemical similarity to calcium and the essential trace metals they may substitute for these metals on their site on proteins [1]. Since the non-essential trace metals do not have the activity promoting effect of the trace metals they replace, the binding inhibits the activity of the functional proteins, i.e., it has a toxic effect. Even the essential trace metals may have a toxic effect if they occur in too high concentrations [1].

Organisms seek to counteract the toxic effect of trace metals by binding them to special metal binding proteins such as metallothioneins (MTs) [1], which are produced by cells as a response to trace metal exposure. This binding reduces the levels of free metals and thus the likelihood of binding of potentially toxic trace metals to functional proteins. When cells have produced large quantities of MT, large amounts of trace metals may be accumulated in the cells, but in the bound state they are considered to be harmless.

Low temperature exposure: effects and protective mechanisms

Freeze-avoiding organisms

Freeze-avoiding insects avoid freezing at low temperatures by means of a high capacity for

supercooling. The high supercooling capacity is the result of a number of physiological changes. First, all nucleating agents are removed from their body fluid, thus reducing their supercooling point from about −10 °C, which is normal for summer insects, to about −20 °C (Fig. 2). Many species reduce their supercooling point further by the accumulation of polyols in their body fluids [20]. The polyols reduce the supercooling points by about twice the corresponding melting point depression [6,7,13,15,25], implying that in insects with multimolal concentrations of polyols the supercooling points may fall below −30 °C, in extreme cases even below −40 °C [26]. Freeze-avoiding insects also produce antifreeze proteins [3], which probably stabilise the supercooled state in part by preventing inoculation of external ice through the body wall [7,8,16], and in part by preventing spontaneous ice nucleation in the highly supercooled body fluids [30].

As the body temperature of an organism drops, the rate of enzymatic processes, including ionic pumps, is reduced. The reduction follows an exponential curve, the curvature of which is given by the so-called Q_{10} value, which is the factor by which the rate changes following a temperature change of 10 °C [19]. For most enzymatic processes the Q_{10} is about 2. For a Q_{10} of 2, a reduction in temperature from +20 to −20 °C implies that the rate of active pumping of ions drops 16-fold, whereas the passive diffusion, which is determined by the absolute temperature, drops by only 15%. Hence, at low temperatures the active extrusion of sodium and calcium from the cells can hardly keep up with the passive influx, and the result could be a fatal reduction of all energy-rich concentration gradients.

Dissanayake and Zachariassen [2] showed that when freeze-avoiding *Rhagium inquisitor* beetles are kept at −10 °C, the difference between extracellular and intracellular sodium concentrations is even greater than at room temperature (see Fig. 3). Similar results were obtained by Hanzal et al. [9]. Hence, the beetles seem to have the capacity to maintain the sodium gradient, in spite of the temperature effects described above.

There may be at least three mechanisms which are responsible for this capacity. At low temperature

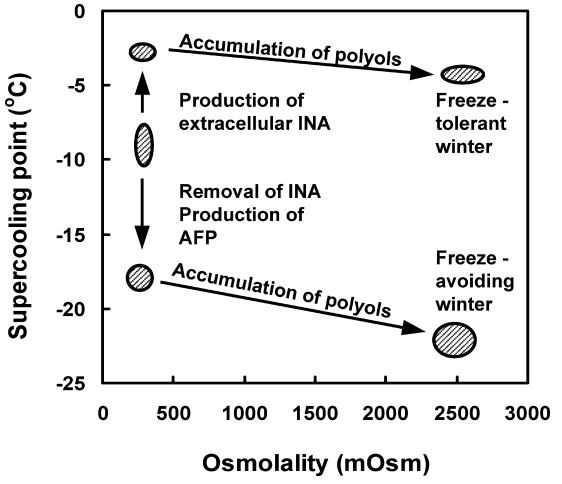

Fig. 2. Supercooling points of summer insects and freeze-avoiding and freeze-tolerant winter insects with underlying mechanisms indicated. Modified after Zachariassen [25].

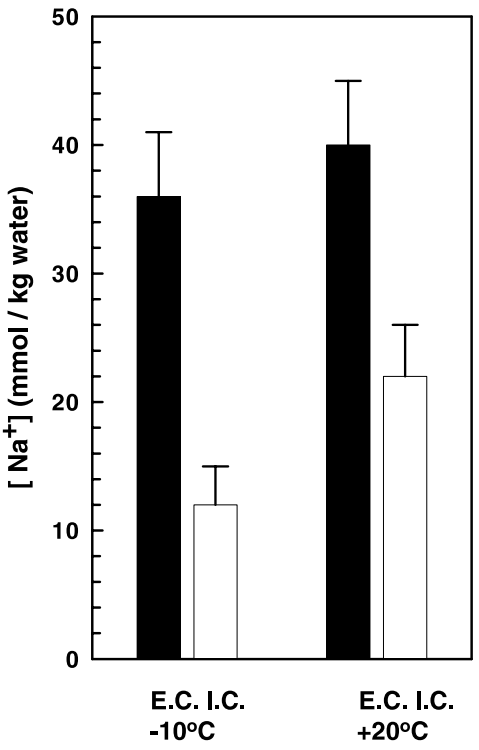

Fig. 3. Extracellular (EC) and intracellular (IC) sodium concentrations of *R. inquisitor* beetles kept at room temperature and at −10 °C. From Dissanayake and Zachariassen [2].

the viscosity of water increases [24]. This will reduce the diffusion rate of ions beyond what can be predicted from the low temperature alone. The accumulation of polyols such as glycerol will make the viscosity even higher [24]. However, unless the glycerol concentration becomes extremely high or the temperature extremely low the reduction in diffusion rate caused by high viscosity is too small to prevent a diffusive elimination of ionic gradients.

There is also experimental evidence indicating that antifreeze proteins from fish may reduce the permeability of calcium through cell membranes [14]. *R. inquisitor* has high levels of antifreeze proteins in the body fluids in the winter, and if the capacity to reduce ionic permeability through membranes applies also to sodium, the antifreeze proteins may be responsible for the maintenance of the sodium gradient at low temperatures. The existence of such an effect in insects should be investigated.

Finally, the diffusion of other solutes into the cells may also contribute. In contrast to the situation in vertebrate plasma, sodium is not a quantitatively dominating solute in *Rhagium* hemolymph [2]. Magnesium is present at far higher concentrations than sodium, and if magnesium (or other important solutes) is allowed to diffuse into the cells at low temperature, it would create an osmotic influx of water, which would dilute intracellular sodium and cause an increase in extracellular sodium concentration. Evidence of a mechanism of this kind comes from the fact that cold-exposed *R. inquisitor* have a higher intracellular magnesium concentration than warm acclimated ones [2]. Furthermore, we have observed that *R. inquisitor* beetles taken directly from their hibernacula in the winter have a remarkably small volume of hemolymph, which increases substantially when the beetles are kept for a few hours at room temperature.

Trace metals may interfere with the cold-hardening mechanisms of insects. Zachariassen and Lundheim [31] found that exposure to copper and cadmium reduced the rate of glycerol removal in cold-exposed *R. inquisitor* larvae kept at room temperature (Fig. 4). The effect on glycerol formation has not been measured, but if enzymes

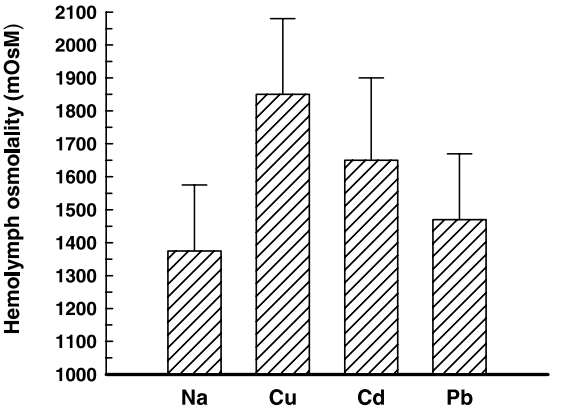

Fig. 4. Hemolymph osmolality of cold-hardy *R. inquisitor* beetles undergoing warm acclimation at 5 °C for 10 days. At the beginning of the acclimation period the beetles were injected with 1 μl samples of chlorides of the respective metals. Numbers on the top of each bar indicate numbers of specimens measured. From Zachariassen and Lundheim [31].

involved in glycerol formation at low temperature were inhibited in a similar way, the metals might reduce the capacity for cold-hardening.

Even protective mechanisms against trace metal injury may affect cryoprotective mechanisms. The metal binding metallothioneins contain about 30% of cystein [1], and so do the antifreeze proteins of many insects [4]. Since cystein occurs at relatively low levels in insects, the consumption of large amounts of free cystein to produce MT may reduce the capacity of insects to produce antifreeze proteins. An alternative strategy for cold-hardiness of metal exposed insects would be to develop antifreeze proteins without a high cystein content or to switch over to freezing tolerance.

Freeze-tolerant organisms

In freeze-tolerant organisms internal injurious freezing of cells or intestine is prevented by a protective extracellular freezing [10]. The extracellular freezing is initiated at a high subzero temperature, before nucleating structures in cells or intestine get the opportunity to trigger freezing in these compartments, and in freeze-tolerant organisms cold-hardening is associated with an increase in supercooling point (Fig. 2). The transition of liquid water to ice in the extracellular

fluid is accompanied by an increase in the solute concentration in the fluid surrounding the ice, and the ice crystals will grow until the concentration of the fluid reaches vapour pressure equilibrium with ice. The lower the temperature, the more ice has to be formed to create vapour pressure equilibrium.

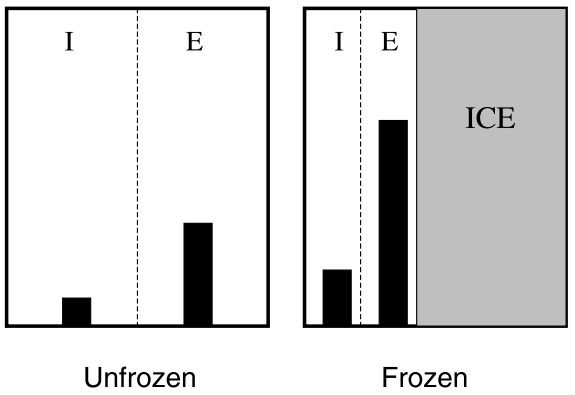

Fig. 5. Intracellular (I) and extracellular (E) concentrations of a hypothetical solute before and immediately after initiation of freezing. Freezing causes no immediate change in concentration quotient but a large increase in concentration gradient. Broken vertical line represents cell membrane. Modified after Kristiansen and Zachariassen [11].

Since cell membranes are permeable to water, the increased extracellular solute concentration causes an osmotic efflux of water and shrinking of the cells. Hence, the intracellular as well as the extracellular fluid will be in vapour pressure equilibrium with ice, and no supercooled fluid compartment will remain in the organism [10]. In this way an injurious intracellular freezing is effectively prevented, regardless of how far down the organism is cooled.

The development of toxic ionic concentrations is counteracted by the accumulation of polyols, which reduce the amount of ice and thus the concentration of solutes by a colligative action. This will displace the formation of toxic ionic concentrations to lower temperatures. There will nevertheless be an increase in intracellular as well as extracellular ionic concentrations, implying that although the concentration quotients remain constant, there will be a strong increase in the concentration gradients across the cell membranes (Fig. 5). As pointed out by Zachariassen et al. [29] and Storey and Storey [21], the changes in ionic concentrations caused by the freezing are likely to affect the energy balance of the organisms.

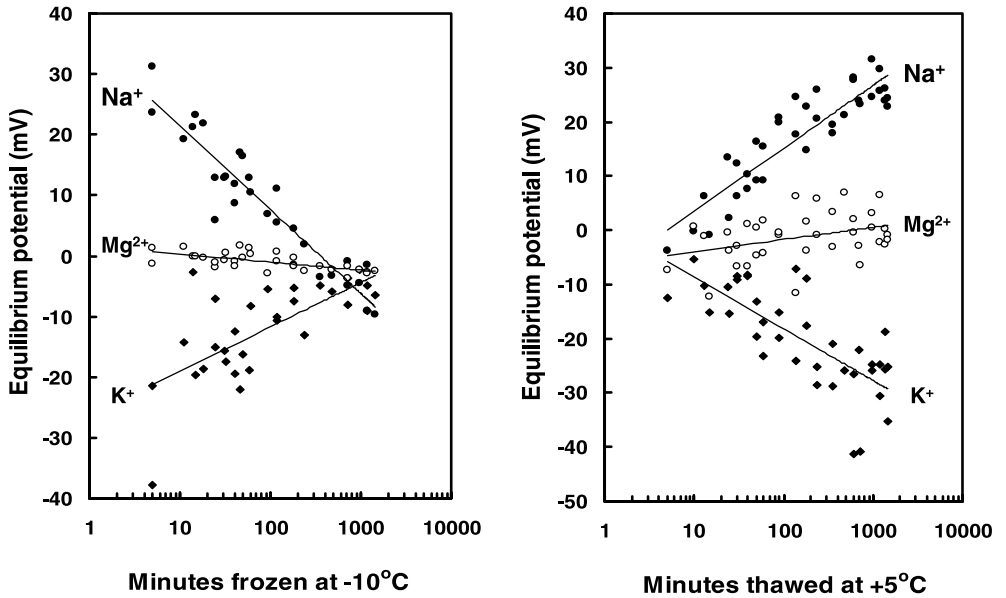

Fig. 6. Equilibrium potentials across cell membranes of sodium, potassium, and magnesium of *Xylophagus cinctus* wood flies during freezing and thawing, plotted as a function of time. From Kristiansen and Zachariassen [11].

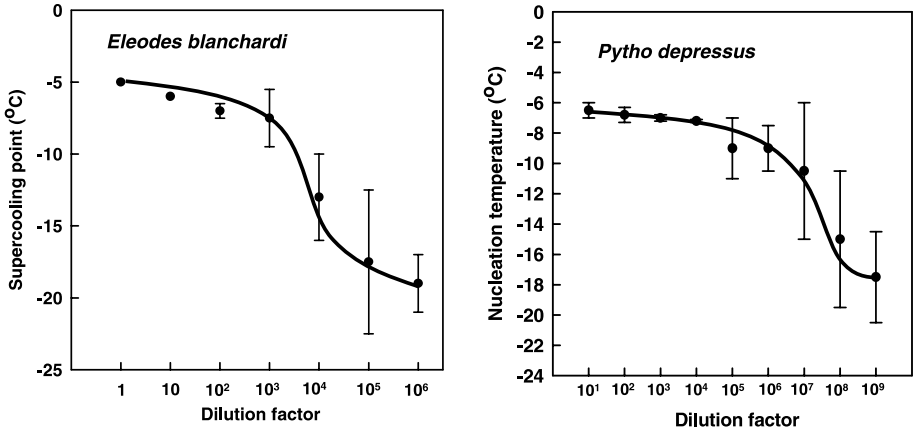

Fig. 7. Effect of isovolumetric dilution of 5 μl samples of hemolymph from freeze-tolerant *E. blanchardi* (left) and *P. depressus* (right) beetles on sample supercooling point. Data taken from Zachariassen et al. [28] and Lundheim [12], respectively.

Kristiansen and Zachariassen [11] showed that in frozen larvae of *Xylophagus* wood flies sodium, potassium, and magnesium diffuse down their concentration gradients and reach electrochemical equilibrium (Fig. 6). Upon thawing the functionally important energy-rich gradient of sodium is re-established quite fast (Fig. 6), suggesting that the frozen organisms have large cellular stores of ATP ready for active transport of the ion. This may suggest that the passive transmembrane diffusion of ions in the frozen insects is associated with a reversal of the ATPases [22], leading to preservation of the energy contained in the sodium gradient by a re-synthesis of large amounts of cellular ATP. Zachariassen et al. [29] observed that immediately after freezing *of Eleodes blanchardi* beetles there was a 100% increase in metabolic rate, which returned to normal after 24 h. This increase may in part reflect the removal of accumulated lactic acid or other anaerobic metabolites from the body fluids, but it may also in part be caused by increased active ionic transport to re-establish ionic gradients.

