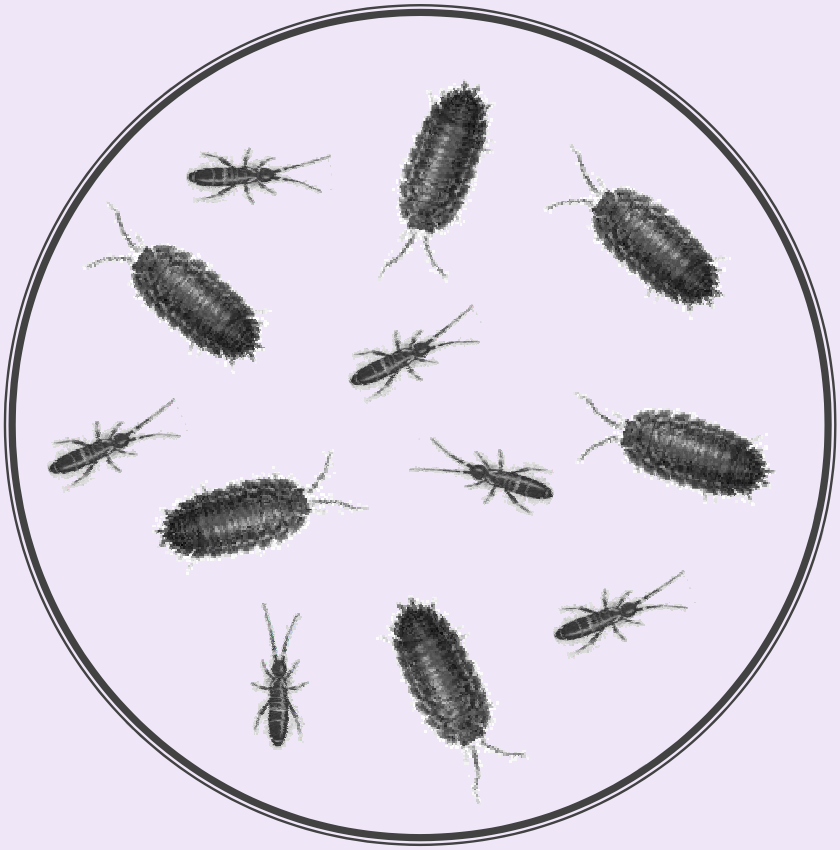
Population genetics of soil invertebrates (Isopoda and Collembola) exposed to metal contamination



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Contents

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Resumo

Os metais encontram-se extensamente dispersos pelos ecossistemas, devido principalmente a actividades antropogénicas, tais como a extracção e a fundição de minério. Nos ecossistemas terrestres, os metais acumulam-se essencialmente no solo e na matéria orgânica. Como tal, organismos que habitam o solo, tais como isópodes, colêmbolos e minhocas, estão directamente expostos à contaminação por metais. Alguns animais conseguem evitar ou limitar a exposição aos metais. Quando expostos, o desenvolvimento de tolerância aos metais pode ocorrer através de alterações fenotípicas ou genéticas. A contaminação por metais pode afectar a diversidade genética, quer em loci neutros, se causarem um decréscimo do tamanho da população, quer em loci selectivos, se causarem a eliminação dos genótipos não tolerantes.

O objectivo principal desta tese foi contribuir para o conhecimento da tolerância aos metais de populações de invertebrados do solo, que habitam locais contaminados, considerando para tal dois casos de estudo. No primeiro, populações do isópode terrestre *Porcellionides sexfasciatus*, amostradas numa mina abandonada, foram usadas para determinar os efeitos da contaminação por metais no desenvolvimento de tolerância e na diversidade genética. No segundo, populações de campo e de laboratório do colêmbolo *Orchesella cincta* foram estudadas para relacionar a variação genética com o fenótipo tolerante e para determinar a influência da regulação da transcrição do gene da metalotioneína (*mt*) na tolerância.

As populações de invertebrados do solo, recolhidas em locais contaminados demonstraram um aumento de tolerância aos metais. Por outro lado, não foi observado um decréscimo da diversidade genética, nem em loci neutros nem selectivos, o que pode ser explicado pelo grande tamanho das populações e pela ocorrência de migrações e/ou mutações. O fenótipo tolerante parece ser influenciado principalmente por factores genéticos que actuam em *cis* na regulação da expressão do gene *mt*.

Summary

Metals became widely spread in ecosystems due to anthropogenic activities, such as mining and smelting of metal ores. In terrestrial ecosystems, the soil-litter compartment is a major sink of metals. Therefore, soil-living organisms such as isopods, collembolans and earthworms, are directly exposed to metal contamination. Some animals may cope with metal contamination avoiding or limiting their exposure. If not they may develop metal tolerance through phenotypic or genetic changes. Metal contamination may affect genetic diversity, at neutral loci, if causing a decrease in population size and at selectable loci, if causing the elimination of genotypes that lack tolerance.

The main objective of this thesis was to contribute to the knowledge of metal tolerance of soil invertebrate populations inhabiting contaminated sites, by considering two case-studies. First, populations of the terrestrial isopod *Porcellionides sexfasciatus*, collected at an abandoned mine area, were used to determine the effects of metal contamination on tolerance development and genetic diversity. Second, field and lab populations of the soil-dwelling collembolan *Orchesella cincta* were studied to link genetic variation to the metal-tolerant phenotype and to assess the influence of transcriptional regulation of the metallothionein (*mt*) gene on tolerance.

Soil invertebrate populations collected at metal-contaminated sites showed increased metal tolerance. On the other hand, no metal-related decrease on genetic diversity both at neutral and at selectable loci was observed, which may be explained by a large population size and the existence of migration and/or mutation events. The metal-tolerant phenotype appeared to be influenced mainly by genetic factors acting in *cis* on *mt* gene expression.

Chapter 1

Introduction

Metals

Metals occur naturally in the environment. Thus, soil may be naturally contaminated, if formed above metal rich-rocks, as for example serpentine soils. Nevertheless, metals became widely spread in ecosystems, due to their increased use in anthropogenic activities, such as mining and smelting of metal ores, but also industrial and agricultural activities. For instance, the use of copper and arsenic as pesticide components has resulted in contamination of agricultural soils (Macnair, 1997; Komárek *et al.*, 2008). Metals can be classified as essential or non-essential. Non-essential metals, such as cadmium (Cd), mercury (Hg) and lead (Pb), have no known biological functions and are toxic even in trace quantities. Although a biological role for cadmium has been discovered in the enzymatic activity of marine diatoms (Lane *et al.*, 2000), in most organisms Cd is known for its high toxicity (Bertin and Averbeck, 2006) and carcinogenicity (Nawrot *et al.*, 2006). Essential metals, like copper (Cu), zinc (Zn) and iron (Fe), are necessary to vital functions of the organisms, such as the immune response, respiration, and enzyme activity (e.g. Irmak *et al.*, 2005). Copper for instance, is an essential element in electron transport, oxygen processing, iron absorption, and enzyme activity (e.g. Barceloux and Barceloux, 1999). However, like non-essential metals also the essential ones are toxic when concentrations exceed a critical level. For example, high levels of Zn in isopods are known to affect respiration, feeding and reproduction activities (Drobne and Hopkin, 1995; and references therein), and high levels of Cu can decrease survival, feeding and reproductive success (Farkas *et al*., 1996; Zidar *et al.*, 2003).

Metal tolerance

In terrestrial ecosystems metals tend to accumulate in the soil (Martin and Coughtrey, 1981) particularly in the organic layer (Jones *et al.*, 1988). Exposure to metals can reduce the survival and reproduction of soil-living organisms which may eventually lead to local population extinction (Bickham and Smolen, 1994). Some organisms are able to avoid or limit their exposure by behavioural actions, such as avoidance. Woodlice may regulate metal intake by avoiding metal-contaminated food (Dallinger, 1977) and/or by diminishing feeding rates (Zidar *et al.*, 2003).

However, when exposure cannot be avoided, tolerance towards metal contamination may be developed. Metal tolerance at the individual level may be defined “as the ability to prevent, decrease or repair adverse effects of metals that have entered the body” (Levitt, 1980) and has been demonstrated for several soil invertebrate species, such as isopods and collembolans (Posthuma and Van Straalen, 1993). In the isopod *Porcellio scaber*, metal tolerance was achieved by adult body-size reduction, earlier reproduction, larger number of offspring, and increased energy allocation to reproduction (Donker *et al.*, 1993a, 1993b). The soil-dwelling springtail *Orchesella cincta* has developed metal tolerance through heritable elevated Cd excretion efficiency (Van Straalen *et al.*, 1987; Posthuma *et al.*, 1992, 1993), lower Cd-induced growth reduction (Posthuma, 1990) and improved survival despite feeding on Cd-contaminated food (Sterenborg, 2003; Timmermans *et al.*, 2005a).

Tolerance may be due to phenotypic adjustments, such as acclimation, maternal effects and phenotypic plasticity. Acclimated individuals are able to cope with a particular contaminant, by induction of physiological detoxification mechanisms after exposure to sub-lethal concentrations. If exposure ceases, individuals will return to their previous physiological state. When the exposed individuals are females, tolerance may be induced also in the offspring, through maternal effects. Such influences may occur before or after birth, and may be caused by transfer of developmental resources that can induce novel variation in offspring in response to the conditions experienced by the progenitors (Badyaev and Uller, 2009). A single genotype may, depending on the environmental conditions, produce multiple phenotypes, the so-called phenotypic plasticity. Plastic responses include changes in behaviour, physiology, morphology, growth, life history and demography, and can be expressed either within the lifespan of a single individual or across generations (Miner *et al.*, 2005; and references therein).

Tolerance may also evolve, at the population level, due to genetic changes (adaptation), which results from natural selection acting on those phenotypes that have an inherited capacity to deal with the contaminants better than other phenotypes.

Population genetic diversity

Metal contamination of soils is considered to be a continuous, strong, directional selective pressure. Thus a reduction on genetic diversity of selectable loci is expected, caused by the elimination of homozygote genotypes that lack the increased tolerance (Van Straalen and Timmermans, 2002). Furthermore, both selectable and neutral loci genetic diversity may be affected by metal pollution, if it acts on other evolutionary processes, mainly genetic drift, migration and mutation. Small populations are particularly sensible to genetic drift (random changes in allele frequencies between generations), since it reduces the available genetic potential by a factor of 1/2Ne (Ne = effective population size) (Wright, 1931). Therefore, if metal pollution causes a decrease in population size, random changes in allele frequencies may be enhanced and genetic diversity decreases. Genetic diversity may, however, be maintained if individuals migrate between metal-contaminated and clean sites. It is accepted that stressful conditions can decrease migration among populations, since individuals will become progressively more restricted to favourable patches in the fragmented habitat (Hoffmann and Hercus, 2000). However, the way toxicant exposure affects gene flow, either increasing or decreasing it, is not easily predictable (Van Straalen and Timmermans, 2002). Mutations are the ultimate source of genetic variation (Hartl and Jones, 1998). Mutation rates may be higher in polluted environments, resulting in increased genetic diversity (Ellegren *et al.*, 1997; Rogstad *et al.*, 2003). However, most mutations are deleterious, which may result in lower population viability and fertility, leading to a bottleneck and consequently to a reduction of genetic diversity (De Wolf *et al.*, 2004). Moreover, selection at one favourable locus may affect other loci, even if they have no direct effect on fitness, through genetic linkage (Van Straalen and Timmermans, 2002). Therefore, the outcome of the effect of metal contamination on genetic diversity will depend on the way evolutionary processes are affected and the genetic network of the genes involved. For instance, genetic diversity appears to be unaffected by metal pollution in the springtail *O. cincta*, given the low amount of genetic differentiation observed among metal-contaminated and reference populations (Frati *et al.*, 1992; Timmermans, 2005). Timmermans (2005) hypothesized that gene flow between these populations counteracts directional selection on metal tolerance.

Moreover, it should be kept in mind, when establishing a link between the observed changes in genetic diversity and metal contamination, that selection, genetic drift, migration, and mutation events are also affected by other environmental stressors and also that these evolutionary processes occur against a historical genetic background (Staton *et al.*, 2001).

Metal tolerance mechanisms

When metals enter the body, their effects may be minimized through the existence of metal-binding proteins and/or accumulating intracellular granules. Isopods are known to accumulate large amounts of several metals, such as Zn, Cu, Cd, and Pb in their hepatopancreas (Hopkin, 1990). The ability of the hepatopancreas to store these elements in insoluble intracellular granules enables concentrations of essential metals such as Zn and Cu to be "buffered" at optimum physiological levels, and for levels of non-essential metals such as Cd and Pb to be maintained below their critical concentrations (Hopkin and Martin, 1982a, 1982b). The capacity to accumulate high amounts of Cu is probably related to the fact that the respiratory pigment of isopods, hemocyanine, has Cu at the active site (Bonaventura and Bonaventura, 1980). However, the storage capacity of the hepatopancreas appears to exceed the physiological requirement for this metal by orders of magnitude (Dallinger and Wieser, 1977).

Another important metal tolerance mechanism is the one involving metallothioneins (*mt*). Metallothioneins are low molecular weight, ubiquitous proteins, with high cysteine content (± 30%) and neither aromatic amino acids nor histidines (Kägi, 1991). Due to their high cysteine content they have a strong affinity for metals, mainly Cd, Cu and Zn (Dallinger, 1996). These proteins are involved in non-essential metals detoxification (e.g. Cd) and essential metals homeostasis (e.g. Cu and Zn) (Dallinger 1996; Hensbergen *et al.*, 1999). Besides being strongly induced by metals, especially Cd, they are also induced by other factors such as oxidative stress (Bertin and Averbeck, 2006). Metallothionein proteins have been isolated in several soil invertebrates, such as snails (Dallinger, 1996), nematodes (Hughes and Stürzenbaum, 2007), isopods (Žnidaršič *et al.*, 2005), earthworms (Stürzenbaum *et al.*, 1998), and collembolans (Hensbergen *et*

al., 1999). A 7.1 kDa *mt* was isolated from *O. cincta*, containing 77 amino acids and 19 cysteines (Hensbergen *et al.*, 1999). It binds 7 or 8 Cd ions in two metal-binding clusters (Hensbergen *et al.*, 2001) and is present mostly in the gut epithelium, were most of the cadmium is stored (Hensbergen *et al.*, 2000).

Metal tolerance has been attributed to the duplication of *mt* genes or to higher *mt* gene expression (e.g. Maroni *et al.*, 1987; Sterenborg and Roelofs, 2003). In *O. cincta*, both higher constitutive and Cd-induced *mt* expression were found in springtails originating from metal-contaminated sites, when compared to reference populations (Sterenborg and Roelofs, 2003; Timmermans *et al.*, 2005a). The expression of a particular gene may be regulated by either *cis*- or *trans*-acting factors, depending on whether it is caused by polymorphisms in binding site structure for transcriptional factors, or in the structure or amount of these factors (Janssens *et al.*, 2009). Janssens *et al.* (2007) suggested that structural differences in *mt* promoter alleles (*cis*-regulation) were responsible for the higher *mt* expression observed in *O. cincta* Cd-tolerant populations. Further studies suggested that *mt* expression was regulated by a combined *cis/trans*-regulatory mechanism (Janssens, 2008; Van Straalen *et al.*, 2011). However, the contribution of *cis*- and *trans*-acting factors to the elevated *mt* expression phenotype associated with metal tolerance is still unclear.

Aim of the thesis

The study of metal tolerance is of evolutionary and ecological importance. From an evolutionary point of view, metal contamination may decrease genetic diversity, impairing a population's capacity to adapt to novel environmental stressors. Furthermore, by acting as a selective force it may lead to population micro-evolution. From an ecological point of view, an understanding of how organisms react to environmental stressors allows the design of more effective ecological risk assessment programs. Therefore, the main purpose of this thesis was to contribute to the knowledge of metal tolerance of soil invertebrate populations inhabiting contaminated sites. Two soil invertebrate species were considered: the terrestrial isopod *Porcellionides sexfasciatus* and the soil-dwelling collembolan *Orchesella cincta*.

Outline of the thesis

Population genetic structure results from an interaction between evolutionary processes, gene flow, genetic drift, mutation and selection that act on a historical genetic background. So, the “normal” genetic structure should be considered before implicating metal contamination on population genetic changes. Therefore, **chapter 2** is a literature survey on the population genetic structure of soil invertebrates, particularly isopods, collembolans and earthworms. These animals are considered to be rather sedentary, with limited migration capacities, consequently they are expected to be found in highly structured populations. Several studies, however, indicated that this may not be so straightforward and that passive dispersal is an important factor to be considered. Populations inhabiting metal-contaminated sites may show increased metal tolerance and reduced genetic diversity. So, in **chapter 3** the existence of metal tolerance in a historically exposed population of the isopod *Porcellionides sexfasciatus* was assessed. Also, the effects of metal contamination on population genetic structure and diversity were analysed. In **chapter 4** the influence of cadmium adaptation on neutral and functional genetic variation in the collembolan *Orchesella cincta* was studied. Also, the influence of transcriptional (*cis/trans*) regulation on the tolerant phenotype was tackled. Finally, in **chapter 5** an integrated discussion of the results of the preceding chapters was made.

Chapter 2

Genetic structure of soil invertebrate populations: collembolans, isopods and earthworms (a review)

Based on: Costa D, Timmermans MJTN, Sousa JP, Ribeiro R, Roelofs D, Van Straalen NM (submitted to Soil Biology and Biochemistry).

Abstract

Soil-living collembolans, isopods and earthworms are considered to be sedentary animals with only limited migration capacities. Therefore, gene flow among populations is expected to be low leading to significant population genetic differentiation due to random drift and local adaptation. With limited gene flow, populations will be more susceptible to genetic drift that may reduce genetic diversity, impairing their capacity to cope with novel environmental conditions. We reviewed the literature to test this expectation. Our survey reveals a clear signature in the current pattern of genetic variation due to post-glacial colonization events. It also reveals that habitus can be a misleading predictor for migration capacity. In some species relatively high gene flow across considerable distances, most likely through passive dispersal, might counteract local genetic adaptation and loss of genetic variation.

Introduction

Population genetic structure is the distribution of genotypes in space and time and is determined by both historical and current evolutionary processes (Hewitt and Butlin, 1997) involving gene flow, genetic drift, mutation and selection (Slatkin, 1987). The absence of migration among populations, either due to the existence of barriers or due to limited dispersal abilities of individuals, results in small gene flow. Under such conditions allele frequencies in each population will start to change independently, resulting in significant genetic differentiation among populations (Hartl and Jones, 1998). Because populations have a finite size, only a subset of parental alleles will be represented among the offspring, causing random fluctuations of allele frequencies over generations and fixation of random alleles due to genetic drift. This may further increase genetic differentiation among populations (Hartl and Jones, 1998).

Without the homogenising effect of gene flow, genetic diversity will decay over time, which might impair the capacity of populations to cope with novel environmental conditions; this may eventually lead to local extinction if the conditions change (Burger and Lynch, 1995; Spielman *et al.*, 2004). Divergence of populations subject to different environmental conditions can also be influenced by

selection. If local directional selection is strong enough, genetic differentiation can occur even in the presence of migration (Ehrlich and Raven, 1969). The accumulation of genetic differences among populations can, however, be counteracted by migration (Slatkin, 1987). In fact, only a small number of individuals exchanging between populations is usually sufficient to prevent the development of high levels of genetic differentiation (Wright, 1943; Slatkin, 1987).

The geographic population structure resulting from drift and dispersal is often represented by the model of "isolation by distance". Under this model a continuous increase of genetic distance between populations is expected with increasing geographic distance (Wright, 1943; Slatkin, 1985). Isolation by distance (IBD) is supported by migration over small distances among neighbouring populations as in a stepping stone model of gene flow (Kimura, 1953) (Figure 2.1). The number of individuals that migrate per generation may be estimated with genetic analysis (Wright, 1943; Slatkin, 1987). Such indirectly derived estimates of dispersal are often considerably greater than direct measurements, which is usually interpreted to indicate that direct methods tend to greatly underestimate dispersal events (Koenig *et al.*, 1996).

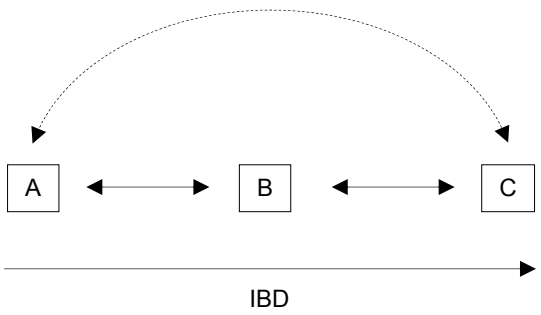

Figure 2.1 Scheme of the isolation by distance (IBD) process. The homogenizing effect of gene flow is due to short-range dispersal among adjacent populations as described by a stepping stone model of gene flow (full arrows). As a result an isolation by distance pattern may be observed, in which distant populations are more differentiated than closer ones. However, in situations where migration occurs mainly over long distances, populations far apart may become more similar to each other than populations in proximity (hatched arrows).

Current evolutionary processes act on a historical genetic background, determining the present population genetic structure (Hewitt and Butlin, 1997). Historical events, for example glaciations, are known to have had a major impact on the distribution of many species (e.g. Hewitt, 1999). It is usually considered that the Quaternary glaciations have influenced the present genetic structure of many species of terrestrial and freshwater habitats worldwide (Hewitt, 2004). During glacial periods numerous species in the Northern hemisphere were restricted to warmer Southern refugia, where populations may have been smaller and under different selection conditions, which would cause populations to diverge genetically (Hewitt, 1996, 1999). Recolonization after the glacial period may have started from several refugia, which may have led to genetically differentiated populations in the recolonized areas (Hewitt, 1996).