When ice is formed in freeze-tolerant organisms, potentially toxic trace metals will also be concentrated in the unfrozen fluid fraction. The development of toxic levels may be prevented if the organisms are able to bind the metals, such that the levels of free metals remain low. There are indications that the extracellular ice nucleating agents have a metal binding function in addition to their nucleating activity. The nucleating agents are present at far greater amounts than is required to nucleate at a high subzero temperature (Fig. 7). In the freeze-tolerant beetle *E. blanchardi* there are a thousand times as many nucleator molecules as is necessary for ice nucleation at the supercooling point of the beetles [28], whereas, in the beetle *Pytho depressus* there are a million times more ice nucleators than is required for ice nucleation [12]. The great surplus of ice nucleators compared to what is required for efficient nucleation indicates that the nucleators have other functions than nucleation, and binding of trace metals may be one reason why the nucleators are present at such high levels. In accordance with this prediction, Zachariassen (unpublished results) has found that the ice nucleators have a high affinity for trace metals.

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Paper II

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Cold hardiness in relation to trace metal stress in the freeze-avoiding beetle *Tenebrio molitor*

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Abstract

The antifreeze proteins (AFPs) are a family of proteins characterised by their ability to inhibit the growth of ice. These proteins have evolved as a protection against lethal freezing in freeze avoiding species. Metal stress has been shown to reduce the cold hardening in invertebrates, but no study has investigated how this type of stress affects the production of AFPs. This study demonstrates that exposure to cadmium (Cd), copper (Cu) and zinc (Zn) reduces the normal developmental increase in AFP levels in *Tenebrio molitor* larvae reared under summer conditions. Exposure to winter conditions, however stimulated the production of AFPs in the metal exposed larvae, and raised the concentrations of AFPs to normal winter levels. The reduced level of AFPs in metal-stressed animals acclimated to summer conditions seems to arise from alterations in the normal gene expression of AFPs. The results indicate that metal exposure may cause freeze avoiding insects to become more susceptible to lethal freezing, as they enter the winter with lowered levels of AFPs. Such an effect cannot be revealed by ordinary toxicological tests, but may nevertheless be of considerable ecological importance.

Keywords: Metal stress; Cold hardiness; Antifreeze protein; Gene expression; *Tenebrio molitor*

1. Introduction

Metals from natural or anthropogenic sources represent an important part of the chemical environment. Trace metals like Cu and Zn are essential for normal growth and metabolism of organisms, but they may also produce toxic effects at high concentrations (Kruk, 1998). Cadmium is a non-essential heavy metal that can be taken up by cells through voltage-gated calcium channels located in the plasma membrane. Intracellularly cadmium accumulates by binding to cytosolic and nuclear material, causing a variety of damaging effects like inhibition of zinc-containing enzymes, lipid peroxidation and disturbance of protein synthesis (Beyersmann and Hechtenberg, 1997). Even though soft bodied animals like springtails and earthworms can take up metal directly, metals mostly enter the food chain via plants (Kabata-Pendias and Pendias, 1992). The metals may cause injury by direct toxic actions, but they have also been shown to cause damage more indirectly by interfering with cold hardening processes important for winter survival in insects (Zachariassen and Lundheim, 1995) and earthworms (Holmstrup et al., 1998; Bindesbøl et al., 2005).

Insects that adaptively allow their body fluids to supercool when they become exposed to low temperatures (freeze avoidance) often produce antifreeze proteins (AFPs) (Zachariassen, 1985; Duman, 2001). This family of proteins is defined by their ability to depress the non-equilibrium freezing point of water while not affecting the melting point. This separation of melting and freezing point is called thermal hysteresis (DeVries, 1971, 1986). The phenomenon is caused by adsorption of antifreeze proteins to the ice crystals (Raymond and DeVries, 1977; Knight et al., 1991; Kristiansen and Zachariassen, 2005). Their function appears to be to promote supercooling by preventing inoculative freezing across the body wall (Gehrken, 1992; Olsen et al., 1998) and masking of ice

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nucleating structures within the body fluids (Olsen and Duman, 1997a, b).

The phenomenon of thermal hysteresis was first described by Ramsay (1964), during his study of the cryptonephridial rectal complex of *Tenebrio molitor*. It was later discovered that this phenomenon was caused by proteins (Grimestone et al., 1968). These proteins have been identified as a family of AFP isoforms which vary both in length and extent of glycosylation (Liou et al., 1999). The thermal hysteresis activity in the hemolymph increases with larval development in *T. molitor* (Graham et al., 2000), but exposure to conditions normally associated with the winter season (e.g. low temperature, desiccation, starvation, short photoperiod) has been shown to further increase the thermal hysteresis activity (Patterson and Duman, 1978; Horwath et al., 1996; Graham et al., 2000).

The purpose of this investigation was to study the effects of exposure to Cu, Zn and Cd on the production of AFPs in the larvae of the freeze avoiding beetle *T. molitor*.

2. Materials and methods

2.1. Insect rearing

A laboratory culture of *T. molitor* larvae (Blades Biological, Edenbridge, Kent, UK) was established and maintained on wheat bran at 25 °C and long day (18:6 h, L:D). The animals were sprayed with distilled water once a week to provide adequate moisture.

2.2. Stress treatment

Animals were exposed to three different concentrations of Cd (0.005, 0.05 or 0.5 mg/g), Cu (0.1, 1 or 10 mg/g) or Zn (0.5, 5 or 50 mg/g) in the food. The metal was administered via the food since ingestion is the most likely route for metal uptake in terrestrial invertebrates with water impermeable skin and wax coating (Vijver et al., 2003). The nine different types of metal-enriched food were prepared by soaking batches of wheat bran (80 g) with aqueous solutions (200 mL) spiked with the necessary amount of chloride salts to give the final concentration of metal on a dry weight basis. The mixture was dried over night at 100 °C. Food for the control groups was prepared in the same manner by adding distilled water without metal.

Larvae chronically exposed to the different metal regimes under summer conditions were obtained by transferring 50 adult beetles to ventilated plastic terrariums ($15 \times 9 \times 11$ cm) situated in a climatic room with 25 °C and long day (18:6 h, L:D). Each terrarium contained 20 g of one of the 10 different wheat bran diets (see above). A piece of paper placed on top of the food provided cover for the animals, and moisture was provided once a day to avoid dehydration. The newly hatched adults (identified by pale cuticle) were allowed to mate and lay their eggs for a one-week period, before they were removed. After a 3-month period the eggs had developed into medium sized larvae. At this stage the larvae were removed and sampled. Winter-acclimated larvae chronically exposed to the same metal regimes were obtained by adding a one-month acclimation period at 4 °C and short day (6:18 h, L:D) after the treatment described for the larvae reared under summer conditions. All treatments were duplicated to provide replication.

2.3. Hemolymph collection, determination of body mass, osmolality and hysteresis activity

Ten larvae were collected from each terrarium. Before sampling, the body mass of the larvae was determined. Hemolymph was collected by making a small incision at the first thoracic segment. The exuding hemolymph was sucked into the attenuated end of a glass capillary tube by means of the capillary force according to a method described by Zachariassen et al. (1982), and the samples were stored at −80 °C until analysis.

Direct determination of the molar AFP concentration was not possible since polyclonal antibodies that cross-react with many of the AFP isoforms were not available. Instead an indirect method was adopted, using the hysteresis activity as a measure of the AFP concentration (see below for detailed description).

Hysteresis activity in the hemolymph was measured using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA). The frozen hemolymph samples were thawed and centrifuged on a Compur micro centrifuge (M110, Compur Elektronik, München, Germany). Approximately 20 nL of hemolymph was placed in the sample holder well and frozen by rapid cooling to −40 °C. The temperature was then slowly raised by approximately 1 °C min^{-1} until the last ice crystal disappeared. This temperature was taken as the sample melting point, from which the osmolality could be calculated. The sample was frozen again, and the temperature was raised slowly until only one small ice crystal was left in the sample. This ice crystal was allowed to stabilize for 1 min before the temperature was lowered slowly (approximately 0.1 °C min^{-1}) until an explosive ice growth was observed. This was taken as the hysteresis freezing point. The difference between the stabilising temperature and the hysteresis freezing point was taken as the hysteresis activity. Each sample was measured repeatedly, until three values of hysteresis activity were obtained that differed maximally by ±5%. The mean of the three measured values was taken as the hysteresis activity of the sample. To reduce the variation in thermal hysteresis due to difference in ice crystal size (Zachariassen and Husby, 1982), only the smallest ice crystals that could readily be identified in the samples were used.

The hysteresis activity is not linearly related to the concentration of antifreeze proteins (Kristiansen et al., 2005; Zachariassen et al., 2002). To compare the AFP

levels among the groups, the hysteresis activities were transformed to relative dilution factor (rDF) according to the method described by Graham et al. (2000). Briefly, a standard curve based on the hysteresis activity at different dilutions (in 0.9% NaCl) of a standard hemolymph sample from *T. molitor* (pooled from winter-acclimated control animals) was used to interpolate the relative concentration. All values were normalized to the mean relative AFP concentration in the summer-acclimated control group. This gave the rDF necessary to reduce the hysteresis activity to that of the summer-acclimated control group. The dilution factor can be converted back to hysteresis activity using the equation: AFP activity (°C) = 4.33 + 1.33ln (rDF).

2.4. Real-time PCR of AFP YL-3 mRNA

Five larvae were collected from each group and used to isolate total RNA. Briefly, the larvae were homogenized in TRIzol reagent and purified as described by the manufacturer (Invitrogen Corperation, Carblad, CA, USA). Contaminating DNA was removed by DNAase *I* treatment (DNAfree kit, Ambion, Austin Texas, USA). Total RNA yield was determined at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Tecnologies, Wilmington, USA). Total cDNA for real-time PCR reactions were generated from 250 ng of total RNA using the iScript™cDNA Syntesis Kit (Bio-Rad) protocol. A standard sample (pooled RNA from winter-acclimated control animals) was used to make a dilution series in DEPC water. Aliquots of these dilutions were included in each run, to later serve as a standard curve for relative quantification during real-time PCR (see below). Total cDNA synthesis was performed twice (in separate runs) on each sample. Primers were designed for the different major AFP isoforms in *T. molitor* using primer design software from Stratagene (http://labtools.stratagene.com/PCRDes/Forms/PCRDHome.php). After initial testing it was decided to focus on AFP YL-3 (gen bank accession no. AF160496) because the primers for this isoform showed high specificity. The set of primers (5′-TGCACTGGTTG TGGAAGTTG-3′ (forward), 5′-TACACGTTTCTGCCC TGACA-3′ (reverse) amplified a 76 base pair sequence from the target cDNA (see Fig. 1).

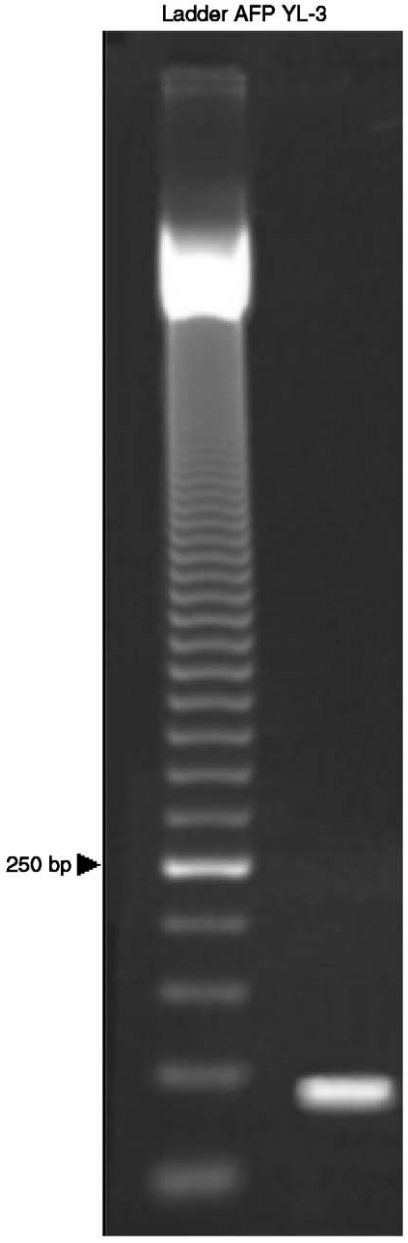

Fig. 1. Real-time PCR product of AFP YL-3 from *T. molitor*. The PCR product was separated by 1% agarose electrophoresis and detected by ethidium bromide staining.

The transcript expression of AFP YL-3 target gene was analyzed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA). Each 25 μL DNA amplification reaction contained 12.5 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad), 400 nM of forward primer and reverse primer, and 1 μL of cDNA as template. The real-time PCR program included an enzyme activation step at 95 °C (10 min), and 40 amplification cycles of 95 °C (30 s), 60 °C (1 min), and 72 °C (30 s). The dissociation curve of the PCR products was obtained by gradual heating of the samples from 55 to 95 °C. Reaction specificity was determined when there was only one peak in the dissociation curve. Controls lacking cDNA template or Taq DNA polymerase were included in each run to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into relative transcript expression using standard plots of Ct-values versus log concentration of the standard sample. The standard plots were generated for the target sequence using known dilutions of the standard sample (see above). Duplicate data obtained for target cDNA amplification in separate runs were averaged and normalized to the mean transcript expression in the summer control group.

To verify that the AFP YL-3 transcript was being amplified, the real-time PCR product was cloned into the pCR®2.1 vector (Invitrogen TA Cloning kit). Positive

clones were grown overnight and harvested for plasmid purification using a GenElute Plasmid Miniprep kit (Sigma). The PCR insert was sequenced in both directions with oligonucleotide sequences corresponding to M13 forward and reverse primers within the pCR®2.1 plasmid as primers for sequencing reaction. The products were sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Department of Biology, NTNU, Norway.

2.5. Statistical treatment

Before statistical treatment the values for rDF and AFP YL-3 mRNA expression were log-transformed. Statistical differences in body mass, osmolality, rDF and gene expression was analyzed with ANOVA using a design with terrarium (two levels) nested within the fixed factors season acclimation (two levels) and metal exposure (seven levels). As no significant difference was found between the replicate terrariums for the different treatments at the 0.25 level, the samples were pooled according to post hoc procedure. Since no significant interaction between season acclimation and metal exposure was found, one-way ANOVA could be used to test the effect of metal exposure under summer and winter-conditions separately. Significant difference obtained by one-way ANOVA was treated further by post priori procedure. To identify differences, pair-wise comparisons of the experimental groups against the control group were performed by using the method of Dunnett (1955), with a family-wise error rate of 0.05. The statistical treatment was performed using S-PLUSS.

3. Results

3.1. Distribution of body mass for the different treatments

Eggs developed into medium sized larvae in all the groups except those that were exposed to the highest levels of Zn (5 and 50 mg/g) and the highest level of Cu (10 mg/g). In these groups none of the larvae developed beyond the 1st instar (see Table 1). Only a small number of larvae (>10) developed in the winter-acclimated groups exposed to 0.1 mg/g Cu and 0.5 mg/g Cd. There was no significant difference in the body mass between the different groups of summer-acclimated animals ($F = 1.22$ with 6 and 133 degrees of freedom, $P = 0.30$). Among the winter-acclimated animals only the copper exposed group showed a significant lower body mass than control group ($F = 2.89$ with 6 and 113 degrees of freedom, $P < 0.05$) (Table 1).