In addition, ongoing evolutionary processes may be affected by more recent events such as environmental stressors (e.g. persistent soil contamination), causing genetic differentiation between exposed and non-exposed populations. Contamination by metals may act as a directional selective pressure that changes allele frequencies by selecting the most tolerant genotypes. Genetic drift effects may also be enhanced as a result of a declined in population size (bottleneck). Increased rate of toxicant-induced deleterious mutations and directional migration out of contaminated areas may contribute to a population size reduction and consequently to a change in allele frequencies. On the other hand, non-deleterious mutations and migration events into the contaminated sites may increase genetic variation (Bickham *et al.*, 2000; Van Straalen and Timmermans, 2002).

We surveyed the available literature on population genetic structure of collembolans, isopods and earthworms to test the hypothesis that gene flow is limited due to their soil-born life style, resulting in significant genetic differentiation (Table 2.1). The influence of metal contamination on population genetic structure was also addressed.

Soil invertebrates

Soil-living collembolans, isopods and earthworms are important in organic matter decomposition processes, nutrient transformation and energy flow in

terrestrial ecosystems (e.g. Drobne, 1997; Rusek, 1998; Kautenburger, 2006). These animals are considered to have limited migration capacities and therefore to be rather sedentary. All life-stages have the same general morphology and there is no stage specifically adapted to dispersal, as observed in other soil organisms (e.g. phoretic nymphs of mites and diapause ("dauer") larvae of nematodes).

Collembolans are generally considered to be unable to disperse over long distances due to the lack of wings and their small body size. Furthermore, many species are dependent on the edaphic environment, where they find the necessary moisture to avoid dehydration (Fanciulli *et al.*, 2009). Sjögren (1997) studied several collembolan species and reported an average dispersal rate of 1.4 cm per week. However, the dispersal capacity varies significantly among species (Hertzberg, 1997) with relatively large epigeic collembolans being more efficient dispersers than edaphic species (Ojala and Huhta, 2001). Dispersal also depends on the availability of food, population density, soil type (Bengtsson *et al.*, 1994), and morphology (Van der Wurff *et al.*, 2003). For instance, the epigeic collembolan *Orchesella cincta* (L.) has well-developed legs and antennae, which indicates mobility (Van der Wurff *et al.*, 2003).

In contrast, isopods are assumed to migrate over longer distances, as for example *Hemilepistus reaumuri* (Audouin) individuals that were found to disperse over a distance of 62-92 m per day (Warburg *et al.*, 1984). For *Armadillidium vulgare* (Latr.) a smaller travel distance, of 1-13 m per day, was recorded (Paris, 1963). However, isopods are also strongly dependent on edaphic conditions and known to select microhabitats according to their moisture preference (Warburg *et al.*, 1984). They furthermore have an inherent tendency to aggregate (Warburg, 1968) and maintain family-based social structures (Linsenmair, 1984), which may limit dispersal.

Earthworms are known to be capable of active dispersal from unsuitable environmental conditions, whether these are soil properties, litter content or population density (Mathieu *et al.*, 2010). However, they are considered to have limited dispersal capacities of only a few meters per year; 2-4 m per year was estimated by Marinissen and Van den Bosch (1992) for two earthworm species (*Aporrectodea caliginosa* Savigny and *Lumbricus rubellus* Hoffm*).* Earthworms actively crawling over the soil surface will disperse considerably faster than species

living in permanent burrows (Zorn *et al.*, 2005).

Dispersal capacity is a life-history trait that is of ecological importance (Clobert *et al.*, 2001; Mathieu *et al.*, 2010) and directly affects the level of gene flow between populations. Considering their limited migration abilities, soil collembolans, isopods and earthworms are expected to be found in highly structured populations.

Population genetic structure of soil invertebrates

The current population genetic structure of several collembolan species has been analysed and related with their phylogeographic history. Cicconardi *et al.* (2010) studied several genetic lineages within the genus *Lepidocyrtus* Bourlet from the North-Western Mediterranean basin. The authors found a noteworthy geographic structure with highly differentiated genetic lineages and no evidences of gene flow, even at relatively small distances. The current distribution of these lineages was attributed to late Miocene paleogeographic events. Several studies on the population genetic structure of the collembolan *O. cincta* have been conducted at various geographical scales. Timmermans *et al.* (2005b) sampled populations across a large part of its European distribution. The results revealed the existence of three population clusters: NW Europe, Central Europe and Italy. The significant division between NW and Central Europe populations, in the absence of a clear geographic barrier, might have a historical reason. The authors suggested that the differentiation between these populations could be caused by recolonization by individuals from different refugial areas during interglacial periods. Moreover, they suggested that the Alps form a barrier that prevents gene flow between Italian and the other analysed European populations. The existence of this Alpine barrier, causing isolation of Italian populations has been observed in other studies, for example with mammals and arthropods (e.g. Taberlet *et al.*, 1998; Hewitt, 1999). This significant population structure in *O. cincta* indicates limited gene flow among populations at a large geographical scale. However, when analysing populations from a smaller geographic area (NW Europe) low population genetic differentiation and high levels of gene flow were observed.

Table 2.1 Soil invertebrate species considered in this literature survey (mtDNA – mitochondrial DNA; AFLP – amplified fragment length polymorphism; TE-AFLP – three enzyme-amplified fragment length polymorphism; RAPD – random amplified polymorphic DNA; ISSRs – inter-simple sequence repeats; * indicates studies comparing reference and exposed populations).

Species	DNA markers	Location	Genetic structure	References
Collembolans				
Lepidocyrtus spp.	mtDNA	NW Mediterranean	differentiation, historical events, no gene low	Cicconardi *et al.* , 2010
Orchesella cincta	mtDNA/AFLP	Europe	differentiation Europe/Italy, limited gene flow differentiation NW/Central Europe, historical events	Timmermans *et al* ., 2005b
	Microsatellites	Netherlands	low differentiation, gene flow	Van der Wurff *et al* ., 2003
	TE-AFLP			
	Microsatellites	NW Europe	low differentiation, gene flow	Timmermans, 2005 *
	Microsatellites	NW Europe	low differentiation, gene flow	Van der Wurff *et al* ., 2005
	Allozymes	NW/SW Europe	low differentiation, gene flow	Frati *et al* ., 1992 *
Orchesella bifasciata	Allozymes	Sweden	low differentiation, gene flow	Travink *et al.* , 1994 *
Tetrodontophora bielanensis	Isozymes	Central/SW Europe	differentiation Central/SW Europe, historical events	Fanciulli *et al* ., 1991
Allacma spp.	Allozymes	Italy	differentiation, historical events	Fanciulli *et al* ., 2009
Tomocerus spp.	Allozyme	Italy	differentiation, historical events	Fanciulli *et al.* , 2000
Pogonognathellus spp.				
Sminthurus viridis	Allozymes	Australia	differentiation, limited gene flow	Roberts and Weeks, 2011
	mtDNA			
	Microsatellites			
Gressittacantha terranova	Allozymes	Antartica	differentiation, limited gene flow	Fanciulli *et al* ., 2001
Folsomia candida	ISSRs	Aquifer	differentiation, distance and limited gene flow	Sullivan *et al.* , 2009
Isotoma notabilis	RAPD	Denmark	differentiation, colonization from surrounding areas	Simonsen *et al* ., 2004 *
Isopods				
Porcellio scaber	Allozymes	Central Europe	low differentiation, historical events, gene flow	Wang and Schreiber, 1999a
Oniscus asellus	Allozymes	Central Europe	low differentiation, historical events, gene flow	Wang and Schreiber, 1999b
Earthworms				
Lumbricus terrestris	RAPD	Germany	low differentiation	Kautenburger, 2006
Dendrobaena octaedra	mtDNA	Canada	differentiation, multiple introduction events	Cameron *et al* ., 2008
	Isozymes	Greenland Canada Europe	differentiation due to historical events	Hansen *et al* ., 2006
	Allozyme	Finland	different genotype distribution, several causes	Haimi *et al* ., 2007 *
Lumbricus rubellus	Allozymes	Faroe Islands	differentiation due to land-use	Enckell *et al* ., 1986
	mtDNA/AFLP	UK	differentiation due to historical events	Andre *et al.* , 2010 *

The existence of high gene flow was first revealed by Van der Wurff *et al.* (2003) who found low genetic differentiation between populations of two Dutch forests separated from each other of about 10 km; no isolation by distance was found. A clear IBD pattern for NW European populations was revealed by Timmermans (2005). His results showed that *O. cincta* populations over a distance up to 60 km were genetically undifferentiated, suggesting the existence of high levels of gene flow over such distances (Figure 2.2; from Timmermans, 2005).

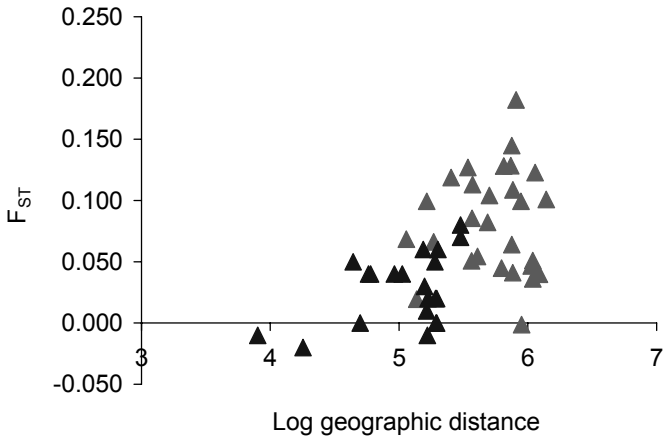

Figure 2.2 Relationship between genetic and geographic distance in the soil-living collembolan *Orchesella cincta*. Geographic distance is measured in m. Genetic distance is expressed as F_{ST}, estimated from six polymorphic microsatellite loci for pairwise comparisons between different populations from North-Western Europe. Light grey triangles: data from Timmermans (2005); dark grey triangles: data from Van der Wurff *et al.* (2005). Figure from Timmermans (2005).

These results can be explained by the moderately high dispersal capacity of *O. cincta*, since species with such a dispersal capacity may be genetically homogeneous at small distances but differentiated over longer distances (Peterson and Denno, 1998). Van der Wurff *et al.* (2005) also found low genetic differentiation and IBD pattern among populations sampled across the Netherlands. *O. cincta*'s tendency to climb trees has been suggested to facilitate wind-driven dispersal over

long distance (Freeman, 1952; Van der Wurff *et al.*, 2003).

A relatively high divergence between populations from Italy and Central Europe and a low differentiation among Central European populations was also found in the collembolan *Tetrodontophora bielanensis* (Waga) *(*Fanciulli *et al.*, 1991). In contrast, Fanciulli *et al.* (2009) showed that *Allacma fusca* (L.) and *A. gallica* (Carl) maintain high levels of genetic differentiation among populations from several regions within Italy. Gene flow estimates indicated that the effective number of individuals that were exchanged between populations were insufficient to overcome effects of genetic drift. The authors also suggested that high levels of genetic differentiation could have been due to bottleneck events during Plio-Pleistocene geological rearrangements that took place in the Italian peninsula. Yet, an isolation by distance pattern was observed for both species, demonstrating the presence of gene flow among geographically close populations, most probably originating from passive dispersal of individuals by either wind and/or animal transport. Fanciulli *et al.* (2000) reported strong population differentiation in four additional Collembola species (*Pogonognathellus flavescens* (Tullberg), *P. longicornis* (Müller), *Tomocerus vulgaris* (Tullberg) and *T. minor* (Lubbock)) caused by Plio-Pleistocene geological rearrangements coupled with genetic drift and limited gene flow. Roberts and Weeks (2011) studied another collembolan species, *Sminthurus viridis* (L.), from several regions in Australia, where it was introduced from Europe in the late 1800s. Results revealed significant population genetic structure, suggesting limited gene flow. Yet, the existence of distant populations less differentiated than closer ones weakened the isolation by distance relationship (Figure 2.1). The observed pattern was explained by the possible existence of long-distance dispersal mediated by human activities. Genetic analysis of *Gressittacantha terranova* Wise from Antarctica revealed three genetically distinct groups with limited gene flow among them. Migration among populations is presumed to be hampered by glaciers that constitute efficient barriers (Fanciulli *et al.*, 2001). Also, *Folsomia candida* Willem populations inhabiting an aquifer were found to be highly structured within an area of about 0.65 km^2 (Sullivan *et al.*, 2009). The genetic differentiation was considered to be caused not only by the distance among populations (an IBD pattern was observed) but also due to the existence of barriers to gene flow. However, it has to be mentioned that the authors

could not rule out local adaptation to have partially caused the observed pattern.

Regarding isopods, Wang and Schreiber (1999a) analysed central European populations of the species *Porcellio scaber* Latr. Low genetic differentiation among nearby locations (10 km) was observed and IBD was found on a larger geographic scale. These authors also analysed the genetic structure of *Oniscus asellus* L. populations collected at several sites in Central Europe (Wang and Schreiber, 1999b), and again found low genetic differentiation among populations. The results of both studies were explained by the fact that the time elapsed since recolonization of Central Europe after Pleistocene glacial periods from Southern refugia was not enough to allow genetic differentiation to occur. Furthermore, the high abundance and therefore presumed large population sizes of these isopod species could buffer the effects of genetic drift. The authors finally suggested that the observed genetic homogeneity among populations could be due to passive dispersal, i.e. mediated by wind, flowing water or human activities. As for the collembolan *O. cincta*, vertical migration towards tree tops might facilitate dispersal by wind in some isopods (Brereton, 1957; Den Boer, 1961).

The population structure of several earthworm species has also been analysed. Kautenburger (2006) studied *Lumbricus terrestris* L. sampled in several locations in Western Germany. A similar genetic structure was observed among neighbouring locations up to about 20 km apart. Populations located at more than 70 km apart were found to be genetically differentiated. However, gene flow among adjacent populations did not lead to a trend of increasing genetic differentiation with geographic distance on a larger scale. The absence of an IBD pattern suggests a complicated pattern of gene flow between earthworm populations.

Human-mediated dispersal of earthworms has been of significant importance for some earthworm species (Edwards and Bohlen, 1996). Cameron *et al.* (2008) studied populations of *Dendrobaena octaedra* Savigny, from Alberta (Canada). The introduction of this species in the boreal forests of Alberta was suggested to be mediated by anthropogenic activities, with the recurrent nature of these introductions resulting in significant population differentiation. No relationship between genetic and geographic distances was found, which strengthens this view and indeed suggests that such “jump dispersal” is of greater importance than diffusive spread and active dispersal. This is consistent with the idea that

earthworms have a limited capacity to autonomously disperse (Marinissen and Van den Bosch, 1992; Sakai *et al.*, 2001). A strong genetic differentiation among Greenlandic and Canadian/European populations of *D. octaedra* was found by Hansen *et al.* (2006), which suggested that dispersal between Greenland and the continental locations has been more restricted than dispersal among continental areas. The authors suggested that populations from Greenland have persisted for a long period, surviving glacial periods in local ice-free refugia. The existence of clones shared among different localities in Greenland was suggested to be the result of (human-mediated) passive transportation. Enckell *et al.* (1986) studied *L. rubellus* from the Faroe Islands. The authors found a weak relationship between genetic and geographic distance and showed that geographical barriers had only slight or no influence on genetic variation between different populations. It's noteworthy to mention that genetic differences appear to be caused by selective effects of land-use (infields versus outfields) and differentiation is mainly determined by environmental conditions.

Genetic structure of metal exposed populations

The soil and litter layer are major sinks for metal contaminants (Martin and Coughtrey, 1981; Jones *et al.*, 1988), and when present soil-living organisms, such as collembolans, isopods and earthworms, are directly exposed to these metals. Therefore, soil invertebrate populations are expected to be genetically affected by metal contamination. Frati *et al.* (1992) compared exposed and reference populations from NW Europe and Italy and found a noteworthy genetic homogeneity in *O. cincta*. This low genetic variation was explained by the relatively short time of recolonization after the Pleistocene glacial period. No evidence of metal contamination effects on population genetic structure was found. The genetic structure of congeneric *Orchesella bifasciata* L. populations was also not affected by metal contamination (Tranvik *et al.*, 1994). The authors suggested that passive dispersal of individuals (mediated by wind, water or other animals) could contribute to maintaining genetic homogeneity. In agreement with these studies Timmermans (2005) found that contamination did not affect gene flow among reference and exposed *O. cincta* populations. Simonsen *et al.* (2004) analysed the collembolan

Isotoma notabilis Schaeffer along a soil copper gradient. Genetic differentiation was observed but this pattern could not be explained by copper contamination. The authors suggested that the obtained results were due to colonisation events from the areas surrounding the field. They suggested that passive dispersal mechanisms, such as wind, would help *I. notabilis* migrate. Haimi *et al.* (2007) evaluated the genetic diversity of the earthworm *D. octaedra* of metal-contaminated soils. Clonal diversity in both contaminated and uncontaminated soils was moderate to high. Although metal contamination seemed to have little effect on clonal diversity, the distribution of genotypes among populations was significantly different. The authors suggested this to be caused by the metal pollution, nevertheless effects of several other factors, including adaptation, lack of migration, and the existence of clone pools or sampling effects could not be excluded. Andre *et al.* (2010) studied *L. rubellus* sampled at an abandoned lead mine. Two distinct lineages were revealed and although this was related to post-glacial colonization events the authors raised a second hypothesis that the different genotypes could display differential responses or tolerance to environmental contaminants.

Conclusions

Despite the limited active dispersal capacities of soil collembolans, isopods and earthworms, populations of several species were found to be genetically undifferentiated over considerable distances. Passive dispersal, either mediated by wind, water flow or animals has been implicated in the maintenance of genetic homogeneity in these cases. Clear evidence of genetic differentiation increasing with distance is seen mainly at a larger scale (e.g. > 60 km in the collembolan *O. cincta*). Population structure of soil invertebrates still reflects historical events, such as glaciations and geological rearrangements. Metal contamination has been shown to have only a limited influence in population genetic structure and results are not always conclusive; when genetic differentiation is observed it may be the result of other, unrelated processes. It is worth mentioning, however, that the lack of genetic differentiation may in some cases be caused by low resolution of the markers used. For instance, where both microsatellites and allozymes have been

used, microsatellite-based analyses seem to be more powerful in detecting differentiation (e.g. compare Frati *et al.*, 1992 and Timmermans *et al.*, 2005b). More information on the genetic variation of soil invertebrates is expected to come from genome-wide polymorphism studies. Such population-genomics approaches may further improve molecular-based estimates of population genetic parameters, such as effective population size, population structure and dispersal rates (Luikart *et al.*, 2003).

Chapter 3

Copper tolerance and genetic diversity of *Porcellionides sexfasciatus* at a highly contaminated mine habitat

Based on: Costa D, Bouchon D, Van Straalen NM, Sousa JP, Ribeiro R (submitted to Ecotoxicology).

Abstract

Mining practices have mobilized several metals such as copper into the environment were they act as toxic threats. Organisms inhabiting metal-contaminated areas may develop metal tolerance, either phenotypically (acclimation, maternal effects and phenotypic plasticity) and/or genetically (adaptation). Through adaptation the most sensitive genotypes are eliminated, causing a shift in allele frequencies, thereby affecting population genetic diversity. In this study, three populations of the terrestrial isopod *Porcellionides sexfasciatus*, collected at an abandoned mine area, were compared to assess the effects of metal contamination on tolerance to lethal and sub-lethal levels of copper, through comparison of survival, avoidance and feeding. The effects of metal contamination on genetic diversity were also considered. Differences in copper tolerance were observed when comparing survival and avoidance behaviour, probably due to different metal contents and also to metal bioavailability. No differences in genetic diversity were found. Moderate levels of genetic differentiation were observed, possibly due to genetic drift; also metal contamination might have contributed to population differentiation.

Introduction

Mining practices mobilize several metals such as cadmium, lead, copper, and zinc into the environment were they act as toxic threats, impairing reproduction, growth and survival of exposed organisms (Fox, 1995). Individuals inhabiting metal-contaminated areas may actively avoid or limit the exposure to toxicants (e.g. Landgon *et al.*, 2001a; Natal-da-Luz *et al.*, 2004). If they fail to avoid exposure, they may develop metal tolerance. The occurrence of metal-tolerant field populations, due to contaminant exposure, has been reported in diverse organisms, including aquatic species (Lopes *et al.*, 2004, 2005), plants (Monni, 2000; Gratão *et al.*, 2008) and soil invertebrates, such as earthworms (Langdon *et al.*, 2001b), ants (Grzés, 2010), collembolans (Posthuma, 1990), and isopods (Donker and Bogert, 1991). Populations may develop tolerance either phenotypically (acclimation, maternal effects and phenotypic plasticity) and/or genetically (adaptation). Through adaptation, the most sensitive genotypes are

eliminated, and this may cause a decrease of population genetic diversity (Van Straalen and Timmermans, 2002). Elimination of sensitive genotypes may occur due to reduced survival and/or diminished reproduction capacities but also through migration events out of the contaminated area (avoidance).

Soil-dwelling invertebrates may be directly affected by metal contamination, since soil is a major sink of metals (Martin and Coughtrey, 1981). Isopods inhabit the upper layer of soil and surface leaf litter where they feed mainly on plant material, thus playing a key role in decomposition (Drobne, 1997). Any change in their feeding rates affects the decomposition process and consequently organic matter and energy cycles through ecosystems (Drobne, 1997). Thus food consumption is a relevant endpoint to study the ecological effects of contaminants in the ecological functions of isopods. Essential metals like copper may have deleterious effects when in high concentrations. In terrestrial isopods, copper is known to be essential for respiration and immune response and to promote digestive processes, however at high concentrations it can decrease survival and reproductive success (Weissenburg and Zimmer, 2003; and references therein).