3.2. Effects of metal exposure on hemolymph osmolality under summer and winter conditions

Winter-acclimation caused a significant increase in the hemolymph osmolality compared to the summer-acclimated animals ($F = 127.32$ with 1 and 258 degrees of freedom, $P < 0.001$), but metal exposure had no significant effect within the summer- ($F = 2.09$ with 6 and 133 degrees

Table 1
Body mass and osmolality in larvae acclimated to summer and winter conditions and exposed to different levels of metals

Seasonal conditions	Metal exposure	Body mass (mg)	Osmolality (mOsm)	Number of animals
Summer: (25 °C, 18:6 h L/D)	Control	65.0±27.3	438±64	2×10
	0.005 mg/g Cd	69.9±34.3	434±42	2×10
	0.05 mg/g Cd	78.3±37.9	427±61	2×10
	0.5 mg/g Cd	75.5±35.5	428±43	2×10
	0.5 mg/g Zn	77.3±35.7	425±20	2×10
	5 mg/g Zn	80.4±38.5	426±56	2×10
	50 mg/g Zn	[a]	[a]	[a]
	0.1 mg/g Cu	57.8±19.1	390±40	2×10
	1 mg/g Cu	[a]	[a]	[a]
	10 mg/g Cu	[a]	[a]	[a]
Winter: (4 °C, 6:18 h L/D)	Control	77.5±32.7	492±59	2×10
	0.005 mg/g Cd	76.6±34.2	528±103	2×10
	0.05 mg/g Cd	59.9±34.0	494±68	2×10
	0.5 mg/g Cd	53.6±27.0	520±17	2×5
	0.5 mg/g Zn	73.8±34.9	526±68	2×10
	5 mg/g Zn	68.2±27.8	516±105	2×10
	50 mg/g Zn	[a]	[a]	[a]
	0.1 mg/g Cu	36.6±10.6 *	478±53	2×5
	1 mg/g Cu	[a]	[a]	[a]
	10 mg/g Cu	[a]	[a]	[a]

Values are means ± SD, and stars indicate statistical differences compared to the respective control group at the 0.05 level.
[a]Data on the larvae exposed to high zinc level (50 mg/g) and medium and high level of copper (1 and 10 mg/g) are not shown as no larvae developed beyond the 1st instar.

of freedom, $P = 0.06$) and winter-acclimated animals ($F = 1.48$ with 6 and 133 degrees of freedom, $P = 0.19$) (Table 1).

3.3. Effects of metal exposure on hysteresis activity under summer and winter conditions

Metal exposure caused a marked reduction in the hemolymph hysteresis activity of the summer-acclimated larvae (see Fig. 2). In the groups exposed to 0.005, 0.05 and 0.5 mg/g Cd, the reduction in hysteresis activity corresponded to a 7.1-, 5.6- and 5.3-fold reduction in the AFP-level, compared to the control group. Exposure to 0.5 and 5 mg/g Zn also reduced the hemolymph hysteresis activity, but the effect was less pronounced, corresponding to a 3.7- and 2.9-fold reduction in the AFP-level. Exposure to 0.1 mg/g Cu also caused a moderate reduction in the hemolymph hysteresis activity, corresponding to a 2.7-fold reduction in the AFP-level. These differences were statistically significant in all cases ($F = 5.63$ with 6 and 133 degrees of freedom, $P < 0.0001$).

As apparent from Fig. 2, all of the winter-acclimated groups showed a higher mean hysteresis activity than the respective summer-acclimated groups. This corresponded to a 2.2-fold increase in the AFP-level of the control group. The groups exposed to 0.005, 0.05 and 0.5 mg/g Cd showed an 8.1-, 10.9- and 5.7- fold increase relative to the observed levels in the respective summer-acclimated groups. Winter-acclimation of the 0.5 and 5 mg/g Zn groups caused a 6- and 4.5-fold increase in AFP-levels, compared to the summer levels. This also applied for the group exposed to 0.1 mg/g Cu, which showed a 5.1-fold increase upon winter-acclimation. There were no significant differences among the groups ($F = 0.94$ with 6 and 113 degrees of freedom, $P = 0.22$).

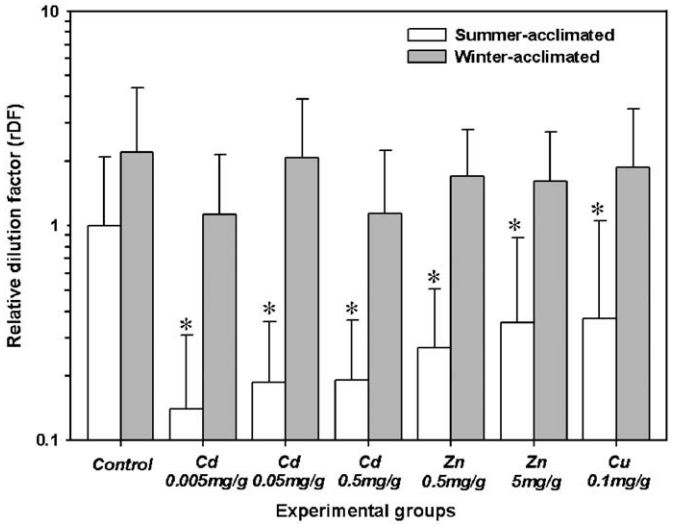

Fig. 2. Effect of metal exposure on the level of AFP under summer and winter conditions in hemolymph from *T. molitor*. Bars show relative dilution factor (rDF) for AFP in larvae during summer and winter conditions. The rDF was obtained by first using the measured hysteresis activity to interpolate a dilution factor from a standard curve and then normalized to the average dilution factor of the summer-acclimated control group. Error bars show the standard deviation and stars indicate statistical differences compared to the respective control group at the 0.05 level. All groups are represented by a sample of 20 animals, except from the winter-acclimated groups exposed to 0.5 mg/g Cd and 0.1 mg/g Cu, which contained only 10 animals.

3.4. Effects of metal exposure regimes on transcript expression of antifreeze proteins under summer and winter conditions

Among the summer-acclimated groups, metal exposure caused a significant reduction in the AFP YL-3 transcript expression in the groups exposed to 0.5 mg/g Cd, 5 mg/g Zn and 0.1 mg/g Cu, when compared to the control group ($F = 2.45$ with 6 and 61 degrees of freedom, $P < 0.05$). Although not statistically significant from control groups ($P < 0.1$), the groups exposed to 0.05 mg/g Cd and 0.5 mg/g Zn also showed a tendency towards reduction in AFP YL-3 transcript level. When comparing the average values of the different groups relative to the control group, Cd appeared to have caused a 1.8-, 3.9- and 26-fold reduction in AFP YL-3 transcript expression in the groups fed with 0.005, 0.05 and 0.5 mg/g, respectively. Similarly, exposure to 0.5 and 5 mg/g Zn appeared to cause a 4.1- and 8.3- fold decrease, and exposure to 0.1 mg/g Cu caused a 23.6 fold reduction in AFP YL-3 transcript level.

The transcript expression of AFP YL-3 in control group did not change after winter-acclimation (see Fig. 3). There was no significant difference in transcript level of AFP YL-3 in any of the metal exposed groups acclimated to winter conditions, when compared to the control group ($F = 0.70$ with 6 and 56 degrees of freedom, $P = 0.65$). The average level of transcript expression in larvae exposed to

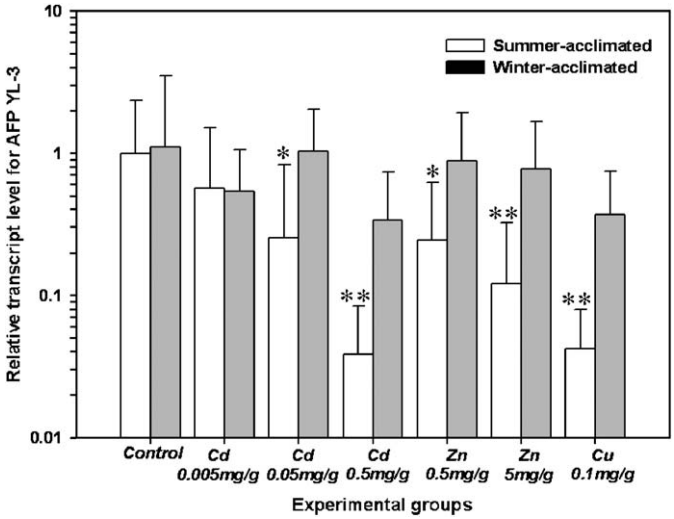

Fig. 3. Effect of metal exposure on the transcript level for AFP YL-3 under summer and winter conditions in *T. molitor*. Values were normalized to the average transcript level in the summer-acclimated control group. Error bars show the standard deviation and stars indicate statistical differences compared to the respective control group at the 0.05 (**) and 0.1 (*) level. All of the groups are represented by a sample of 10 larvae.

0.005 mg/g Cd was similar for warm and cold-acclimated larvae. In all the other metal exposed groups the average winter level of transcript expression was increased when compared to the corresponding groups under summer conditions. In the groups fed 0.05 and 0.5 mg/g Cd there was a 4.1- and a 9-fold increase compared to summer levels, respectively. The larvae from the groups exposed to 0.5, 5 mg/g Zn and 0.1 mg/g Cu also showed 3.7-, 6.5- and 8.7-fold increases in transcript level compared to summer levels, respectively.

4. Discussion

4.1. Distribution of body mass for the different treatments

The occurrence of only small 1st instar larvae in the groups exposed to the two highest levels of Cu (1 and 10 mg/g) and in the groups exposed to the highest level of Zn (5 mg/g) clearly indicates that the level of chronic metal stress in these groups was too high to sustain normal larval development. This is not surprising since these levels were far above the normal range for copper (0.005–0.025 mg Cu/g dry weight) and zinc (0.02–0.4 mg/g dry weight) in plants (Reeves and Baker, 2000). The small number of larva obtained after winter-acclimation in the groups exposed to 0.5 mg/g Cd and 0.1 mg/g Cu may have been the result of increased mortality due to toxic stress. The level of cadmium was certainly far above the normal range for this metal in plants (0.0001–0.003 mg Cd/g dry weight) (Reeves and Baker, 2000). However, it cannot be excluded that the low number of larvae obtained in the two former groups was cased by cannibalism, as this phenomenon has been reported in *T. molitor* (Weaver and McFarlane, 1990).

4.2. Effects of metal exposure on hysteresis activity under summer and winter conditions

The presence of hysteresis activity in summer-acclimated larvae was not surprising, as this phenomenon is normal in *T. molitor* (Patterson and Duman, 1978; Horwath et al., 1996; Graham et al., 2000). The level of hysteresis activity observed in the control group (~4.3 °C) is approximately 0.9 °C higher than Graham et al. (2000) reported for *T. molitor* reared under similar conditions. This difference may be due to use of smaller seed crystals during our measurements, as the hysteresis activity has been shown to be negatively related to the ice fraction (Zachariassen and Husby, 1982). As apparent from Fig. 2, the metal stress under summer conditions was accompanied by a pronounced reduction in AFP-levels, when compared to the control group. The levels of AFPs have been reported to increase with larval development in *T. molitor* (Graham et al., 2000). However, since there was no significant difference in the average body mass between the groups, differences in development cannot explain the reduced activity observed among the metal stressed groups.

4.3. Effects of metal exposure on transcript expression of antifreeze proteins under summer and winter conditions

Even though only the groups that received the highest level of metal exposure (0.5 mg/g Cd, 5 mg/g Zn, 0.1 mg/g Cu) showed significantly reduced transcription levels of AFP-YL3 under summer conditions, the results give the overall impression that the low levels of AFP's in the metal stressed animals were caused by reduced gene expression. Differential regulation of transcription factors of AFP isoforms through the year has been described in the cold tolerant beetle *Dendroides canadensis* (Andorfer and Duman, 2000). Thus, even though the YL-3 transcript codes for one of the most abundant AFP isoforms in *T. molitor* hemolymph (Liou et al., 1999), other transcription factors may be differently regulated. Still, the three-fold increase among the common AFP isoforms associated with winter-acclimation observed from purification profiles and mass spectra of the hemolymph from *T. molitor* (Graham et al., 2000), suggests that YL-3 is representative for the transcriptional regulation of AFPs in this species. Also, no new AFP isoforms were detected in the hemolymph after cold exposure, suggesting coordinated regulation of the isoforms during winter-acclimation (Graham et al., 2000).

The increase in hemolymph hysteresis activity following winter-acclimation of the control group (from 4.3 to 5.4 °C, corresponding to a 2.2-fold increase in AFP-level) is consistent with observations from other studies (Patterson and Duman, 1978; Graham et al., 2000). This increase was not accompanied by an increase in the level of transcript for YL-3. However, similar discrepancies between hysteresis activity and transcription levels were also observed in late winter beetles of *D. canadensis*, suggesting that AFPs have increased half-life during winter, reducing the need for continual expression (Andorfer and Duman, 2000). Winter-acclimation increased the hysteresis activity substantially in all the metal exposed groups, suggesting AFP-levels comparable to those in the winter-acclimated control group. The fact that metal exposed animals also showed increased expression of YL-3 AFP, suggests that the increased AFP level was linked to increased gene expression of AFPs. However, as noted by Graham et al., (2000), increased transcript levels of AFPs during winter conditions, may also come from increased mRNA stability at lower temperatures.

The presence of hysteresis activity in *T. molitor* larvae under summer conditions has been proposed to be a protection against a sudden temperature decrease in the autumn or the spring (Patterson and Duman, 1978; Graham et al., 2000). In this context the reduced hysteresis activity observed in metal stressed larvae indicates disturbance of the normal cold hardening process. Even though the metal exposed larvae seem to be able to compensate by producing more AFPs when transferred to winter conditions, this may involve a time lag before the levels are restored to normal. During this period the larvae may have a reduced capacity to inhibit lethal freezing,

leaving them more vulnerable to low temperatures. Presence of hysteresis activity during summer conditions is not a phenomenon exclusive to *T. molitor*, but it is also found in about half of the insect species known to produce AFPs (Duman et al., 1982). In these species metal stress may cause a similar reduction in summer levels of AFPs as observed for *T. molitor* in this study.

Another possible role for AFPs during summer conditions may be water conservation. In a study of the water conserving cryptonephridal complex in *T. molitor*, Ramsay (1964) found especially high hysteresis activity in the perirectal space, suggesting that these AFPs might be involved in water reabsorption. Acclimation to low humidity has been shown to increase both the hysteresis activity and the transcription level of AFPs (Patterson and Duman, 1978; Graham et al., 2000) and this was accompanied by a higher capacity to survive low humidity conditions (Patterson and Duman, 1978).

Even though the changes in AFP levels seem to be explained by altered levels in the gene expression of AFPs, it should be noted that other factors may influence the AFP activity. Low molecular mass substances, endogenous proteins and ice nucleators have been reported to enhance the activity of AFPs in vitro (Wu and Duman, 1991; Olsen and Duman, 1997a; Li et al., 1998), and the presence of varying levels of such factors in the hemolymph may thus modify the observed AFP activity. Low molecular mass solutes are probably not important in *T. molitor* since this species does not produce high amounts of glycerol (Patterson and Duman, 1978), and the low hemolymph osmolality excludes the presence of significant levels of other types of polyols (see Table 1). Also, the high hysteresis activities observed in vitro for purified AFPs from *T. molitor* ($>5\,^{\circ}C$) suggests that activators are probably not so important in *T. molitor* (Graham et al., 1997). In a recent study Wang and Duman (2005) reported that some of the different isoforms of AFPs from *D. canadensis* can activate each other. Such interactions may also be important in *T. molitor*, but probably more so in other species that produce significant amounts of polyols, as addition of 0.5 M glycerol was required to get the strongest effect (Wang and Duman, 2005).

Combating toxic stress by detoxification and excretion may be energetically costly for organisms (Calow, 1991). The reduced AFP levels in summer-acclimated beetles exposed to metals could result from a trade-off situation, where production of AFPs is down-regulated to supply an increased energy demand for detoxification. It is likely that juvenile hormone and other hormones are involved in this process. Winter cues like short photoperiod and low temperature have been shown to increase the level of juvenile hormone in *T. molitor* (Xu et al., 1992). Also, juvenile hormone has been shown to increase the production of AFP by the fat body in both *T. molitor* and *D. canadensis* resulting in increased hysteresis activity (Xu and Duman 1991; Xu et al., 1992).

It has been proposed that metal stress may lead to competition for cystein as production of metal binding proteins like metallothioneins, with up to 30% cystein, may deplete the pool of cystein and thereby reduce the ability to produce AFPs (Zachariassen et al., 2004). As the AFPs in *T. molitor* contain 20% cystein (Graham et al., 1997; Liou et al., 1999), this might be a possible scenario. However, the observed increase in hysteresis activity among the metal stressed animals after winter-acclimation does not indicate any competitive situation for cystein. The concentration of AFPs in the hemolymph of *T. molitor* is estimated to be $\sim$1–2 mg mL^{-1} (Graham et al., 1997), and may therefore be too low to cause a competitive situation. Alternatively, metal-binding proteins with low cystein may be produced as an alternative to metallothioneins. The latter alternative is supported by results, which indicate that *T. molitor* produces a novel metal binding protein without cysteine, in response to Cd exposure (Pedersen, unpublished observations).