This study aimed at verifying if a historically exposed population of a soil-living organism was more tolerant to lethal and sub-lethal levels of copper than less contaminated populations, through the comparison of survival, avoidance and feeding in laboratory exposures. Moreover, the effects of metal contamination on genetic diversity were assessed using neutral markers and the correlation between genetic diversity and copper sensitivity was evaluated. Copper was chosen because previous chemical analysis revealed that it is present in elevated amounts (over one order of magnitude) in the historically contaminated area relatively to other nearby sites. Genetic diversity was estimated by Random Amplified Polymorphic DNA (RAPD; Williams *et al.*, 1990); RAPD markers have been used in several ecotoxicological studies (e.g. Theodorakis *et al.*, 2006; Deng *et al.*, 2007), to assess the effects of anthropogenic contaminants on population genetic diversity. This method does not require previous DNA sequence information and so is adequate to be used with less studied species, such as the terrestrial isopod *Porcellionides sexfasciatus*.

Metal tolerance at this mining area has already been found in plants (Freitas *et al.*, 2004) and aquatic invertebrates (Lopes *et al.*, 2004, 2005), however till now no

metal tolerance studies have been performed with soil invertebrates.

Material and Methods

Study site

This study was conducted at an abandoned cupric-pyrite mine, Mina de São Domingos, located in Southeast Portugal (37°40′ N, 7°29′ W). This region is part of the Iberian Pyrite Belt (IPB) that has an extension of 250 km length and 30-60 km width (an area of 12 500 km^2). It comprises the regions of Alentejo (Portugal) and Andalusia (Spain) (Pereira *et al.*, 2004). Along with massive amounts of pyrite, there are also deposits of manganese and iron and veins of copper, antimony, lead, and barium (Oliveira and Oliveira, 1996). Most of the mines along the IPB are abandoned and cause negative impacts on the environment. Mina de São Domingos was intensively explored between 1859 and 1966 with the extraction of copper, iron, sulphur, and zinc. The abandonment of the mine left behind old structures with a high level of corrosion, ruins of industrial buildings, a deep pit filled with highly acidic water, dams and numerous diverting and channelling streams with characteristic reddish-yellow banks and tons of mine tailings (Lopes *et al.*, 1999). Natural vegetation in this area is dominated by *Quercus ilex* and *Eucalyptus* spp. trees and by *Lavandula stoechas* and *Genista hirsuta* shrubs (Freitas *et al.*, 2004). Three sampling sites were defined in the mine area: Santana de Cambas - SC (37°37′56″ N, 7°31′06″ W), Tronco - TR (37°40′55″ N, 7°30′54″ W), and Corte do Pinto - CP (37°42′10″ N, 7°27′31″ W). Soil and litter samples were collected and metal concentrations determined by DRAPN (Porto, Portugal), according to Natal-da-Luz *et al.* (2011).

Model organism and sampling

This study was performed with *Porcellionides sexfasciatus* (Koch), an isopod species found underneath stones and in the case of CP site almost exclusively in dry cow excrements. Other species of isopod were present, but only *P. sexfasciatus* was common to all three sampling sites. Animals were collected by hand, in spring and taken to the lab where they were kept in soil from the

respective sampling site, at 20ºC ± 2 and a photoperiod of 16 hours light: 8 hours dark.

Ecotoxicology tests

One lethal and two sub-lethal (avoidance and feeding) ecotoxicological tests were performed with copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$, 99% purity, from Fluka, Buchs, Switzerland), mixed into soil or leaves. Preliminary tests were made to establish copper concentrations to be used in the following experiments.

For the survival experiment about 100 animals from each site were placed individually in test containers with contaminated OECD soil (5% organic matter; OECD, 2009) and were frequently observed (every hour during the 1st twelve hours, then frequency was gradually reduced along the test). The nominal copper concentration used was 10 000 µg Cu/g soil dry weight. The test was performed until all animals died. Isopods were preserved in ethanol for later DNA extraction.

Approximately 30 animals per site were used to test the avoidance response. Isopods were exposed to a clean and a contaminated (nominal concentration of 100 µg Cu/g soil dry weight) OECD soil (5% organic matter) for 24 hours; observations were made after 6 and 24 hours.

Alder leaves (*Alnus glutinosa*) contaminated with 500 µg Cu/g dry leaf (nominal concentration) were used to measure weight increase, food consumption, and food assimilation efficiency over a 28 days exposure (Donker and Bogert, 1991) of 15 isopods from each population. Leaves were cut into small discs (approximately 12 mm) and dry weight was recorded. Leaf discs were contaminated with the copper solution and dried overnight at room temperature. Dry contaminated leaves were weighed, re-hydrated and given to the isopods. Every week faecal pellets and remaining food were removed and weighed, and new contaminated leaves were given to the isopods. Animals were individually weighed at the beginning and at the end of the test.

DNA extraction and RAPD amplification

DNA was extracted from isopod muscles, gonads, and nervous tissue, according to Kocher *et al.* (1989). RAPD amplification (Williams *et al.*, 1990) was performed with two primers (R2: 5´-TGCCGAGCTG-3´ and R12: 5´-

TCGGCGATAG-3´). Each reaction mix contained 4 µl 5x Buffer, 1 U Taq polymerase, 0.01 nmol primer, 2.15 mM dNTP and 1 µl template DNA in a total volume of 20 µl. Amplification was performed with 35 cycles of 94ºC for 1 min, 35ºC for 1 min and 72ºC for 1.30 min, preceded by a denaturation step at 94ºC for 2 min, and a final step at 72ºC for 5 min. Amplification products were visualized in a 2.5% agarose gel. Bands were scored semi-automatically (manual adjustments were made whenever necessary) with GelAnalyzer2010a software (available from www.gelanalyzer.com). To avoid unbiased estimates of heterozygosity, only loci where the frequency of null alleles (band absence) was higher than 3/N (N= total sample size) were included in the analysis (Lynch and Milligan, 1994). To assure reproducibility of banding patterns positive and negative control samples were included.

Data analysis

Copper survival data was fitted to a logistic model to estimate the median lethal time (LT50) according to the equation: survival= (maximum / (1+ (time/ LT50) ^ slope)). Survival curves were compared using the likelihood ratio test. Avoidance behaviour at 6 and 24 hours was analysed with chi-square test. Results from the feeding experience were used to calculate, feeding parameters, such as isopod weight increase, food consumption and food assimilation efficiency. Weight increase was determined as the final weight of the isopod minus the weight at the beginning of the test (Donker and Bogert, 1991). Weekly food consumption was measured as the difference in the initial and final weight of the disc leaves, food assimilation was calculated as the food consumption minus the faeces production (Donker and Bogert, 1991). Results of the four weeks were summed (Donker and Bogert, 1991) and total food consumption and total food assimilation efficiency (summed food assimilation / summed food consumption *100) were determined. Feeding parameters (isopod weight increase, total food consumption and total food assimilation efficiency) were compared with one-way ANOVA. Previously, assumptions of normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene´s test) were verified. Post-hoc comparisons were made with Newman-Keuls test. Comparison of the survival curves, chi-square tests and ANOVA were performed with STATISTICA 7.0 software (StatSoft, Tulsa, OK, USA).

RAPD markers were considered to be in Hardy-Weinberg equilibrium and were scored as presence (1) or absence (0). DNA fingerprint was then converted into a binary matrix that was used to assess populations genetic diversity, through estimation of Shannon information index (I) and expected heterozygosity (He); significant differences were tested with Kruskal-Wallis test. Total genetic variance was partitioned among and within populations with an Analysis of Molecular Variance (AMOVA); significance was determined with a permutation test (999 permutations). Pairwise Φ_{PT} (PhiPT, analogous to F-statistics – F_{ST}) were estimated, via AMOVA, to assess genetic differentiation among populations. The number of migrants per generation (Nm) was estimated according to Wright (1943), replacing F_{ST} by Φ_{PT}. Isolation by distance was tested by plotting pairwise $\Phi_{PT}/ (1-\Phi_{PT})$ and Nei´s genetic distance against ln-transformed geographic distance; significance was tested with Mantel test (999 permutations). Furthermore, a Principal Component Analysis (PCA) was done, to more effectively analyse genetic distance patterns. Population genetic analyses were performed with GENALEX 6.4 software (Peakall and Smouse, 2006). To confirm that the studied RAPD loci behaved as neutral markers Ewens-Watterson test for neutrality was performed using POPGENE 1.32 software (Yeh *et al.*, 1997).

To determine the association between LT50, genetic diversity and soil and litter copper concentrations, Pearson correlations were used. Normality was tested with the Kolmogorov-Smirnov test. Correlation analysis was performed with STATISTICA 7.0 software.

Results

Soil and litter metal content

All selected areas presented considerably high metal contamination both in soil and in litter (Table 3.1). The highest metal concentrations, mainly copper, iron, zinc, cadmium and lead, were found in the SC site.

Table 3.1 Soil and litter metal concentrations (mg/kg) and pH (measured in H_20) from all sampled areas (TR – Tronco; CP – Corte do Pinto, SC – Santana de Cambas).

	Soil			Litter		
	TR	**CP**	**SC**	**TR**	**CP**	**SC**
Cu	34	66	933	22	31	302
Fe	54663	53013	94413	31068	23568	50935
Mn	979	1530	179	1500	1331	341
Zn	77	44	320	64	29	283
Cd	< 2.8	< 2.8	3.1	<2.8	<2.8	4
Cr	28	23	22	43	24	27
Pb	54	<45	3276	<45	<45	1192
Co	54	58	50	29	34	37
Ni	50	59	28	39	34	33
pH	6.1	6.0	4.5	5.1	5.6	5.6

Ecotoxicology tests

The highest and the lowest median lethal time (LT50) values were found at the least contaminated sites, TR and CP respectively (Table 3.2).

Table 3.2 LT50 values (hours) with corresponding standard error (S.E.) and 95% confidence intervals (C.I.) for the survival of different *Porcellionides sexfasciatus* populations exposed to 10 000 µg Cu/kg soil dry weight, estimated with a logistic model.

	LT50	**S.E.**	**C.I.**
TR	9.96	0.256	9.44-10.5
CP	14.7	0.470	13.8-15.7
SC	11.4	0.415	10.6-12.3

Survival curves (Figure 3.1) comparisons revealed significant differences ($p < 0.05$) in LT50 values among all populations.

Avoidance behaviour was significantly different ($p < 0.05$) between TR and SC animals, when considering the responses after 6 hours. When considering just the response after 24 hours no differences were found among populations (Figure 3.2).

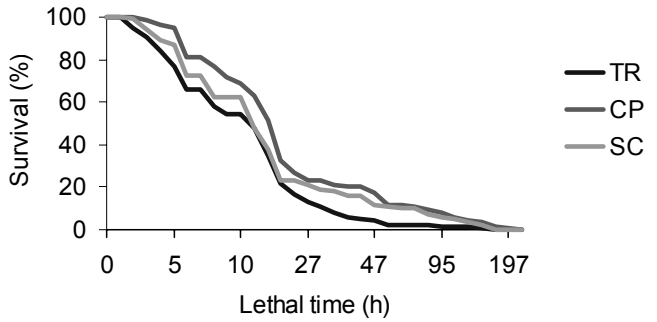

Figure 3.1 Survival along time (hours) of different *Porcellionides sexfasciatus* populations exposed to 10 000 µg Cu/g soil dry weight.

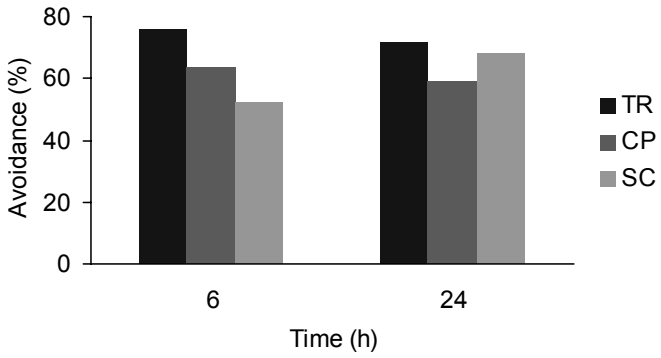

Figure 3.2 Avoidance responses of different *Porcellionides sexfasciatus* populations exposed to 100 µg Cu /g soil dry weight. Observations were made after 6 and 24 hours exposure.

Most isopods from TR (75%) and CP (94%) had an increased weight after the 28 days exposure to contaminated food, while just 53% of SC animals gained weight. Significant differences ($p < 0.05$) were found between CP and SC isopods weight increase (Table 3.3). Considering total food consumption and total food assimilation efficiency no differences ($p = 0.32$ and $p = 0.70$, respectively) were found among populations (Table 3.3).

Table 3.3 Feeding experiment parameters (mean ± standard deviation) of different *Porcellionides sexfasciatus* populations exposed to 500 µg Cu/ g dry leaf (WI - isopod weight increase; FCt – total food consumption; FAEt – total food assimilation efficiency).

	WI	FCt	FAEt
TR	0.819 ± 1.93	35.0 ± 10.5	24.7 ± 19.7
CP	1.40 ± 1.07*	33.0 ± 6.42	29.4 ± 17.9
SC	0.163 ± 0.750*	30.7 ± 7.59	30.7 ± 25.5

* $p < 0.05$

Population genetic analysis

A total of 57 loci were analysed, from which 74% were polymorphic. Both genetic diversity indices were slightly lower at the most contaminated site, SC (I = 0.262 ± 0.031; He = 0.162 ± 0.022), than at TR (I = 0.345 ± 0.032; He = 0.219 ± 0.023) and CP (I = 0.276 ± 0.033; He = 0.174 ± 0.023), although no significant differences were detected with Kruskal-Wallis test ($p = 0.12$). AMOVA results showed that 78% of total genetic variance was explained by variation within populations, while variation among populations explained 22% ($\Phi_{PT} = 0.224$; $p < 0.05$). Pairwise Φ_{PT} comparisons revealed the existence of significant differentiation among all populations (Table 3.4). Gene flow (Nm) varied between 1.03 and 0.701 (Table 3.4). Mantel test showed no correlations between genetic and geographic distances.

Table 3.4 Nei's genetic and geographic distances (km), Φ_{PT} and Nm (number of migrants) between all *Porcellionides sexfasciatus* populations.

	Nei`s genetic distance	Geographic distance	Φ_{PT}	Nm
TR vs CP	0.06	6.66	0.195*	1.03
TR vs SC	0.05	5.47	0.212*	0.929
CP vs SC	0.07	10.2	0.263*	0.701

* $p < 0.05$

In the PCA plot, axis 1 (explaining 30.1% of total variance) separated SC from

CP and to a lesser extent from TR. Axis 2 (explaining 22.9% of total variance) separated CP from TR population (Figure 3.3). The genetic distance pattern observed in the PCA plot was in agreement with the pairwise Φ_{PT} results. The Ewens-Watterson test, run over all populations, confirmed that most loci were neutral (93%).

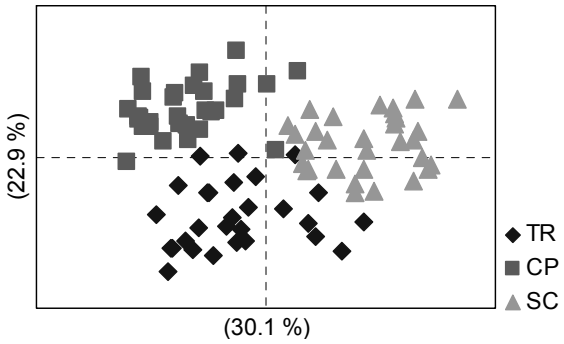

Figure 3.3 Principal Component Analysis biplot based on standardized genetic distance data for different *Porcellionides sexfasciatus* populations.

Survival and genetic diversity

No correlations were found between LT50, genetic diversity and copper concentrations ($p > 0.11$).

Discussion

The selected areas presented considerably high metal concentrations both in soil and in litter as expected due to their location in the IPB area. Soil is naturally metal enriched all across the whole mine area (Pereira *et al.*, 2004, 2006). Tronco and Corte do Pinto sites presented similar levels of soil and litter metal concentrations, while Santana de Cambas had a higher metal contamination due to the mining activities (contamination at this site occurred mainly by wind deposition).

Considering metal concentrations, it would be expected that Tronco and Corte

do Pinto populations would present similar sensitivity to copper. A possible explanation for the higher copper tolerance (higher LT50) observed in Corte do Pinto population may be the fact that this area was used as a pasture and that most isopods were found and collected from dry excrements and not directly from the soil. Isopods at this site may have been further exposed to metals in the excrements. The high metal content in plants (Freitas *et al.*, 2004) most probably eaten by cattle may have lead to high metal contents in the excrements. Furthermore, the use of copper supplements (or other veterinarian pharmaceuticals) that may have been given to cattle could have also contributed to the higher copper tolerance. However, this hypothesis cannot be confirmed at the moment. Ecotoxicity results from Corte do Pinto stressed the importance of having complete information about sampling sites to be able to attribute a cause to test results, and also the difficulty of isolating environmental stressors.

Since the soil and litter of Santana de Cambas presented the highest metal concentrations, it was expected that the isopods from this site would have increased copper tolerance when compared with the other populations. The higher copper tolerance observed in Corte do Pinto population compared to Santana de Cambas may be due to differences in metal bioavailability, since the aging of metals in soil tends to immobilize them and make them less available than freshly added metals (Sauvé, 2002). Comparing Tronco and Santana de Cambas, this population had a higher LT50 value and a later avoidance response, indicating an increased copper tolerance. These results are in agreement with those obtained for aquatic organisms by Lopes *et al.* (2004, 2005) that, studying the freshwater cladocerans *Daphnia longispina* and *Ceriodaphnia pulchella* from the same abandoned mining area, found increased copper tolerance in the contaminated populations. Increased metal tolerance was also found at other historically contaminated sites. Donker and Bogert (1991), studying the terrestrial isopod *Porcellio scaber* from a zinc smelter area and a lead mine site, and Posthuma (1990), with the collembolan *Orchesella cincta* from various contaminated areas, found increased cadmium tolerance. Also, Langdon *et al.* (2001b) found that the terrestrial oligochaetes *Lumbricus rubellus* and *Dendrodrilus rubidus* from abandoned mining areas were resistant both to arsenate and copper.

Total food consumption and total food assimilation efficiency did not differ

among populations and most isopods were able to gain weight despite feeding on Cu-contaminated food. Therefore, it appears that the ecological function of isopods, as comminutors, is maintained at this highly contaminated habitat. However, the fact that in the most contaminated population nearly half of the isopods were not able to increase weight along time may be of concern.

No significant differences in genetic diversity using neutral markers were found among populations. The lack of reduced genetic diversity (Van Straalen and Timmermans, 2002) was also observed in other studies using similar techniques. Martins *et al.* (2009), studying *D. longispina* from the same abandoned mining area, did not find evidence for genetic erosion. Also, Timmermans (2005) studying *O. cincta* from historically contaminated sites did not observe a contaminant-related decrease in genetic diversity. It should be mentioned that the capacity of neutral markers, such as RAPD loci, to identify contamination-induced changes on genetic variation seems to be limited, since a decrease in genetic diversity will only be detected when population size is reduced and gene flow is restricted (Hoffmann and Willi 2008). For instance, in a *D. longispina* case-study, no decreased genetic variation was observed with AFLP loci (Martins *et al.*, 2009), although when considering selectable traits, such as tolerance to lethal levels of copper, genetic erosion was observed with the elimination of the most sensitive individuals from the contaminated populations (Lopes *et al.*, 2004, 2006).

Populations in our study showed moderate levels of genetic differentiation (Φ_{PT} = 0.224). Wright (1978) considered the range between 0.150 and 0.250 to indicate moderate differentiation. Population differentiation may be caused by several factors, such as geographic isolation, habitat fragmentation, genetic drift, and local selective pressures and may be counteract by gene flow. The number of migrant individuals (Nm) between populations was low, and except between the two less contaminated populations, TR and CP, smaller than 1 (Nm ≥ 1 prevents genetic differentiation due to genetic drift, Slatkin, 1987). Isopods are considered to be inefficient active dispersers, since migration seems to be limited to the crawling capacity and to passive dispersal events. Given that no isolation by distance pattern was observed, population differentiation may be explained by genetic drift and/or local selective pressures. Since Nm was smaller than 1 (between SC vs. TR and CP populations) genetic drift may explain the genetic differentiation observed.

In addition, metal contamination, acting as a selective pressure, might have contributed to population differentiation. It is generally considered that selection is a more powerful (and directional) evolutionary force, since large populations are not very susceptible to genetic drift and tend to maintain their original degree of genetic variance (Merrel, 1981). Results showed that despite being closer, Tronco and Santana de Cambas populations have a higher pairwise Φ_{PT} value, and consequently a lower Nm, than the two less contaminated populations, that are slightly more distant. Santana de Cambas was the more differentiated population, this may suggest the existence of metal effects on population genetic structure.

Additional studies, including more sampling sites and endpoints, such as reproduction, should be performed to confirm the existence of increased tolerance on terrestrial isopods from the historically contaminated site.

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Chapter 4

Influence of adaptive evolution of cadmium tolerance on neutral and functional genetic variation in *Orchesella cincta*

Based on: Costa D, Mariën J, Janssens TKS, Van Gestel CAM, Driessen G, Sousa JP, Van Straalen NM, Roelofs D (submitted to Ecotoxicology).

Abstract

Adaptation to environmental toxicants, such as metals, can affect population genetic diversity, both at neutral and selectable loci. At the transcriptional level, evolution of metal tolerance is possible due to the existence of polymorphisms in the *cis*-regulatory sequences of stress-responsive genes such as the metallothionein gene (*mt*). This study determined the influence of cadmium adaptation on genetic diversity of soil-living *Orchesella cincta* (Collembola) populations in neutral (microsatellites and AFLP) and in functional (*mt* promoter) markers. Also, the influence of *cis*- versus *trans*-acting factors on increased tolerance was addressed. No reduced genetic diversity was observed in two tolerant populations compared to five sensitive populations, either in neutral or in selectable markers. Extensive migration and/or mutation events along with a large population size may explain the high genetic diversity measured. The metal-tolerant phenotype seems to be influenced mainly by genetic factors acting in *cis* on *mt* gene expression. The results suggest that the higher *mt* expression in tolerant populations is due to some exclusive promoter genotypes, such as those with the D2 allele, which are found mainly in tolerant populations. However, more studies are needed to clearly unravel the influence of *cis/trans*-regulatory evolution in tolerant populations.