5. Conclusions

The main finding of this study was that metal stress causes a reduction in the hemolymph hysteresis activity in *T. molitor* larvae acclimated to summer conditions. The reduction in hysteresis activity was accompanied by reduced levels of AFP YL-3 transcription, suggesting reduced levels of AFPs. Winter-acclimation of metal stressed larvae restored both the level of AFP YL-3 mRNA and hemolymph hysteresis activity to normal levels. The reduced hysteresis activity may have fitness consequences in the context that constitutive expression of AFPs in summer-acclimated animals is considered to serve a protective role against lethal freezing during frost episodes in the autumn and spring.

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Paper III

Pedersen S.A., Kristiansen E., Andersen, R.A., Zachariassen, K.E. Isolation and preliminary characterization of a Cd-binding protein from *Tenebrio molitor* (Coleoptera). Preliminary accepted in *Comp Biochem Physiol C.*

Title: Isolation and preliminary characterization of a Cd-binding protein from *Tenebrio molitor* (Coleoptera)

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Abstract

The effect of cadmium (Cd) exposure on Cd-binding ligands was investigated for the first time in a beetle (Coleoptera), using the mealworm *Tenebrio molitor* (L) as a model species. Using a Cd-saturation technique, it was found that Cd-exposure resulted in a doubling of the Cd-binding capacity of the protein extracts. Analysis showed that the increase was mainly explained by the induction of a Cd-binding protein of 7134.5 Da, with non-metallothionein attributes. Amino acid analysis and *de novo* sequencing revealed that the protein has an unusually high content of the acidic amino acids aspartic and glutamic acid that may explain how this protein can bind Cd, even without cysteine residues. Similarities in the amino acid composition suggest that the protein belong to a group of little studied proteins that is often referred to as “Cd-binding proteins without high cysteine contents”. This is the report on isolation and peptide sequence determination of a “Cd-binding protein without high cysteine contents” from a coleopteran.

Keywords: Cadmium; Cd-binding protein; Coleoptera; *De novo* sequencing; Metal detoxification; *Tenebrio molitor*

1. Introduction

Cadmium (Cd) is a heavy metal without any known biological function. It has caused concern as an environmental contaminant due to its extreme toxicity and ability to cause mutations (McMurray and Tainer, 2003). In insects Cd is not regulated and enters cells via mediated transport through Ca^{2+}-channels and probably through non active ionopores (Braeckman et al., 1999). Inside the cells Cd binds to cytosolic and nuclear materials, causing a variety of damaging effects, including inhibition of zinc-containing enzymes, lipid peroxidation and disturbance of protein synthesis (Beyersmann and Hechtenberg, 1997). Detoxification of accumulated trace metals is generally achieved via sequestration in membrane enclosed vesicles (granules) which form a part of the lysosomal fraction in cells (Dallinger, 1993), and through binding to metallothioneins (MTs) in the cytosol (Korsloot et al. 2004).

While MTs appear to be ubiquitous in vertebrate tissues (Hamer, 1986), in insects these proteins have only been unequivocally identified in *Drosophila* flies (Lastowski-Perry et al., 1985; Mokdad et al., 1987) and in the springtail *Orchesella cincta* (Hensbergen et al., 1999). However, as pointed out by Stone and Overnell (1985), several insects and other invertebrates have “Cd-binding proteins without high cysteine contents”. For instance, the pronounced Cd resistance in the aquatic larvae of the stonefly *Pteronarcys californica* seems to be due to a Cd-binding glycoprotein with only ~ 2 % cysteine (Clubb et al., 1975). Similarly, Martoja et al. (1983) found that Cd-exposure induced a Cd-binding glycoprotein with low cysteine content (2.1 %) in the locust *Locusta migratoria*. In the woodlice *Porcellio scaber* Dallinger et al. (1993) found a Cd-binding protein with low cysteine content (2.9 %), showing an amino acid composition very similar to that of the two Cd-binding glycoproteins. This far only a limited number of species have been investigated with regards to metal binding proteins, and sequence information on “Cd-binding proteins without a high cysteine contents” from insects is still missing. Hence, the nature and importance of these proteins in detoxification of metals remains unresolved.

Even though beetles (Coleoptera) constitute one third of all known insects species, and one forth of all known species altogether, little is known about how they protect themselves against metals in the environment. Lindqvist and Block (1995) found that larvae of the beetle *Tenebrio molitor* were unable to control the body composition of Cd when they were exposed to the metal through the diet. They also found that most of the accumulated Cd was excreted during the metamorphosis (Lindqvist and Block 1995), indicating that some form of sequestration was involved. Preliminary studies in our lab have indicated that an increase in the Cd-binding capacity takes place following exposure to Cd in *T. molitor*, suggesting that coleopterans have proteins that assist in the detoxification of Cd. The aim of this study was to isolate and characterise the ligand(s) responsible for the increase in Cd-binding capacity in *T. molitor*.

2. Material and methods

2.1. Exposure conditions

Commercially obtained *T. molitor* larvae (Blades Biological, Edenbridge, Kent, UK) were kept in ventilated plastic terrariums (25 x 15 x 20 cm). A layer of wheat bran (3 cm) covered the terrarium floor. One group of 500 animals was fed wheat bran contaminated with 0.5 mg Cd $\cdot g^{-1}$ dry weight ("Cd-exposed group") while another group of 500 animals were feed non-contaminated food ("control group"). The contaminated food was prepared by mixing 80 g of wheat bran with 65.24 mg Cd chloride ($CdCl_2$ x 2 H_20, Merck) dissolved in 200 mL of distilled water and drying it over night at 90ºC.

The terrariums containing the animals, were kept in a climatic chamber (25 °C, L/D: 18/6) for a period of one month. A piece of paper on top of the wheat bran provided cover for the animals, and food was renewed once a week. The paper was gently soaked with water daily to avoid dehydration. After three weeks of exposure the animals were allowed to empty their guts by removing the food 48 h before they were collected. They were stored inside 50 mL test tubes at -20ºC until further processing. After the treatments

the mean body mass (± SD) of the larvae in the Cd-exposed group and the control group were 128.5 ± 0.3 mg and 130.3 ± 0.3 mg, respectively.

2.2. Cadmium analysis

Six subsamples of approximately 0.3 g each were collected from the Cd-contaminated food and the non-contaminated food and dried to constant mass at 90ºC. Also, six animals were randomly taken from both the Cd-exposed group and from the control group. The dry body mass (± 0.1 mg) was determined after freeze drying. All samples were acid digested using nitric acid (0.54 mL of 65 %) and hydrogen peroxide (0.27 mL of 20-40 %) on a heating plate (85ºC, 1 h). The samples were cooled and diluted to a final volume of 13 mL using deionized water (>18 MΩ cm^{-1}, Milli-Q) and analyzed on a high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS). Standard reference material (Bovine liver (15776), US National Institute of Standards and Technology) was analyzed to verify the efficiency of the digestion and the calibration of the instrument. The measured concentration of Cd in the reference material (0.514 ± 0.010 µg g^{-1}, n = 6) was 2.8 % above the certified value. The reagent blanks were generally constant and negligible (< 1 % of the concentration in the solutions).

2.3. Cd-binding capacity (Cd-BC)

The Cd-BCs of the animals were measured by a Cd-saturation method described by Bartsch et al. (1990), originally designed to measure the concentration of MT in tissue samples. In this technique Cd and a small amount of radiotracer (^{109}Cd) bind to proteins whereupon excessive amounts of Cd are removed by the cationic exchanger Chelex 100 (Bio-Rad). The only deviation from the original method, apart from using homogenates of whole animals instead of tissue samples, was the preparation of protein extracts. In this study 10 volumes of buffer, instead of 4 volumes, were added per gram of fresh weight sample during the homogenization. The ^{109}Cd content was determined by using a gamma counter (COBRA II Auto-gamma, Packard, GMI, Minnesota, USA). The Cd-BCs of the samples were calculated using the following equation:

µg Cd bound / g fresh body mass = $(CPM_{(s)} - CPM_{(bkg)}) \times 29.56/CPM_{(tot)} \times 16.39$

where $CPM_{(s)}$: counts per minute in sample, $CPM_{(bkg)}$: counts per minute in the background sample and $CPM_{(tot)}$: counts per minute in the total counts sample. The constant 29.56 refers to the amount of Cd (µg) added per millilitre of sample solution, and the constant 16.39 refers to the sample dilution during the preparation of protein extracts and during the assay procedure.

2.4. Preparation of protein extracts

The frozen animals were homogenized together in a glass tube with 7 volumes of degassed ice-cold Tris buffer (1 mM phenylthiocarbamide, 1mM phenylmethylsulphonylfluoride, 20 mM Tris/HCl, pH 7.6) using an Ultra Turrax T18 homogenizer (T 18 basic, IKA, Wilmington, USA) at 30,000 rpm for 1 min. The resulting supernatants after heat treatment (80ºC, 13 min) and centrifugation (30,000 *g*, 10 min, 0ºC) were immediately frozen and stored at -20ºC. This procedure was applied on animals from both the Cd-exposed group and the control group.

2.5. Marking of Cd-binding ligands with ^{109}Cd

Cadmium-binding ligands in the protein extracts were marked using a procedure modified from the Cd-saturation method described by Bartsch et al. (1990). Five volumes of protein extract were mixed with 2 volumes of $CdCl_2$ solution with a small amount of radiotracer (^{109}Cd)(0.66 mM Cd in 20 mM Tris/HCl, pH 7.6, 0.5 mCi/ mg Cd) and incubated under continuous mixing using a Rotator Drive (STR-4, Stuart Scientific, Jencons) at 10 rpm for 3 min. One volume of Chelex 100 resin (66 % in 10 mM Tris/HCl, 85 mM NaCl, pH 7.6) was added, and the mixture was incubated for 3 min under continuous mixing using the rotator drive. The Chelex 100 resin was removed by centrifugation at 10 000 *g* (15 min, 4ºC) and the collected supernatant was filtered using a 0.45-µm filter (GHP Arodisc, Pall Gelman Sciences). Addition of acetonitrile to the

protein extracts was omitted since large proteins already had been removed by heat treatment (see above).

2.6. Ion exchange

The radioactively marked homogenates (~ 40 mL) were directly applied to an ion exchange column (XK-16/20 packed with Q Sepharose High Performance, Amersham Biosciences, Uppsala, Sweden) using a protein purification system (AKTA prime with a 50 mL super-loop, Amersham Biosciences). Proteins were eluted for 140 min at a flow rate of 1 mL min^{-1} in a linear gradient of buffer A (20 mM Tris/HCl, pH 7.6) to 55 % buffer B (1 M NaCl, 20 mM Tris/HCl, pH 7.6). Absorbance was measured at 214 nm (peptide bonds). Contents of ^{109}Cd was determined in all collected fractions (1 mL) using the gamma counter. Fractions that gave a peak in ^{109}Cd activity were pooled and concentrated to ~ 0.5 mL using a Macrosep 1K Omega centrifugal concentrator with a 1 kDa cut-off (Pall Life Sciences).

2.7. Size exclusion chromatography

The concentrates from the ion-exchange were applied to a size exclusion column (XK-16/70 packed with Superdex 30 (separation range 1 – 10 kDa), Amersham Biosciences) and eluted using a running buffer (200 mM NaCl, 20 mM Tris/HCl, pH 7.6). Absorbance was measured at 214 nm and ^{109}Cd-concentration was determined in all collected fractions (0.5 mL) using the gamma counter. The void volume (V_0) was determined using blue dextran (2,000 kDa) while the elution volume (V_e) of the pure standards aprotinin (6512 Da) and glutathione (307 Da) were used to obtain a selectivity curve for the size exclusion column (see Andrews, 1965). This relationship was used to find the apparent molecular mass of the protein eluting in the ^{109}Cd-peak. The fractions corresponding to the peak in ^{109}Cd (69-71 mL) were pooled and concentrated to ~ 0.5 mL using Macrosep 1K Omega. The salt was removed by diluting the concentrates to 10 mL using deionized water and repeating the concentration step three times. Aliquots of the dialysates were freeze-dried (Hetovac VR-1, HetoHolten, Allerad, Denmark).

2.8. High performance liquid chromatography

The purity of the ^{109}Cd-peak from the size exclusion step was analyzed using high performance liquid chromatography (HPLC). An aliquot of the ^{109}Cd peak was applied to a HPLC system (Thermo Separation Products, Spectra Series P200 with an UV100 unit) equipped with a reverse phase column (Vydac Protein and peptide C18 column) and eluted using a 5 – 90 % gradient of buffer B_1 (30 % trifluoroacetic acid (TFA) (0.1 % w/v) and 70 % acetonitrile) in buffer A_1 (0.1 % TFA w/v). The absorbance was registered at 222 nm (Biotechnology Centre of Oslo, University of Oslo, Norway).

2.9. Mass spectrometry

2.9.1. Mass determination

A freeze dried aliquot of the ^{109}Cd-peak from the size exclusion step was dissolved in 20 μL distilled water (milli-Q), and 0.5 μL of this solution was mixed into 0.5 μL sinaminic acid solution (1.5 mg mL^{-1}), containing 1/1 (v/v) water – acetonitrile with 0.1 % TFA, and pipetted on to a sample plate. After the samples had dried at room temperature, the plate was inserted into a mass spectrometer (Reflex IV, Bruker Daltonics) and the mass spectrum was determined by MALDI-ToF as previously described (Kristiansen et al., 2005).

2.9.2. Trypsin fingerprint and *de novo* sequensation

A freeze dried aliquot of the ^{109}Cd-peak from the size exclusion step was reduced and alkylated using dithiothreitol (Amersham Biosciences) and iodoacetamid (Sigma Aldrich). Trypsin Porsine (Promega) was used to digest the protein, and the resulting fragments were N-terminus derivatizated using Ettan CAF-MALDI sequencing Kit

according to the manufacturers instructions (Amersham Biosciences). The sample was spotted onto a sample plate with matrix (2.5-dihydroxybenzoic acid). The spotted sample was first analyzed using MALDI-ToF (Ultraflex, Bruker Daltonics) to generate a peptide mass fingerprint, and sequence information was obtained using the MS/MS-mode of the instrument (PROBE, Proteomic Unit at University of Bergen, Bergen, Norway).

2.10. Amino acid composition

A freeze dried aliquot of the ^{109}Cd-peak from the size exclusion step was hydrolyzed in 6N HCl (24 h at 108-110ºC) and subjected to amino acid analysis in an amino acid analyzer (Model 421, Applied Biosystems) according to standard procedure (Biotechnology Centre of Oslo, University of Oslo, Norway). To avoid oxidation of cysteine and methionine the hydrolysis was carried out in vacuum.

2.11. Statistical treatment

To compare means between the Cd-exposed group and the control group Student's *t* test was used. All data were log-transformed prior to statistical treatment.

3. Results

3.1. Cd analysis and measurement of Cd-BC

Analysis of the food used to feed both the control and the Cd-exposed group revealed only small differences compared to the nominal concentration (Table 1). The final Cd concentration in the animals from the Cd-exposed group was approximately thousand times higher than the level in the control animals ($p < 0.001$, $t = 6.39$, $DF = 10$). The Cd-BCs were approximately doubled in animals from the Cd-exposed group when compared to individuals from the control group ($p < 0.001$, $t = -5.21$, $DF = 18$).

3.2. Anion exchange chromatography

The anion exchange chromatogram of heat treated protein extracts from the control and the Cd-exposed animals revealed almost identical elution profiles in several parallel runs (data not shown). In the control animals (Fig. 1A), Cd concentrations in the collected fractions, measured by the radiotracer ^{109}Cd, showed that little of the Cd (~5 %) was associated with the proteins that passed through the column during both sample application (Ve = 25-35 mL) and washing (Ve < 115 mL). Most of the Cd was associated with the component(s) appearing as a shoulder of the main absorbance peak (Ve = 90 mL). However, in the Cd-exposed group (Fig. 1B) a distinct Cd-peak in fractions eluting between 100-105 mL was apparent, which could not be detected in the control group.