Introduction

Adaptation to environmental toxicants can affect population genetic diversity. Metal contamination, for instance, may act as a directional selective pressure towards a more tolerant population, eliminating the most sensitive genotypes and diminishing population genetic diversity (Van Straalen and Timmermans, 2002). Environmental pollution will affect variation of selectable loci mainly if alleles at such loci have a large effect on the phenotype. It may affect variation at neutral loci if there is a strong decrease of population size due to environmental pollution (Hoffmann and Willi, 2008). In this study both types of genetic variation were investigated. As a selectable locus, the genetic variation of transcriptional regulation in the metallothionein (*mt*) gene of *Orchesella cincta* (Collembola) was considered. Transcriptional regulation is a fundamental component of the

genotype-phenotype interaction (Wray *et al.*, 2003). The presence of metal-tolerant phenotypes found in populations inhabiting metal-contaminated areas has been proposed to occur by transcriptional regulation, probably caused by polymorphisms in *cis*-regulatory sequences of the metallothionein gene (Sterenborg and Roelofs, 2003; Janssens, 2008). The collembolan species *O. cincta* has developed metal tolerance through heritable elevated cadmium (Cd) excretion efficiency (Van Straalen *et al.*, 1987; Posthuma *et al.*, 1992, 1993), lower Cd-induced growth reduction (Posthuma, 1990) and improved survival despite feeding on Cd-contaminated food (Sterenborg, 2003; Timmermans *et al.*, 2005a).

Metallothioneins are small ubiquitous proteins, with high cysteine content (± 30%) and no aromatic amino acids. They are involved in metal detoxification through the binding of essential metals like copper and zinc and also non-essential metals as cadmium (Dallinger 1996; Hensbergen *et al.*, 1999). They are strongly induced by metals, especially cadmium, but also by other factors such as oxidative stress (Bertin and Averbeck, 2006). In the springtail *O. cincta, mt* gene occurs as a single copy (Sterenborg and Roelofs, 2003) and is expressed mostly in the gut epithelium, where most of the cadmium is stored (Hensbergen *et al.*, 2000).

Genetic diversity among *O. cincta mt* protein coding sequence was identified, however, no association was observed between a particular *mt* protein coding allele and metal tolerance (Timmermans *et al.*, 2007). At the transcriptional level both higher constitutive and Cd-induced *mt* expression were found in springtails from metal-contaminated sites, when compared to reference populations (Sterenborg and Roelofs, 2003; Timmermans *et al.*, 2005a). Elevated *mt* expression was implicated in the cadmium tolerance mechanism observed in *O. cincta* (Sterenborg and Roelofs, 2003; Janssens *et al.*, 2007). Roelofs *et al.* (2006) showed that there is scope for selection on high expresser phenotypes, because a significant part of the *mt* transcriptional variability was found to be heritable in a reference population.

Nine alleles were identified in the *mt* promoter (*pmt*) sequence of *O. cincta* that differ from each other in their general structure, likely via recombination events, as reported by Janssens *et al.* (2007). Using a luciferase reporter assay, these authors were able to show that the different promoter alleles were differentially induced by Cd and oxidative stress. Also, *pmt* allele frequencies differed

significantly in animals from metal-contaminated and reference sites. The frequency of a highly Cd-inducible promoter allele increased in metal-tolerant populations. Further studies suggested that *mt* expression was regulated by a combined *cis/trans*-regulatory mechanism (Janssens, 2008; Van Straalen *et al.*, 2011). However, the contribution of *cis*- and *trans*-acting factors to the elevated *mt* expression phenotype associated with metal tolerance is still unclear.

In addition to selectable loci, effects of pollution may also extend to neutral loci if it causes a severe decrease of population size. Until now, studies on *O. cincta* have suggested that genetic diversity in proteins and neutral markers is not affected by metal contamination, given the low level of genetic differentiation observed among metal-tolerant and reference populations (e.g. Frati *et al.*, 1992; Timmermans, 2005). Timmermans (2005) suggested that moderate gene flow between populations prevents genetic differentiation and counteracts possible local bottlenecks due to pollution.

The previous studies have considered either neutral or selectable markers separately in population genetic studies of metal tolerance. Here, both markers were combined: neutral markers, microsatellites and AFLP (Amplified Fragment Length Polymorphism) with a selectable genetic marker, the *mt* promoter. The main objective of the present study was to link genetic variation to the Cd-tolerant phenotype both at the organismal level (survival on Cd-contaminated food) as well as at the molecular level (*mt* mRNA abundance). The inclusion of laboratory subpopulations of *O. cincta* with distinct *pmt* genotypes allowed us to gain more insight about the influence of *trans*-acting factors on *mt* expression.

Materials and Methods

Model organism and sampling

Orchesella cincta (L.) field animals were collected at three sites: one reference, Roggebotzand (ROG), and two metal-contaminated sites, Plombières (PLO) and Stolberg (STO). ROG (The Netherlands) is a pine forest on reclaimed land (N52º34´17´´, E5º47´56´´), PLO (Belgium) an open woodland on an abandoned lead-zinc mine (N50º44´04´´, E5º59´02´´) and STO (Germany) a forest in a lead

smelter area (N50º45´38´´, E6º14´15´´) (Van Straalen *et al.*, 1987; Timmermans *et al.*, 2005a). Soil and litter metal contents are present in Table 4.1 (data from Janssens, 2008). Animals were sieved from the litter above a white tray and collected with an aspirator. The collected animals were transported to the lab in PVC jars with a moist bottom of plaster of Paris. Once in the lab, animals were transferred to larger culture containers with a plaster of Paris bottom, fed with algae (mostly *Desmococcus* spp.) present on twigs collected at the reference site and placed in a climate room (temperature: 20ºC; relative humidity: 75%; light/dark period: 12/12 hours). Experiments were done with F1 individuals.

Four lab populations (Lab1, Lab2, Lab3, Lab5) originated from a laboratory culture founded with a mixture of animals from different non-polluted places in Europe, mainly Poland (Pilica: N50°29´05´´, E19°39´30´´), Sweden (Ringarum: N58°21´23´´, E16°13´37´´) and The Netherlands (Roggebotzand: N52°34´17´´, E05°47´37´´ and Amsterdamse Waterleidingduinen: N52°1´36´´, E04°33´11´), were also used. For further details about these lab populations see Driessen *et al.* (2007).

Table 4.1 Soil and litter total metal concentrations (mg/kg) and pH (measured in H_2O) from field sampled areas (PLO – Plombières; STO – Stolberg; ROG - Roggebotzand). Data from Janssens (2008).

	Soil			Litter		
	PLO	**STO**	**ROG**	**PLO**	**STO**	**ROG**
Cu	3416	519	512	1246	740	9
Fe	10858	5848	643	50177	7308	1654
Zn	19094	2791	130	4629	1142	58.5
Cd	30.5	41.2	0.2	9.6	41.6	0.8
Pb	17057	4641	47.3	10962	6778	86.5
Ni	26.3	52.8	7.4	60.1	12.4	1.7
pH	6.3	5.1	7.6	5.8	4.8	4.8

Survival experiment

Cadmium tolerance was determined for the three field populations (PLO, STO and ROG) and the four lab populations (Lab1 to Lab5) by measuring survival time

during metal exposure, as described by Timmermans *et al.* (2005a). For each treatment (clean and cadmium-contaminated food) twenty animals per population were used. Animals within the same size range (2-3 mm) were randomly selected. The nominal Cd concentration in the food was 400 µg Cd/g dry algae, added as a chloride salt ($CdCl_2$.2½ H_2O, purity ≥ 98% from Fluka, Buchs, Switzerland). Animals were observed every day and mortality was scored. For each observation time, survival was corrected for control mortality (5-30%) using the Henderson-Tilton correction (Henderson, 1955). Corrected survival data was then fitted to a logistic model to estimate the LT25 (lethal time at which 25% mortality was observed), and the slope of the survival curve using the equation: survival = (maximum / (1+ (0.25/ 0.75)*(time/ LT25) ^ slope)). The two parameters were estimated simultaneously by least squares approximation. Survival curves were compared using a likelihood ratio test, with STATISTICA 7 software (StatSoft, Tulsa, OK, USA).

DNA extraction and microsatellites amplification

DNA from single individuals was extracted with the Wizard SV Genomic DNA purification system (Promega, Madison, WI, USA) and stored at -20ºC until further analysis. *O. cincta* was screened for variation at each of seven microsatellite loci previously developed by Van der Wurff *et al.* (2001) and Mariën (unpublished data). Forward primers were 5`-labelled with a fluorescent dye (see Table 4.2 for primer sequence).

Table 4.2 Primer sequences used for *Orchesella cincta* microsatellite loci amplification (Van der Wurff *et al.*, 2001; Mariën, unpublished data).

Name	Forward labelled primer	Reverse primer
***Oc* -GT**	GTGTCTAATGGATGGGTTCG	GTTCGAACTCAACTCTGCTCGC
***Oc* -GA**	ACGATGATCGTCATGATCAAC	TGATCCGTGACTTTTTCTGG
***Oc* -CT7**	GGTCGATTTATGGAATGTGTCAC	GTTCCGCGATTTCTTTCACAGG
***Oc* -CT6**	CGATCTCACTTTATGCTACTTTGC	GTTACAACCTCACTTTGATCTATATGAAC
***Oc* -GA8**	GCTCCTTGCTACTCTCGTTTG	GTTGGGTTTCGGTCATAGATGCTTAG
***Oc* -TG7**	GGTGCACAAGAGTAAGTACTACAGTG	GTTCAGCCTGGTCATAGCTGC
***Oc* -TGA5**	GTCCTCTTGAAACCTTGAGTAAAG	GTTCCACATCCAAACACAACTAAATC

Amplification was performed in a multiplex PCR reaction that contained: 2.5 µl 10x Buffer, 1.25 U Taq polymerase, 1.6 mM dNTP, 0.2 µM primers (*Oc*-GT, *Oc*-GA, *Oc*-CT7, *Oc*-GA8, *Oc*-TG7), 0.3 µM primer *Oc*-TGA5, 0.4 µM primer *Oc*-CT6 and 1 µl template DNA, making a total reaction volume of 25 µl. The following temperature program was used: 95ºC for 5 min, followed by 30 cycles of 95ºC for 15 s, 55ºC for 30 s and 72ºC for 60 s, and a final step at 72ºC for 30 min. Amplification products were visualized on an automated sequencer ABI 3100 (Applied Biosystems, Carlsbad, CA, USA), using GeneScan 500 LIZ® Size Standard (Applied Biosystems) as a size marker. Alleles were scored with GeneMapper 3.7 software (Applied Biosystems).