3.3. Size exclusion chromatography

The elution profile of the Cd-peak concentrate from the ion exchange showed an absorbance peak corresponding to the void volume of the column (V_0 ~50 mL), indicating that the majority of proteins in the concentrate had a molecular mass ≥ 10 000 Da (nominal cut off for the column). Cadmium concentrations in the collected fractions, detected as radioactive ^{109}Cd, showed that only a minor amount of the Cd was associated with proteins eluting in the void volume. In the control group the Cd was distributed evenly among the collected fractions, with no apparent distinct Cd-peak (not shown). However, the Cd-exposed group (Fig. 2) showed a distinct peak in Cd concentration with a V_e ~ 67.5 mL. This peak was also reflected by a small but corresponding peak in absorbance at 214 nm. The apparent molecular mass, based upon the elution coefficient of known molecular marker proteins, was ~ 7500 Da.

3.4. Reverse phase chromatography

An aliquot of the pooled fractions from the Cd-peak collected during the size exclusion chromatography were separated using reverse phase chromatography. As apparent from the elution profile (insert I, Fig. 2), only one distinct absorbance peak could be detected near the end of the gradient. This suggested that the metal-binding protein was successfully separated from the rest of the protein components after the two previous chromatographic steps.

3.5. Mass spectrometry, trypsin fingerprint and de novo sequencing

Mass spectrometry of the Cd-peak from the size exclusion chromatography revealed the presence of a single component with a molecular mass of 7134.5 Da (insert II, Fig. 2). The mass spectra obtained after tryptic digest (not shown) showed no similarity with other described proteins when using the peptide mass fingerprinting tools Aldente (http://www.expasy.org/tools/aldente/) and Mascot (http://www.matrixscience.com/search_form_select.html). The sequence information obtained by *de novo* sequencing of four of the peptide fragments in the tryptic digest is shown in Table 2. Two alternative combinations of the latter part of the sequences fit with the observed fragmentation pattern for peptides 1071.6, 1238.8 and 1699.7 Da, and can therefore not be distinguished. This may possibly be due to repeat motifs inside the protein. Blast of the fragment sequences (http://www.ncbi.nlm.nih.gov/BLAST) failed to give any significant match with other described proteins.

3.6. Amino acid composition

The amino acid composition of the *T. molitor* Cd-binding protein determined in the present work is given in Table 3. Assuming that the amino acid of lowest abundance is represented only once in the sequence, the combined mass of the identified amino acids is 6781.76 Da. Considering the crudeness of the present procedure, the similarity of this combined mass and the experimentally determined mass (7134.5 Da) is reasonably good.

The most abundant amino acids were Asx (Asp + Asn) and Glx (Gln + Glu) which constituted 13.3% and 37.7% of the total residues, respectively. The protein also seems to contain modest amounts of the aromatic amino acids tyrosine, tryptophan and phenylalanine in addition to histidine (1.9 %). No cysteine, however, was detected.

3.7. N-terminal sequencing

Repeated attempts of N-terminal sequencing by the Edman-technique failed, suggesting that the protein was N-terminally blocked. Internal sequencing using cyanobromide to cut within the protein also failed.

4. Discussion

Analysis of the protein extracts using ion-exchange and size exclusion chromatography shows that a Cd-binding protein of 7134.5 Da is mainly responsible for the doubling in Cd-BC observed following Cd-exposure of *T. molitor* (Table 1.). This protein is absent or present in very low titre in the control animals. Simple addition of radiotracer (^{109}Cd) directly to the protein extracts prior to the chromatographic procedures gave poor in-between run reproducibility, as the distribution of the radiotracer between different protein peaks was strongly dependent on the amount of radiotracer added, indicating "spillover" of Cd to proteins with lower affinity (data not shown). However, removal of unbound and loosely bound Cd, by the addition of a chelating resin (Chelex-100), solved these problems and also provided information on the amount/binding-capacity of the Cd-ligands.

The molecular mass obtained by mass spectrometry of the isolated Cd-binding protein (7134.5 Da) is somewhat lower than the apparent molecular mass (7500 Da) estimated from the size exclusion chromatography. Although this difference may be due to the crudeness of the estimate from the gel-filtration procedure, it is a phenomenon often seen among proteins with an elongated shape like e.g. MTs (Kägi and Nordberg, 1979). The

heat treatment (80°C, 13 min) performed on the protein extracts prior to the chromatography shows that the protein is heat stable. N-terminal sequencing of this protein was not successful, suggesting that the protein may be N-terminally blocked. The peptide finger print and four peptide sequences obtained from the tryptic digest failed to show similarities with described proteins using on-line software, suggesting that the Cd-binding protein is novel.

The Cd-binding protein from *T. molitor* shows some characteristics that are typical for MTs (e.g. Cd-affinity and inducibility, low molecular mass, heat stability). However, the four partial sequences that were determined by *de novo* sequencing showed no cysteine residues (Table 3). This is confirmed by the amino acid composition analysis (Table 2), which also showed no presence of cysteine. Additionally, aromatic amino acids like tryptophan, tyrosine and phenylalanine were also present in moderate levels in the amino acid composition analysis (1-2 residues of each). Since a high content of cysteine (~30 %), and no/low content of aromatic amino acids are some of the key characteristics of MTs (Kägi and Nordberg, 1979), it can be concluded that the inducible Cd-binding protein isolated in the present work is not a MT.

The amino acid composition of the Cd-binding protein from *T. molitor* shows similarities with Cd-binding proteins previously described in some other insects (Clubb et al., 1975; Martoja et al., 1983; Dallinger, 1993) referred to as “Cd-binding proteins without high cysteine contents” in a review by Stone and Overnell (1985). To make comparisons convenient, the amino acid compositions of three such proteins are summarized in Table 2, along with that of the inducible Cd-binding protein from *T. molitor*. Besides a low apparent molecular mass (5-20 kDa) and a high proportion of acidic amino acids, the proteins also share a low content of cysteine and a substantial content of aromatic amino acids and histidine.

The absence of cysteine residues in the Cd-binding protein from *T. molitor* means that other groups than sulfhydryls must be responsible for the Cd-binding. Potential candidates for the complexation of Cd are the acidic amino acids glutamic- and aspartic-

acid which constitute 35 % of the amino acids in the *de novo* sequences that gave a distinct fragmentation pattern (letters not underlined in Table 2). Investigations of non-MT-like metal-binding proteins have shown that the negatively charged carboxyl groups of aspartic- and glutamic-acid residues may provide binding sites for metals like Cd. One example is the barnacle *Megabalanus volcanus*, where two peptides of 1703 and 2004 Da are produced in response to Cd-exposure (Togi et al., 1998a, b). These two peptides have no cysteine residues, but a high proportion of acidic amino acids (33%), which by means of NMR have been shown to bind Cd unspecifically and reversibly (Togi et al., 1998b). Also, Nagano et al. (1984) described an inducible Cd-binding protein from the alga *Chlorella ellipoidea* with 52 % glutamic acid.

Whether the isolated Cd-binding protein is a normal low titre constituent without metals that becomes induced in Cd-exposed animals due to functional inactivation from complexing Cd, or if this complexation is a functional property of the protein, can not be concluded. The last scenario opens the possibility of the Cd-binding protein acting in concert with MTs in the detoxification of metals. Presence of MTs in the tissues of *T. molitor* may have been obscured by the Cd-binding protein isolated in the present work, since Cd-concentrations in collected fractions were used to detect metal binding proteins.

Cysteine is considered to be semi-essential, since it must be derived from the essential amino acid methionine (Cohen, 2004). *In vitro* studies on the effect of cysteine addition on MT production and survival in Cd-resistant cell lines from *D. melanogaster* have shown that cysteine may become a limiting factor under certain circumstances (Debec et al., 1985). Such a situation, which might favour metal-binding proteins of the “low cysteine type”, may exist in some freeze avoiding invertebrates from cold areas. Animals from these species rely on production of cysteine rich antifreeze proteins (17-19 %, see Duman et al., 1998; Liou et al., 1999) that promote supercooling and allow them to avoid lethal freezing during the winter season (Zachariassen, 1985; Duman, 2001). In a recent study chronically exposure of *T. molitor* to Cd, Cu or Zn during winter acclimation was found to have no apparent effect on the capacity to produce antifreeze proteins (Pedersen et al. 2006). The finding in the present study, that an inducible Cd-binding protein of the

"low cysteine type" is responcible for most of the Cd-binding in *T. molitor*, possibly serving as a supplement to MT, may explain why no reduction in the capacity to produce antifreeze proteins could be observed.

Including the present work, Cd-binding proteins of the "low cysteine type" has so far been identified in tree insect orders (see Clubb et al., 1975; Martoja et al., 1983). Considering the modest number of studies on metal binding proteins from insects, it seems likely that similar proteins are present in more insect species. However, more information about the structure, dynamics and internal localisation of these proteins is necessary before a functional role in detoxification can be assigned these proteins.

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Fig. 1. Anion exchange of heat treated supernatant from control (A) and Cd-exposed (B) larvae of *T. molitor*. Upper panel: Absorbance profile at 214 nm. Lower panel: ^{109}Cd profile. Buffer: 20 mM Tris pH 8.6. Gradient: 0 - 600 mM NaCl. Flow rate: 1 mL min^{-1}. Fraction size: 1 mL. Fractions eluting between 100 and 105 mL were pooled for further separation using size exclusion chromatography (indicated by horizontal bars).

Fig. 2. Elution profile on size exclusion chromatography of concentrate from the Cd-peak in ion exchange separated. Upper panel: Absorbance profile at 214 nm. Lower panel: ^{109}Cd profile (counts min^{-1} mL^{-1}). Buffer: 20 mM Tris pH 8.6 containing 250 mM NaCl. Flow rate: 0.5 mL min^{-1}. Fraction size: 1 mL. Symbols (▼) indicate elution in parallel runs of the molecular mass standards (from left to right): Aprotinin (6512 Da), glutathione (307 Da). Insert I: Elution profile from reverse phase chromatography of aliquot from Cd-peak (fractions 67-71, indicated by horizontal). Solid line: Absorbance profile at 222 nm. Broken line: 5-90 % buffer B (A = 0.1% trifluoroacetic acid, B = 30 % A + 70 % acetonitrile). Insert II: Mass spectra obtained from an aliquot of the Cd-peak.

Table 1

Content of Cd in the food and in the whole body of *T. molitor* larvae (mean ± SD). Accumulation factor relative to actual Cd-content in food is listed in brackets below Cd-content in animals. Values are related to dry mass except for the Cd-binding capacity in the protein extracts which is related to the wet body mass. Number of replicates is shown in brackets and statistical difference from control group ($p < 0.001$) is indicated by asterisk (*).

	Nominal Cd-content in food ($\mu g\ g^{-1}$)	*Actual Cd-content in food ($\mu g\ g^{-1}$)*	*Cd-content in animals ($\mu g\ g^{-1}$)*	*Cd-binding capacity ($\mu g\ g^{-1}$)*
Control	0	0.43 ± 0.01 (6)	0.25 ± 0.06 (6) (0.58)	26.69 ± 11.10 (10)
Cd-exposed	500	503.01 ± 0.92 (6)	258.05 ± 72.76 (6)* (0.51)	62.20 ± 19.01*(10)

Table 2

Partial amino acid sequence of *T. molitor* Cd-binding protein. The sequence information was obtained by MALDI-TOF using CAF-reagens subsequent to reduction, alkylation and trypsin-digest. The peptide mass was obtained by subtracting the extra mass from the CAF reagens (136 Da). Isoleucine and leucine can not be distinguished by this technique (I/ L). The underlined letters printed in italics indicate alternative combinations of amino acids that fit the fragmentation patterns observed, and are therefore uncertain.

Peptide mass (Da)	Sequence
1071.6	AD(I/L)AEQA(*I/L)A*K *A(I/L)*
1199.6	EG(I/L)(I/L)DPAWEK
1238.8	N(I/L)EDHD(I/L)*RW*R *DN(I/L)*
1699.7	(I/L)EQVDDE(I/L)EDS*MPK* *(I/L)ER*

Table 3

The amino acid composition of *T. molitor* Cd-binding protein and representative isoforms from other insect species. The number of residues of each amino acid is indicated in brackets. All other numbers are percentage of total number of residues in the proteins.

	Coleoptera ψ *T. molitor* (This work)	Locusta† *Locusta migratoria*	Plecoptera‡ *Pteronarcys californica*	Isopoda* *Porcellio scaber*	Fruit fly§ *Drosophila melanogaste*
Cys (C)	–	2.1	1.9	2.6	25.0
Asx (N/D)	13.25 (7)	10.9	4.9	17.7	7.5
Glx (Q/E)	37.7 (20)	12.9	15.9	19.8	5.0
Ser (S)	3.8 (2)	9.0	3.45	7.6	15.0
Gly (G)	7.5 (4)	10.9	12.9	15.1	17.5
His (H)	1.9 (1)	2.2	3.8	–	–
Arg (R)	1.9 (1)	2.0	3.1	–	–
Thr (T)	3.8 (2)	9.0	2.3	6.2	2.5
Ala (A)	7.5 (4)	11.0	4.2	8.7	7.5
Pro (P)	3.8 (2)	11.0	1.3	6.2	5.0
Tyr (T)	1.9 (1)	–	2.2	–	–
Val (V)	3.8 (2)	4.3	4.5	3.7	–
Met (M)	1.9 (1)	–	0.3	–	2.5
Ile (I)	1.9 (1)	2.2	1.3	3.6	–
Leu (L)	3.8 (2)	4.1	3.1	3.9	–
Phe (F)	1.9 (1)	2.1	1.7	–	–
Lys (K)	1.9 (1)	6.3	29.4	4.9	12.5

ψ (This paper.)

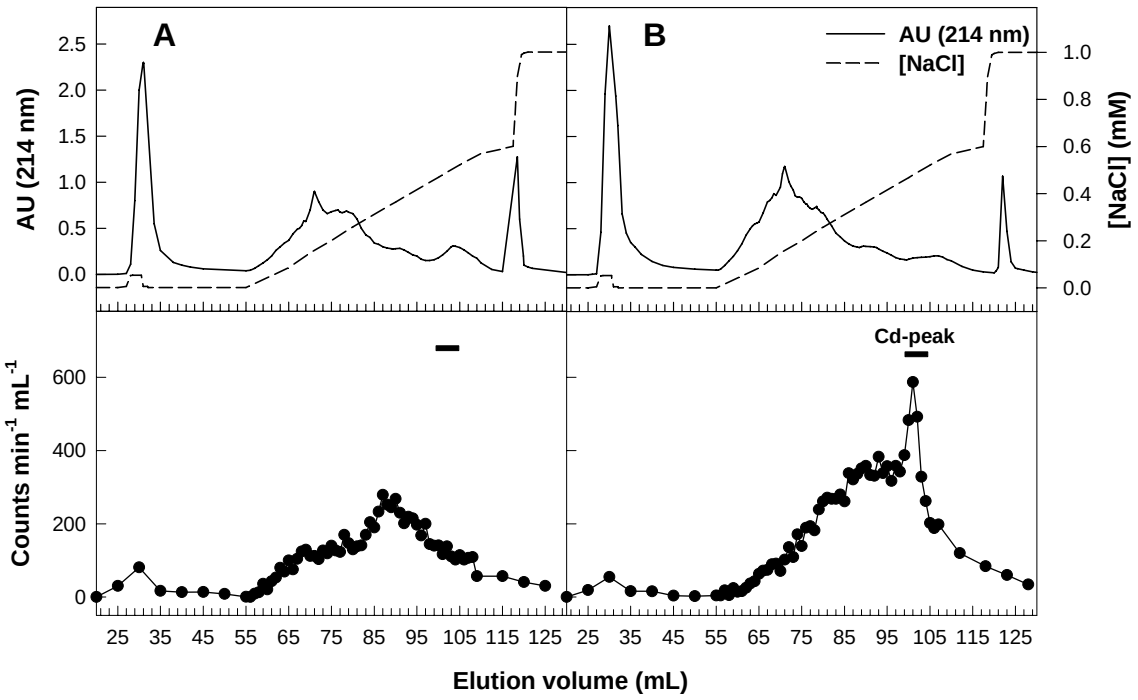
† (Martoja et al., 1983)

‡ (Clubb et al., 1975)

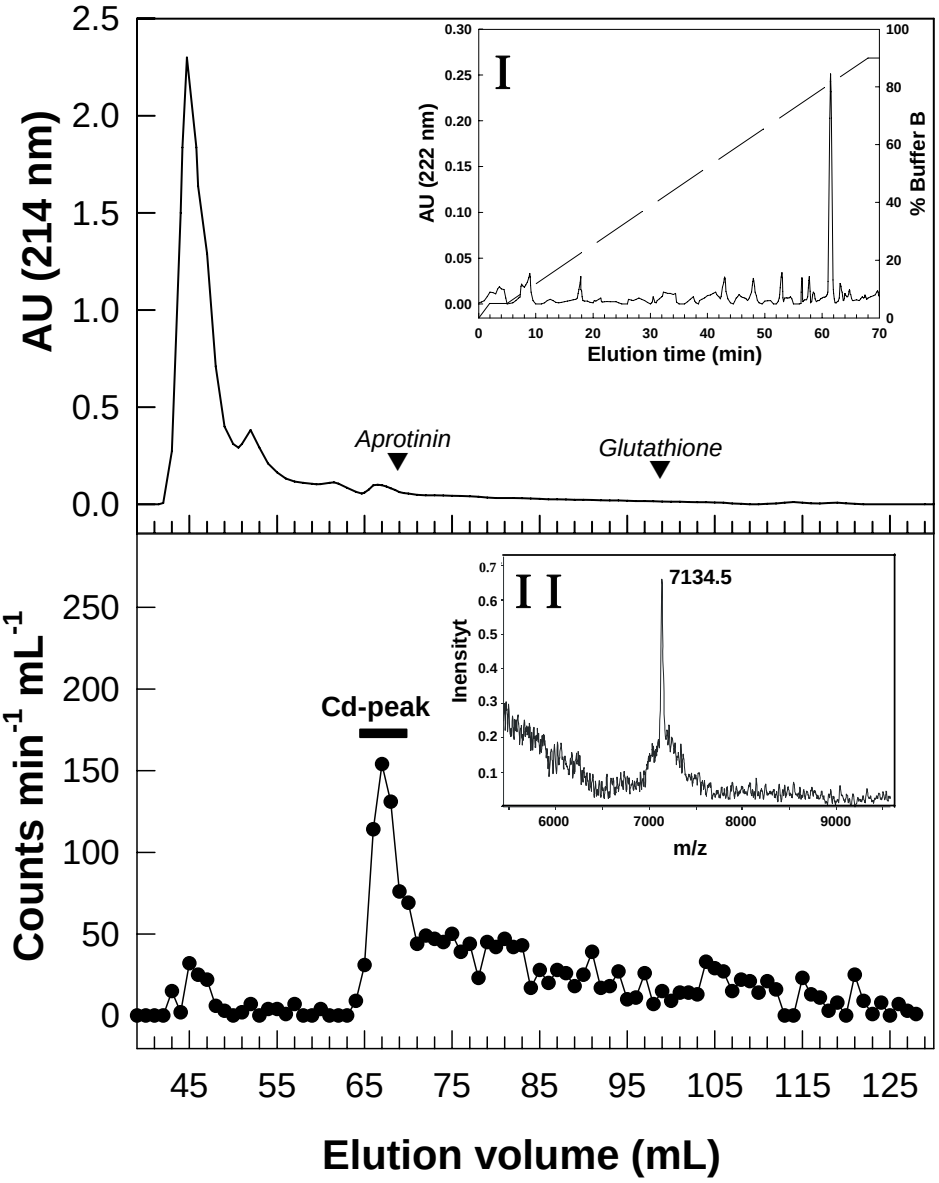
* (Dallinger, 1993)

§ (Lastowski-Perry et al., 1985)

Fig. 1.


A
B
AU (214 nm)
[NaCl]
AU (214 nm)
[NaCl] (mM)
Cd-peak
Counts min-1 mL-1
Elution volume (mL)

1 Fig. 2.


AU (214 nm)
Aprotinin
Glutathione
I
AU (222 nm)
% Buffer B
Elution time (min)
Counts min-1 mL-1
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m/z
Elution volume (mL)

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Paper IV

Pedersen S.A., Kristiansen E., Andersen, R.A., Zachariassen, K.E. Cadmium exposure of the beetle *Tenebrio molitor* (L): Internal distribution of Cd and induction of a Cd-binding protein in the gut content. Manuscript.

Title: Cadmium exposure of the beetle *Tenebrio molitor* (L.): Internal distribution of Cd and induction of a low cysteine Cd-binding protein in the gut content.

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Abstract

In terrestrial arthropods binding of cadmium (Cd) to metallothionein (MT) and non-MT proteins with low contents of cysteine have been observed. We recently reported that Cd-exposure induced a Cd-binding protein of the low cysteine category in *Tenebrio molitor*. In this study we have examined the molecular distribution of Cd within extracts of different tissues and compartments of Cd-exposed *T. molitor*. The low cysteine Cd-binding protein was found to be induced within the gut content where it could be detected after 4-8 days of exposure. No increase in Cd-binding capacity, suggesting no accumulation of MTs, was found in extracts of the gut wall tissue of the exposed larvae. Incorporation of Cd in the gut wall tissue stabilized after day 8 at a rather low level compared to the other organs. There was a statistical trend towards Cd being incorporated in the gut content in a manner that was disproportionally high compared to the amount of Cd in the gut wall tissue. The possible role of the low cysteine Cd-binding protein in reducing the uptake of Cd in the tissues is discussed.

Keywords: Cadmium; Cd-binding protein; Coleoptera; Metal detoxification; *Tenebrio molitor*

1. Introduction

Cadmium (Cd) is an extremely toxic and mutagenic non-essential metal of concern as an environmental pollutant (McMurray and Tainer, 2003). It has the ability to form lipid soluble inorganic complexes (Gutknecht 1981) which may easily cross the microvilli border of the digestive tract in animals. Cd is not actively taken up in insects, and enters the cells via mediated transport through Ca^{2+}-channels and non-active ionopores (Braeckman et al., 1999). Inside the cells, Cd may cause damage by binding to cytosolic and nuclear material, with effects such as inhibition of zinc-containing enzymes, peroxidation of lipids and disturbance of protein synthesis (Beyersmann and Hechtenberg, 1997).

In terrestrial arthropods food is normally the main source of metals, and the wall of the digestive tract seems to be the major organ for storage of metals (Aoki et al., 1984; Hensbergen et al., 1999; Lindqvist et al., 1995; Maroni and Watson, 1985; Suzuki et al., 1984). Detoxification of accumulated trace metals is generally achieved by storage sequestration in membrane enclosed vesicles (granules), which form a part of the lysosomal fraction in mid-gut epithelial cells (Ballan-Dufrancais, 2002; Dallinger, 1993), and through binding to metallothioneins (MTs), in the cytosol (Korsloot et al., 2004). Some arthropods have efficient mechanisms to eliminate the sequestered metals. In the fruit fly *Drosophila melanogaster*, apical extrusion of Cd from the epithelial cells have been observed (Lauverjat et al., 1989), while in the springtail *Orchisella cincta*, ~35% of the total Cd burden is eliminated during each moult, by renewing the midgut epithelium, and shedding the old (Posthuma et al., 1992).

When exposed to Cd, some arthropods have been shown to produce Cd-binding proteins with low cysteine content (Clubb et al., 1975; Martoja et al., 1983; Dallinger, 1993). An inducible

Cd-binding protein was recently isolated from whole animal extracts of the mealworm larvae *T. molitor* (Pedersen: Unpublished). Amino acid analysis and *de novo* sequencing revealed that the protein (7134 Da) contained no cysteine residues, but displayed unusually high content of glutamic and aspartic acid (Pedersen: Unpublished). Apart from the fact that they become induced by Cd-exposure, and that they may bind Cd, little is known about these non-metallothionein proteins. Which tissue(s) they are associated with, and a functional role for these proteins in detoxification of metals remains to be shown. In the present study we have examined the location and time dependency for the induction of Cd-binding proteins during dietary exposures to Cd in larvae of the beetle *T. molitor*. To complement these studies the whole body distribution of Cd was also examined.

2. Materials and methods

2.1. Exposure conditions

Commercially obtained *Tenebrio molitor* larvae (Blades Biological, Edenbridge, Kent, UK) were exposed to wheat bran contaminated with 0.5 mg Cd g^{-1} dry weight. The food was prepared soaking 80 g of wheat bran with 200 mL of a cadmium chloride solution (65.24 mg $CdCl_2 \cdot 2H_20$ and 20 µL of a ^{109}Cd stock (3.7 KBq mL^{-1}, Perkin Elmer, Wellesley, MA, USA)), followed by drying over night at 90 ºC. The radiotracer ^{109}Cd was added to allow Cd content in larvae, dissected tissues and eluted fractions to be determined from the radioactivity measured on a γ-counter (COBRA II Auto-gamma, Packard, GMI, Minnesota, USA). To verify homogenous distribution of Cd in the bran and determine the ratio between non-radioactive Cd and ^{109}Cd (assuming a nominal concentration of 0.5 mg Cd g^{-1}), counts per minute was measured in five subsamples (~ 0.5 g each) of the contaminated bran. The

radiotracer was omitted from the food of the larvae where the gut tissue was intended for Cd-saturation treatment (see below for description). The larvae were kept at 25 ºC (L/D: 18/6 h) in ventilated plastic terrariums (25·15·20 cm^3) with a layer of Cd-contaminated food (~ 3 cm) covering the floor. After the 16-day exposure period some exposed larvae were also transferred to non-contaminated food and held there for 4 and 8 days. In this experiment, moulting or pupating larvae were excluded. Food was renewed once a week. A piece of paper resting on top of the bran provided cover for the animal. The paper was gently soaked with water daily to avoid dehydration.

A second group of larvae were exposed to Cd by direct injections (1 µL g^{-1} fresh weight) of a cadmium solution (1 µL 25mM $CdCl_2 \cdot 2H_20$, 1.5% NaCl, spiked with ^{109}Cd) into their body cavities. These larvae were offered uncontaminated food during the 16 day incubation period following the injection.

2.2. Analysis of Cd-binding protein(s) in the whole alimentary tract, gut wall tissue and gut content

Larvae were sampled at different points (3, 6, 12 hrs, 1, 2, 4, 8, and16 days) during the course of the 16-day exposure period to Cd. At each time point the whole alimentary tracts (tissue and content) were dissected out of 20 larvae. This was done by loosening the distal end of the alimentary tract by cutting into the middle of the last segment with a fine pair of forceps. The alimentary tract could then be removed through the posterior end of the larvae by gently dislodging the larvae's head with a pair of tweezers. The 20 larvae that had been injected with Cd were processed identically. Additionally, after 16 days of dietary exposure, 20 alimentary tracts were dissected in isosmotic solution (1.5% w/v NaCl), so that the gut tissue and the gut

content could be collected separately. The dissected alimentary tracts, gut tissues and gut contents were collected in micro tubes (1.5 mL) and stored at -20°C until further processing.

While cooled on ice, the whole alimentary tract and gut wall tissue was homogenized in micro tubes (1.5 mL), with the use of plastic pistils , using 4 x volumes of degassed buffer (20 mM Tris/HCl, pH 7.6, 1mM phenylmehtylsulphonylfluoride (PMSF), 1mM phenylthiocarbamide). Supernatants obtained after heat treatment (80°C, 10 min.) and centrifugation (12 000 x g, 10 min., 4°C) were stored as 100 µL aliquots at -20°C prior to future processing by chromatographic methods.

Molecular association of Cd with proteins in the aliquots was examined using size exclusion and ion exchange chromatography. For size exclusion chromatography (XK-16/20 column packed with Sephadex G-75, Amersham Biosciences, Uppsala, Sweden) the flow rate of the elution buffer (20 mM Tris/HCl, 250 mM NaCl, pH 7.6) was 0.5 mL $min.^{-1}$. Ion exchange chromatography (Hi Trap Q HP, 1 mL, Amersham Biosciences) was performed using a flow rate of 1 mL $min.^{-1}$ in a linear gradient of buffer A (20 mM Tris/HCl, pH 7.6) to B (1 M NaCl, 20 mM Tris/HCl, pH 7.6). Fractions of 0.5 mL were collected in all runs, and absorbance was measured at 214 nm (absorbance of peptide bonds). Radioactivity (CPM) in the collected fractions, measured by γ-counting, was converted into µg Cd mL^{-1}.

2.3 Marking of Cd-binding ligands in extracts of the gut wall tissue with ^{109}Cd.

The gut wall tissue of twenty larvae exposed 16 days to uncontaminated and Cd-contaminated food (0.5 mg Cd g^{-1} d.w.), respectively, was dissected out and used to prepare extracts (see above for description). The Cd-binding ligands in the extracts were marked using a procedure

modified from the Cd-saturation method described by Bartsch et al. (1990). The extracts (0.5 mL) were incubated (10 rpm, 3 min.) with 0.2 mL of a $CdCl_2$ solution (0.66 mM Cd in 20 mM Tris/HCl, pH 7.6, 0.5 mCi mg^{-1} Cd). Following the incubation, 0.5 mL of a Chelex 100 (Bio Rad) suspension (66 % in 10 mM Tris/HCl, 85 mM NaCl, pH 7.6) was added. After another incubation step (10 rpm, 3 min.), and centrifugation at 10 000 g (15 min., 4°C), the supernatants was collected. The supernatants (0.7 mL) were analysed using ion exchange chromatography (see above for description). The radioactivity (CPM) of the collected fractions (1 mL) was measured by γ-counting.

2.3. Analysis of the internal distribution of Cd during exposure

2.3.1. Whole body autoradiography

To locate the most important tissue(s) with regard to accumulation of Cd, whole body autoradiography (WBARG), as described by Ullberg et al. (1982), was performed on larvae after 16 days of exposure. Briefly, each larva was rinsed (0.1% EDTA in distilled water) to remove any Cd that could be adsorbed to the body surface, before total radioactivity was determined, using the γ-counter. Generally, the final radioactivity of the larvae was ~ 10,000 CPM. The larvae were enclosed in 10% gelatine and fixated by cooling. The embedded larvae were flash-frozen in liquid-N_2 and fixated onto microtome stages using a freezing gel (Microm Laborgeräte, Walldorf, Germany). Saggital sections (20 µm thick) of the larvae were prepared using a cryostat (Microm HM 500 O, Microm Laborgeräte). The sections were collected using a tape transfer technique (Neschen, Tamro MedLab AS), freeze dried and applied to X-ray film (Kodak BioMax XAR-film). The film was exposed for one month at -82ºC, before processing as recommended by the manufacturer. Sections corresponding to

1 those used for autoradiography were dried and stained (fixated in 4% formaldehyde, rinsed in
2 water, stained in Mayer's Hemalum solution (Merck), rinsed in water, stained in eosin (0,5%
3 solution, Merck), rinsed in ethanol (100%) and cleared in xylene, each step 10 sec.). Digital
4 photos of representative autoradiograms and corresponding tissue sections were inverted and
5 enlarged using Adobe Photoshop 4.0.
6
7 *2.3.2. Determination of Cd content by γ-counting*
8
9 Ten larvae were collected at the different time points (2, 4, 8, and16 days) during the exposure.
10 After rinsing (as described above), total Cd content in the whole larvae (WL_{Cd}) were
11 determined by γ-counting. The gut content was removed after dissection (see description
12 above) prior to measurement of the Cd content in the gut wall tissue (GW_{Cd}) and remaining
13 organs (RO_{Cd}) by γ-counting. This allowed estimation of both the Cd content the whole
14 alimentary tract (AT_{Cd}) and Cd content in the gut content (GC_{Cd}) using the following
15 equations:
16
17 1) $AT_{Cd} = WL_{Cd} - RO_{Cd}$
18 2) $GC_{Cd} = AT_{Cd} - GW_{Cd}$
19
20 *2.4. Statistical treatment*
21
22 The amount of Cd in the different tissues at different days during the course of the Cd-
23 exposure period was analysed using ANOVA. Any statistically significant effects were further
24 analyzed using Tukey's test for *post hoc* comparisons of means. A Pearson correlation was

used to analyse the relationship between the ratio of mg Cd in gut content / mg Cd in gut wall tissue and the exposure time. All data were log-transformed prior to statistical treatment.

3. Results

Size exclusion analysis of extracts from the whole alimentary tract and the gut content of *T. molitor* larvae exposed for 16 days showed similar elution profiles for Cd, although the Cd concentrations were generally somewhat lower in the collected fractions from the gut content (Fig. 1A). In both types of extracts most of the Cd was distributed as a Cd-peak with an elution volume (Ve ~ 19 mL) somewhat smaller than observed for aprotinin (6,500 Da, Ve ~ 19.5 mL), thus indicating the presence of Cd-binding ligand(s) of ~ 7000 Da. No Cd-peak that corresponded to the one observed in extracts of the whole alimentary tract and the gut content was apparent in the extract of the gut wall tissue. Instead, most of the Cd was distributed as a broad peak that eluted faster than the Cd-peak observed from the whole alimentary tract and the gut content, indicating the presence of Cd-binding ligand(s) with a molecular weight > 7000 Da.

Size exclusion analysis of extracts from the whole alimentary tract of *T. molitor* larvae exposed for 3 hrs – 16 days showed that the distribution of Cd changed as the exposure period increased (Fig. 1B). In larvae that were sampled between 3 hours and 2 days of exposure, most of the Cd in the extracts eluted early (V_e ~15 mL) as a broad peak, similar to the Cd-profile observed in extracts from the gut wall tissue. In larvae that were sampled between 4 – 16 days of exposure, most of the Cd in the whole alimentary tract eluted later (V_e ~ 19 mL), indicating the presence of a Cd-binding ligand with an apparent molecular weight of ~ 7000 Da.

Ion exchange analysis of extracts from the whole alimentary tract and the gut content of larvae exposed for 16 days, showed similar elution profiles for Cd (Fig. 2A). The Cd concentrations collected during elution of the gut content extract were generally lower than for the corresponding fractions collected for the whole alimentary tract. Both displayed a distinct peak in Cd concentration in fractions 27 and 28. In the elution profile from the gut wall tissue extract, a small Cd-peak was apparent around fraction 7-8. However, no Cd-peak was apparent in fraction 27 and 28, as observed in the extracts of the whole alimentary tract and the gut content. Also, no peak was apparent in the elution profile of the extracts of whole alimentary tracts from larvae exposed to Cd by injection.

Ion exchange analysis of extracts from the whole alimentary tract of *T. molitor* larvae exposed for 3 hrs – 16 days showed that the distribution of Cd changed during the course of the exposure period (Fig. 2B). A distinct Cd-peak (around fraction 27-28) was not apparent before day 4 of the exposure. This peak became even more distinct in the extracts sampled after 8 and 16 days of exposure.

The distribution of Cd in chelex-treated extracts of the gut wall tissue was analysed using ion exchange chromatography to see if Cd-exposure might cause an increase in the Cd-binding capacity of this tissue. As apparent from Fig. 3, there was little difference between control and Cd-exposed animals with respect to total binding capacity (i.e. the sum of radioactivity in the different eluted fractions) in the extracts of the gut wall tissue. In extracts of the gut wall tissue from the control larvae the Cd eluted as distinct peak around fraction 8-9, and less pronounced shoulder around fractions 13-15. In the extracts of the gut wall tissue from the

Cd-exposed larvae the main Cd-peak eluted a little later (i.e. fractions 9-10), and the shoulder, that could be seen in the control animals, was less pronounced.

The distribution of silver grains observed on the film following WBARG of larvae exposed to Cd for 16 days, corresponded to the location of the alimentary tract on the stained reference sections (Fig. 4), thus indicating that most of the Cd was associated with the alimentary tract.

As shown in Fig. 5, the increase in Cd body burden during the exposure period was mainly due to incorporation of Cd in the gut content. Here, the Cd content increased throughout the exposure period and contributed to ~ 77% of the total Cd body burden at day 16. The accumulation of Cd in the gut wall tissue and the rest of the body (fat body, Malphigian tubules, muscle and cuticula) contributed to only ~11 and ~13% of the total Cd body burden at day 16, respectively. Eight days after the exposed larvae were transferred to uncontaminated food, the reduction in Cd content was less prominent in the gut wall tissue and the rest of the body (62 and 44% of initial content, respectively) than in the gut content (~ 80% of initial content).

As shown in Fig. 6, there was a statistical trend ($P = 0.07$) that relatively more Cd was taken up in the gut content than in the gut wall tissue (the ratio between Cd in the gut content and Cd in the gut wall tissue increased) as the exposure period progressed.

4. Discussion

A distinct Cd-peak appeared in both the size exclusion and ion exchange profiles of extracts from both the whole alimentary tract and the gut contents of Cd-exposed larvae (Figs. 1, 2).

The Cd-peak is consistent to a Cd-binding protein of 7134 Da that was recently isolated from whole larvae extracts of Cd-exposed *T. molitor* larvae (Pedersen: unpublished). The peak was absent in extracts of exposed larvae where the alimentary tract had been dissected out (data not shown) and in gut wall tissue of exposed larvae (Figs. 1, 2). From this it may be concluded that the site for induction of the 7134 Da Cd-binding protein in Cd-exposed *T. molitor* larvae is the gut content.

The internal location of Cd-binding protein of the low cysteine type has not been investigated previously in terrestrial arthropods. The discovery that the Cd-binding protein in *T. molitor* seems to be induced in the gut content may therefore be considered interesting. The protein appears to be slowly induced since its distinctive peak was not apparent in extracts of the whole alimentary tract until after 4 and 8 days of exposure, when analysed by ion exchange and size exclusion chromatography, respectively (Figs 1, 2). Since no characteristic Cd-peak could be detected in ion exchange profiles of the whole alimentary tract extracts from *T. molitor* larvae exposed by injection of Cd it seems that induction of the protein depends on oral administration (Fig. 2A).

Gut wall tissue has been identified as the main organ for MT induction in both *Drosophila melanogaster* and *Orchesella cincta* (Durliat et al., 1995; Hensbergen et al., 2000). The less pronounced Cd-peak observed in the elution profile from the gut wall tissue of *T. molitor* (Figs. 1, 2) could therefore be interpreted as a contribution from MTs. Since each MT molecule can bind up to 7 Cd ions (Hensbergen et al., 2000; Kägi and Vallee, 1960) a potential induction of MT in the gut tissue might be expected to cause a marked increase in the Cd-binding capacity. However, analysis of binding capacity in gut tissue extracts of Cd-

exposed and control larvae revealed no marked change (Fig. 3). Thus, no accumulation of MTs seem to occur in the gut tissue during detoxification of Cd in *T. molitor*.

As revealed by both autoradiography (Fig. 4) and dissection (Fig. 5), most of the accumulated Cd was located in the alimentary tract after the 16 days exposure period. At the end of this period the gut content contributed to ~77% of the total amount in the whole larvae. This could, however, be expected since gut clearance was not performed on the animals prior to dissection. Transferring the animals to uncontaminated food showed that the larvae quickly eliminated the Cd associated with the gut content (~80% reduction compared to initial content after 4 days). Since moulting or pupating larvae were excluded from the study, elimination of Cd through the faeces is the most likely explanation for this phenomenon.

Comparison between the different organs showed that Cd was evenly distributed between the gut wall tissue and the remaining organs throughout the exposure period (Fig. 5). This is different to results on the distribution of Cd in other studies where ≥ 90 % of the Cd has been found to be associated with the gut following Cd-exposure. For instance, after thorough clearing of Cd-contaminated food from the gut in the two dipterans *D. melanogaster* and *Sarcophaga peregrina*, 96 and 90 % of the total body burden of Cd was recovered in the gut tissue, respectively (Aoki et al., 1984; Maroni and Watson, 1985). The observation by Durliat et al. (1995), that a marked induction of MT was only apparent in the gut tissue of Cd-exposed *D. melanogaster*, has been taken as an explanation for the dominant role of this tissue in Cd-binding. Interestingly, the elution profile of the gut tissue from *T. molitor* in the present work displayed no marked Cd-peak that could support a pronounced induction of MT.

The observation that Cd-exposure caused no apparent increase in the Cd-binding capacity of the gut tissue (Fig. 3) is consistent with the observed tendency ($P = 0.07$) towards gut content incorporating relatively more Cd than the gut wall tissue as the exposure period progressed (Fig. 6). This could be explained by an increase in the elimination of Cd from the epithelial cells and/or a reduction in the uptake of Cd from the gut content. The present observation, that a Cd-binding protein is induced in the gut content, between day 4-8 (Figs 1B and 2B), around the time as the accumulation of Cd in the gut wall tissue stabilized (Fig. 5B), supports the idea of a reduced uptake of Cd. Chelation of Cd by the Cd-binding protein could contribute to reduce the free concentration of Cd in the gut content and thus reduce the uptake of Cd into epithelial cells.

In a previous study we found no reduction in the capacity to produce cysteine rich antifreeze proteins (AFPs) when *T. molitor* larvae, chronically exposed to metals (Cd, Cu or Zn), were acclimated to winter conditions (Pedersen et al., 2006). This was surprising as Zachariassen et al. (2004) had proposed that metal exposure, by causing accumulation of cysteine rich MTs, might deplete the cysteine pool and thus hamper the production of cysteine rich AFPs in freeze avoiding insects such as *T. molitor*. The observation that no accumulation of MTs seems to take place in Cd-exposed *T. molitor* suggest that the larva's cysteine pool may not have been depleted in the study of Pedersen et al. (2006), and therefore no reduction in the capacity to produce AFPs should be expected.

Since the Cd-binding protein from *T. molitor* is located in the gut content, it cannot be excluded that it may be produced by the bacteria or fungi that make up the microflora of the gut. If it turns out that the Cd-binding protein originates from the gut microflora it would be a new example of beneficial effects of microsymbionts in insects.

To summarise, accumulation of MTs does not seem to take place in the defence against Cd toxicity in *T. molitor*. A Cd-binding protein, distinct from the MTs, was found to become induced in the gut content after 4-8 days of dietary exposure to Cd. As the exposure period progressed, there was a statistical trend towards gut wall tissue incorporating less Cd than the gut content. These observations are consistent with the idea that the functional role of the Cd-binding protein may be to reduce the uptake of Cd by lowering the free concentration of Cd in the gut content.

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Figure captions

Fig. 1. A: Size exclusion profiles from extracts of the whole alimentary tract (●), the gut wall tissue (○) and the gut content (▼) of *T. molitor* larvae exposed to 0.5 mg Cd g^{-1} d.w. through the diet for 16 days; absorbance at 214 nm (– – –); corresponding Cd concentrations (——). B: Size exclusion profiles of extracts prepared from whole alimentary tracts of larvae sampled at different time points during the course of the 16-day exposure period for 0.5 mg Cd g^{-1} d.w.

Fig. 2. A: Ion exchange elution profiles from extracts of the whole alimentary tract (●), gut wall tissue (○), gut content (▼) of *T. molitor* larvae after 16 days of dietary Cd (0.5 mg g^{-1} d.w.) exposure; corresponding Cd concentration (——); [NaCl] in elution buffer (– – –). Elution profile of the extract from the whole alimentary tract (Δ) of larvae exposed by injection of Cd into the body cavity is also included. B: Elution profiles of extracts prepared from whole alimentary tracts of larvae sampled at different time points during the 16 day exposure period for 0.5 mg Cd g^{-1} d.w.

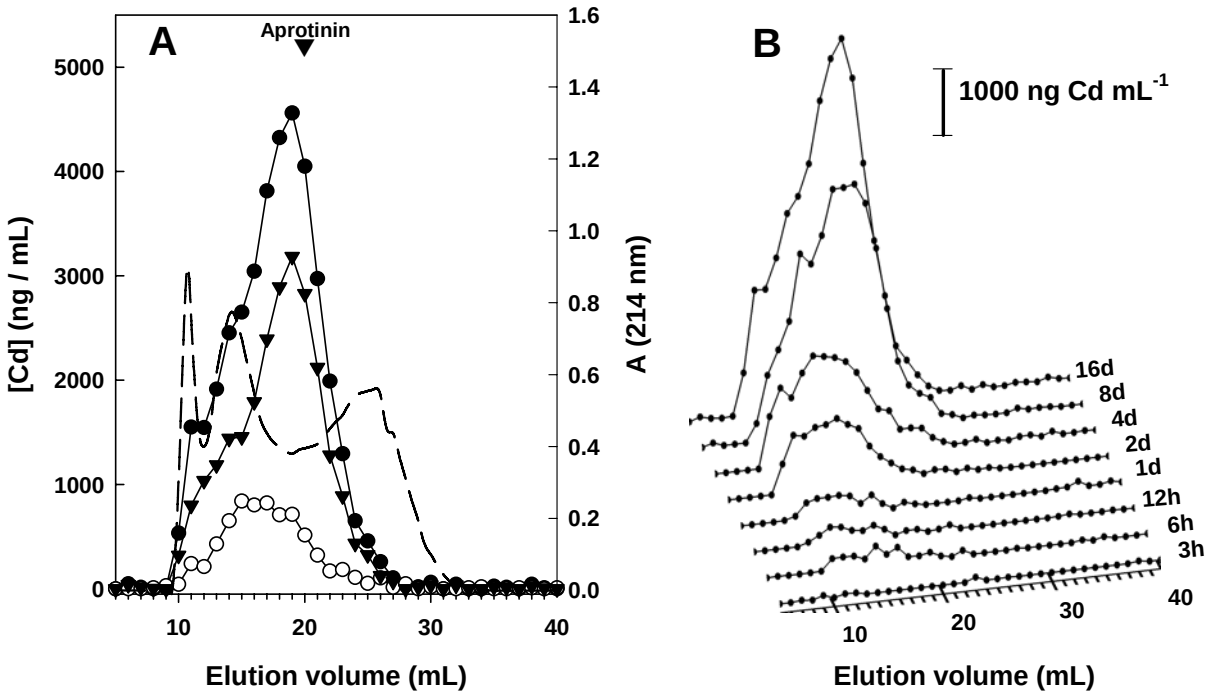
Fig. 3. Ion exchange profile of chelex-treated extracts of gut wall tissue from control and Cd-exposed (0.5 mg Cd g^{-1} d.w.) larvae.

Fig. 4. Whole body autoradiography of *T. molitor* after 16 days of exposure to food contaminated with 0.5 mg Cd g^{-1} and spiked with radioactive ^{109}Cd. A: Autoradiograms. S: Stained tissue sections corresponding to the autoradiograms.

Fig. 5. A: Distribution of Cd in whole animals and in animals without alimentary tract (fat body, Malphigian tubules, muscle and cuticutla). B: Distribution of Cd in gut content and gut wall tissue. Closed symbols: Cd levels after exposure to food supplemented with Cd (0.5 mg Cd g^{-1}). Open symbols: Cd levels after transfer to uncontaminated food in larvae pre-exposed to Cd (0.5 mg Cd g^{-1}) for 16 days. Asterisk (*) indicate significant differences ($P < 0.05$) compared to other time points that are listed behind the asterisk.

Fig. 6. Log-transformed ratio between the amount of Cd in the gut content and gut wall tissue as a function of days of dietary Cd-exposure. Each point represents the mean of 10 larvae and the error bars show the standard error.

Fig. 1.


A
Aprotinin
[Cd] (ng / mL)
A (214 nm)
Elution volume (mL)
B
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16d
8d
4d
2d
1d
12h
6h
3h
40
Elution volume (mL)

Fig. 2.

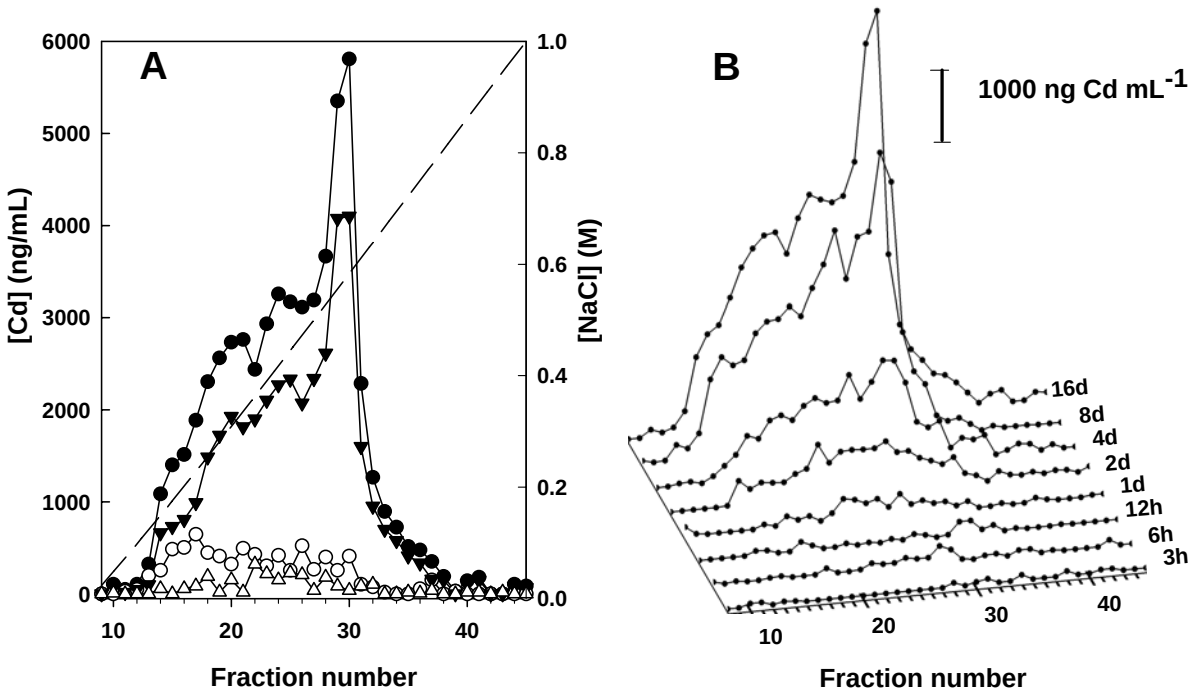

A
[Cd] (ng/mL)
[NaCl] (M)
Fraction number
B
1000 ng Cd mL-1
16d
8d
4d
2d
1d
12h
6h
3h
Fraction number

Fig. 3.

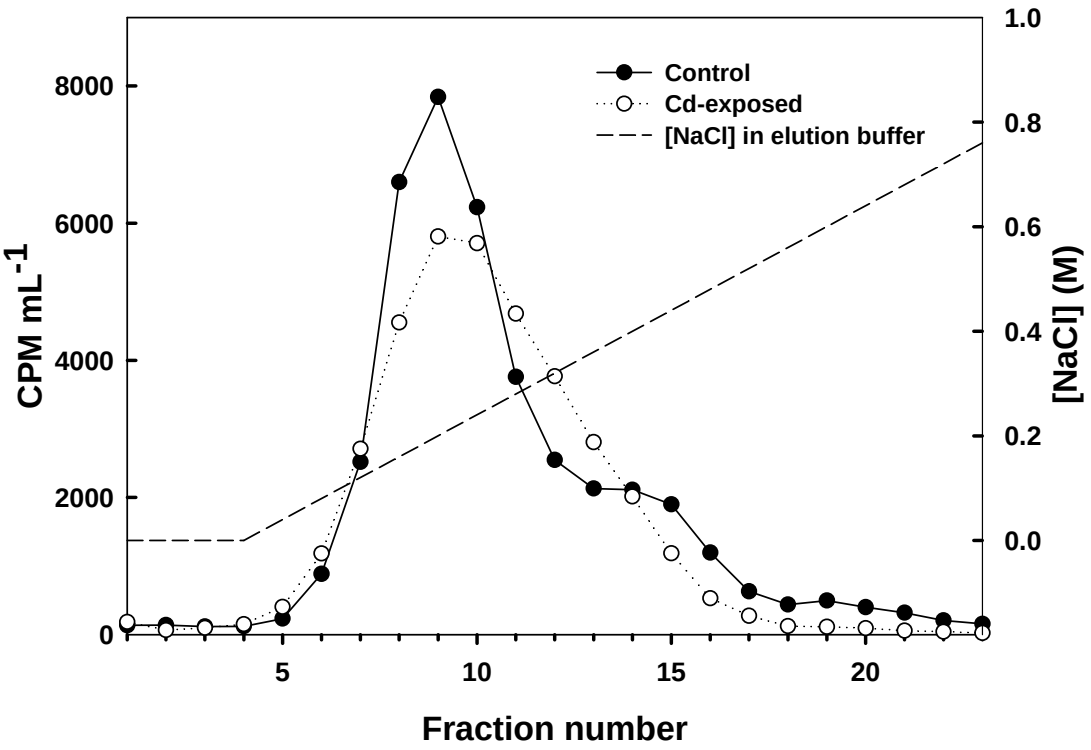

Control
Cd-exposed
[NaCl] in elution buffer
CPM mL-1
[NaCl] (M)
Fraction number
0
2000
4000
6000
8000
0.0
0.2
0.4
0.6
0.8
1.0
5
10
15
20

Fig. 4.

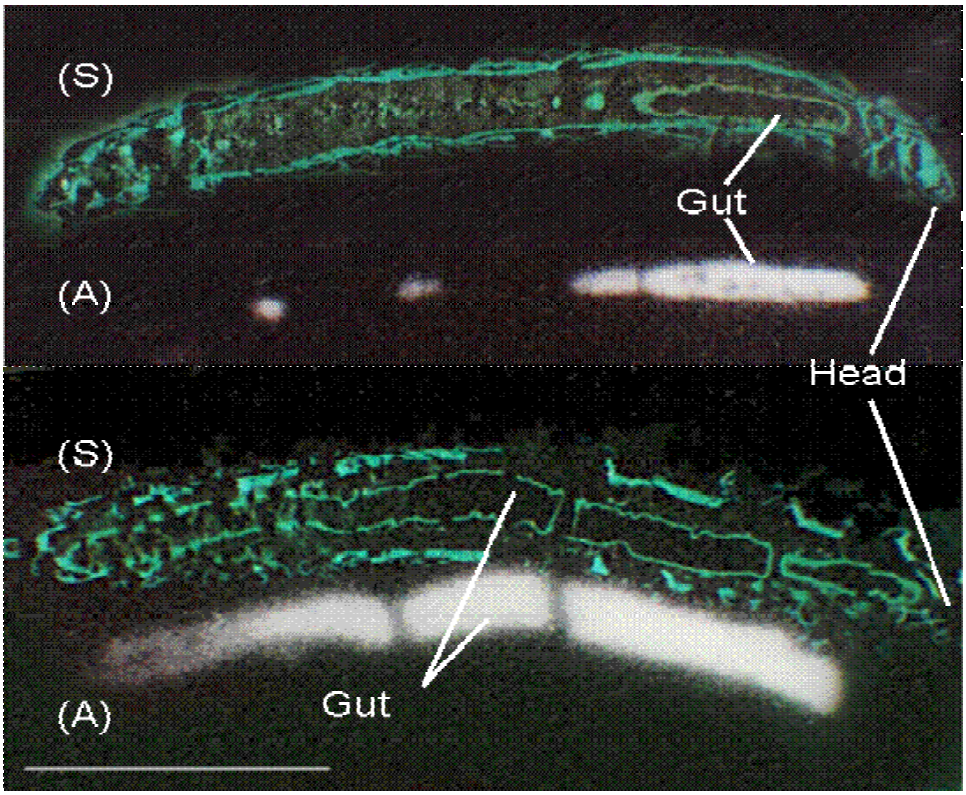
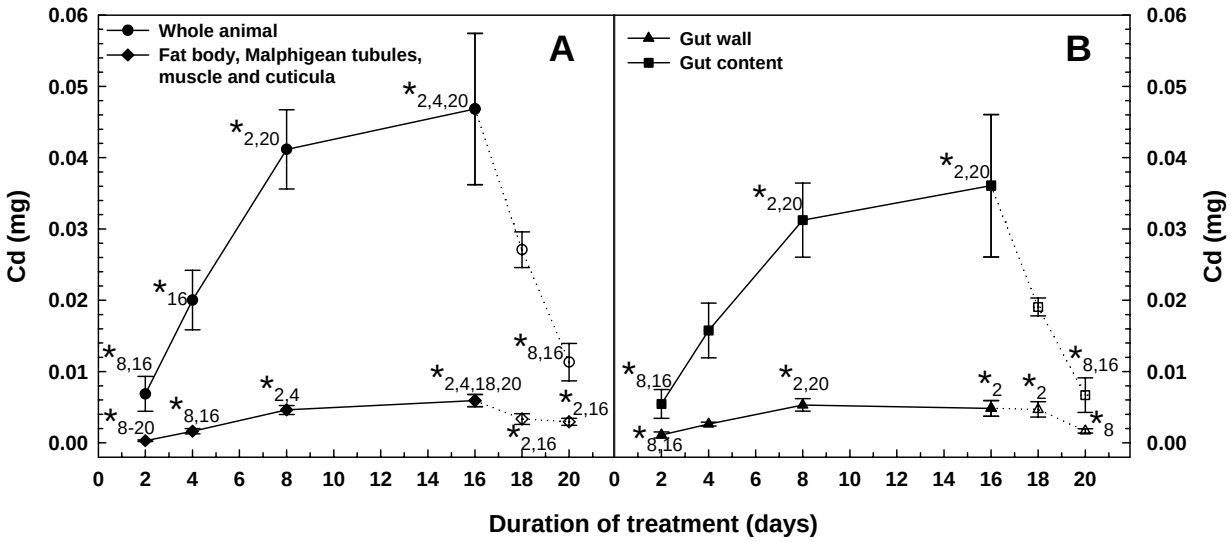


Fig. 5.


Whole animal
Fat body, Malphigean tubules, muscle and cuticula
A
Gut wall
Gut content
B
Cd (mg)
Duration of treatment (days)
*8,16
*16
*2,20
*2,4,20
*8-20
*8,16
*2,4
*2,4,18,20
*8,16
*2,16
*2,16
*8,16
*2,20
*2,20
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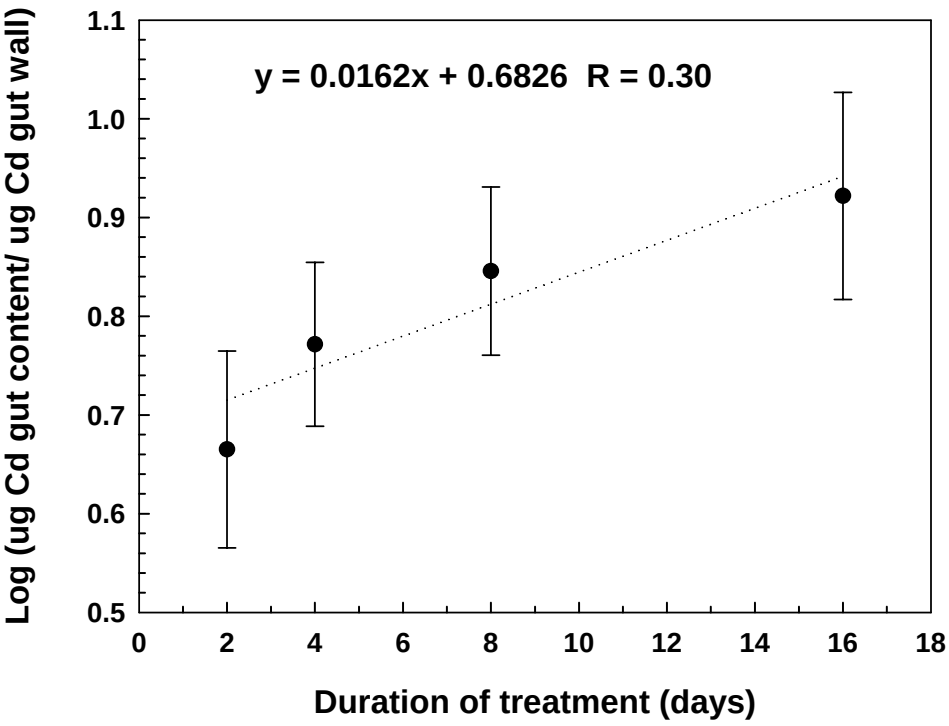
*2,20
*2
*2
*8
*8,16

Fig. 6.


y = 0.0162x + 0.6826 R = 0.30
Log (ug Cd gut content/ ug Cd gut wall)
Duration of treatment (days)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
2
4
6
8
10
12
14
16
18

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook *Corvus frugilegus.*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.
1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit *Parus montanus*.

1987 Jarle Inge Holten	Dr. philos Bothany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway
1987 Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987 Bjørn Åge Tømmerås	Dr. scient. Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
1988 Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988 Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
1988 Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
1988 Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.).
1989 John W. Jensen	Dr. philos. Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.
1989 Helga J. Vivås	Dr. scient. Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*.
1989 Reidar Andersen	Dr. scient. Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation.
1989 Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture,
1990 Bengt Finstad	Dr. scient. Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
1990 Hege Johannesen	Dr. scient. Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
1990 Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990 Arne Johan Jensen	Dr. philos. Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams.
1990 Tor Jørgen Almaas	Dr. scient. Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
1990 Magne Husby	Dr. scient. Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*.
1991 Tor Kvam	Dr. scient. Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway.
1991 Jan Henning L'Abêe Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.
1991 Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991 Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants

1991	Trond Nordtug	Dr. scient. Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods.
1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos. Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism.
1991	Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient. Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient. Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos. Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
1992	Arne Vollan Aarset	Dr. philos. Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient. Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient. Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient. Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek.
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient. Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*.
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers

1994 Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994 Morten Bakken	Dr. scient. Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*.
1994 Arne Moksnes	Dr. philos. Zoology	Host adaptations towards brood parasitism by the Cockoo.
1994 Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1995 Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions.
1995 Hanne Christensen	Dr. scient. Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*.
1995 Svein Håkon Lorentsen	Dr. scient. Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition.
1995 Chris Jørgen Jensen	Dr. scient. Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995 Martha Kold Bakkevig	Dr. scient. Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
1995 Vidar Moen	Dr. scient. Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations.
1995 Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden.
1996 Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae.
1996 Ola Ugedal	Dr. scient. Zoology	Radiocesium turnover in freshwater fishes
1996 Ingibjørg Einarsdottir	Dr. scient. Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines.
1996 Christina M. S. Pereira	Dr. scient. Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
1996 Jan Fredrik Børseth	Dr. scient. Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics.
1996 Gunnar Henriksen	Dr. scient. Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region.
1997 Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae.
1997 Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters.
1997 Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming.
1997 Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture.

1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.
1997	Torgeir Nygård	Dr. scient. Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors.
1997	Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway.
1997	Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
1997	Rolv Lundheim	Dr. scient. Zoology	Adaptive and incidental biological ice nucleators.
1997	Arild Magne Landa	Dr. scient. Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*.
1997	Jarle Tufto	Dr. scient. Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos. Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos. Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient. Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins.
1998	Thor Harald Ringsby	Dr. scient. Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity.
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro.
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach.
1998	Bente Gunnveig Berg	Dr. scient. Zoology	Encoding of pheromone information in two related moth species
1999	Kristian Overskaug	Dr. scient. Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999	Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)

1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway.
1999	Ingvar Stenberg	Dr. scient. Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis.
1999	Trina Falck Galloway	Dr. scient. Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
1999	Marianne Giæver	Dr. scient. Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*.
1999	Ingrid Bysveen Mjølnerød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient. Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard

2000	Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient. Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient. Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient. Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*

2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*).
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations.
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius

2005	Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Ander Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	Phd Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	PhD Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	phD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	phD Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	phD Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	phD Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	phD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	phD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system