AFLP amplification

AFLP amplification was performed with 10 µl of genomic DNA previously extracted as described above. The AFLP procedure was performed according to Vos *et al.* (1995) adjusted for the use of fluorescent labelled primers (Timmermans, 2005). The pre-amplification reaction was performed with primers with one selective base (EcoRI-A and MseI-C) and the final selective amplification PCR with the primer combination EcoRI-AC/ MseI-CGT. Amplification products were run in the ABI 3100 automated sequencer using GeneScan 500 LIZ® Size Standard as a size marker. The AFLP banding pattern was analysed using the software package GeneMapper 3.7. Presence was scored for bands with ≥ 100 fluorescence units and within the range of 100-500 bp (Vekemans *et al.*, 2002). All bins were visually checked and adjusted whenever necessary. This resulted in 114 scored loci.

PCR-RFLP

Individual springtails were genotyped for the *pmt* locus using five restriction enzymes (*Cla* I, *Ssp* I, *Tau* I, *Taq* I and *Mnl* I) according to Janssens *et al.* (2008). The PCR-RFLP (PCR-Restriction Fragment Length Polymorphism) products were visualized by 2% agarose gel electrophoresis and the restriction banding pattern was manually scored.

RNA/DNA extraction and RT-PCR analysis

Metallothionein expression of approximately twenty animals per population

(PLO, STO, ROG, Lab3, and subpopulations from Lab3 and Lab5) was quantified with quantitative RT-PCR (Real-Time PCR), according to Roelofs *et al.* (2006). RNA extraction was performed using the SV Total RNA Isolation System (Promega), with a slight change in order to permit DNA extraction of the same individual. Therefore, every animal was individually crushed in liquid nitrogen and 250 µl SV RNA Lysis Buffer was added. From this, 75 µl was transferred to another tube for DNA extraction, leaving 175 µl for RNA extraction. The remaining procedure was as described by Promega. At the end, RNA was diluted in 50 µl nuclease-free water. The 75 µl remaining volume was used to extract DNA as previously described. DNA was diluted in 50 µl nuclease-free water and used to genotype the *mt* promoter following the protocol described above. For cDNA synthesis and RT-PCR the protocol described by Roelofs *et al.* (2006) was applied. The Q-Gene module was used to determine mean normalized expression values (MNE) of three pseudo-replicate Ct (cycle threshold) values (Muller *et al.*, 2002). A maximum standard error (S.E.) between technical replicates of up to 20% was allowed and samples with higher S.E. were either discarded or re-amplified.

Statistical analysis

Population genetic analyses were performed with GENALEX 6.4 software (Peakall and Smouse, 2006). To determine population genetic diversity, the Shannon information index (I) and expected heterozygosity (He) were estimated; significant differences (in neutral markers) were tested with a Kruskal-Wallis test. An AMOVA (Analysis of Molecular Variance) was performed to quantify the variance within and among populations; significance was determined with a permutation test (999 permutations). Pairwise F_{ST} (F-statistics) and Φ_{PT} (PhiPT, analogous to F_{ST}; for AFLP loci) were estimated, via AMOVA, to assess genetic differentiation among populations. Deviations from Hardy-Weinberg equilibrium (microsatellites and *pmt* loci) were tested through the X^2 test. Differences in allele frequency among populations were tested with a G-test.

To determine the association between survival and genetic diversity, Pearson correlation analyses between LT25 values and diversity indices and Spearman correlation analyses between LT25 and *pmt* allelic frequencies were performed. Significant values were corrected with the Benjamini-Yekutieli false discovery rate

method (Narum, 2006). To represent the studied populations according to their survival time and genetic diversity a Principal Component Analysis (PCA) was performed.

Differences in constitutive and induced *mt* expression within and among populations were tested with a one-way ANOVA. Previously, data was fourth-root transformed to meet the assumptions of normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene´s test). A Newman-Keuls post-hoc test was performed to compare the means. Pearson correlation analyses were performed with survival, *mt* expression and genetic diversity. Significant values were corrected with Benjamini-Yekutieli false discovery rate method (Narum, 2006). One PCA analysis was run to distinguish the studied populations according to their phenotypes (increased survival and higher *mt* expression) and genetic diversity, and a second PCA analysis was made to distinguish the *pmt* genotypes (Lab3 subpopulations) according to the association between *mt* expression and *pmt* allelic frequencies. Analyses of variance and correlations were performed with STATISTICA 7 software (StatSoft, Tulsa, OK, USA). Principal Component Analyses were performed in Canoco for Windows 4.5 software (Ter Braak and Smilauer, 2002).

Results

Survival experiment

Reference populations reached 25% mortality (LT25) after 4 to 21 days; Lab3 population was the most sensitive one (Table 4.3). In contrast, LT25 was significantly prolonged in metal-contaminated populations, PLO and STO, up to 30 days. Comparison of the survival curves (Figure 4.1) revealed significant differences ($p < 0.05$) among all populations except between the two tolerant ones, PLO and STO.

Table 4.3 LT25 values (days) with corresponding standard error (S.E.) and 95% confidence intervals (C.I) for the survival of different *Orchesella cincta* populations exposed to 400 µg Cd/g dry algae, estimated with a logistic model. PLO and STO are tolerant populations, ROG and Lab1 to Lab5 are reference populations.

	LT25	S.E.	C.I.
PLO	30.4	1.80	26.8 - 34.1
STO	30.1	1.50	27.0 - 33.2
ROG	20.9	1.10	18.6 - 23.2
Lab1	9.03	0.71	7.38 - 10.7
Lab2	7.27	0.34	6.48 - 8.06
Lab3	4.43	0.66	2.86 - 6.00
Lab5	11.2	0.49	10.1 - 12.3

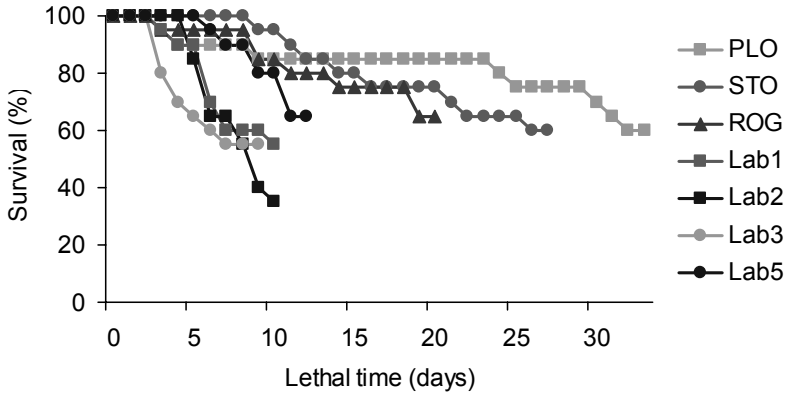

Figure 4.1 Survival along time (days) of different *Orchesella cincta* populations exposed to 400 µg Cd/g dry algae. PLO and STO are tolerant populations, ROG and Lab1 to Lab5 are reference populations.

Population genetic analysis

Population genetic diversity estimates from microsatellites, AFLP and *pmt* loci are presented in Table 4.4. Genetic diversity indices estimated from variation in microsatellite loci and the *pmt* locus where higher in tolerant populations (PLO and STO). With AFLP all populations showed a similar diversity. No significant differences were detected on neutral loci diversity.

Table 4.4 Genetic diversity estimated from microsatellites, AFLP and *pmt* loci of different tolerant (PLO, STO) and reference (ROG, Lab1 to Lab5) *Orchesella cincta* populations. Number of analysed animals (n), Shannon information index (I), expected heterozygosity (He) and *pmt* allele frequencies (A1 to F; in %).

	Microsatellites			**AFLP**			***pmt* locus**										
Populations	**n**	**I**	**He**	**n**	**I**	**He**	**n**	**I**	**He**	**A1**	**A2**	**B**	**C**	**D1**	**D2**	**E**	**F**
PLO	8	0.688	0.379	8	0.271	0.176	46	1.88	0.828	26.1	10.9	21.7	7.61	15.2	12.0	3.26	3.26
STO	8	0.632	0.374	8	0.253	0.165	41	1.61	0.782	30.5	17.1	6.10	20.7	22.0	3.66	0.000	0.000
ROG	8	0.527	0.314	8	0.243	0.159	51	1.45	0.718	44.1	15.7	0.000	6.86	21.6	0.000	0.980	10.8
Lab1	7	0.216	0.134	8	0.212	0.138	8	0.000	0.000	100	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Lab2	8	0.440	0.276	7	0.182	0.117	9	0.349	0.198	88.9	0.000	11.1	0.000	0.000	0.000	0.000	0.000
Lab3	8	0.477	0.296	8	0.296	0.194	40	0.920	0.543	61.3	0.000	25.0	0.000	13.8	0.000	0.000	0.000
Lab5	8	0.470	0.281	8	0.196	0.130	40	0.848	0.401	76.3	3.75	0.000	8.75	8.75	0.000	0.000	2.50

The G-test applied to *pmt* allele frequency data revealed significant differences among all populations except when comparing Lab1 vs. Lab2 and Lab5. The AMOVA analyses revealed that 84% (microsatellites and AFLP) and 89% (*pmt*) of total genetic variance was explained by variation within populations, while variation among populations explained 16% (microsatellites and AFLP) and 11% (*pmt*). Variation among populations was significant (F_{ST} microsatellites = 0.157; F_{ST} *pmt* = 0.114; Φ_{PT} AFLP = 0.163; $p < 0.05$). Pairwise population F_{ST} values (Table 4.5) revealed the existence of genetic differentiation among all populations, considering at least two genetic markers.

Significant departures from Hardy-Weinberg (H-W) equilibrium were detected in STO (GT and GA8 microsatellite loci; the observed heterozygosity was lower than expected). No deviation from H-W equilibrium was detected in the *pmt* locus.

Table 4.5 F-statistics pairwise comparisons (F_{ST} - microsatellites and *pmt*; Φ_{PT} - AFLP) between all *Orchesella cincta* populations. PLO and STO are tolerant populations, ROG and Lab1 to Lab5 are reference populations.

Pairwise comparisons	Microsatellites F_{ST}	AFLP Φ_{PT}	*pmt* F_{ST}
PLO vs STO	0.076*	0.020	0.025*
PLO vs ROG	0.071*	0.100*	0.055*
PLO vs Lab1	0.214*	0.128*	0.294*
PLO vs Lab2	0.106*	0.214*	0.219*
PLO vs Lab3	0.052	0.089*	0.092*
PLO vs Lab5	0.048	0.210*	0.195*
STO vs ROG	0.094*	0.053*	0.025*
STO vs Lab1	0.248*	0.114*	0.295*
STO vs Lab2	0.209*	0.211*	0.233*
STO vs Lab3	0.153*	0.069*	0.126*
STO vs Lab5	0.094*	0.213*	0.172*
ROG vs Lab1	0.146*	0.185*	0.219*
ROG vs Lab2	0.264*	0.289*	0.165*
ROG vs Lab3	0.197*	0.131*	0.088*
ROG vs Lab5	0.105*	0.247*	0.099*
Lab1 vs Lab2	0.365*	0.262*	0.050
Lab1 vs Lab3	0.325*	0.096*	0.175*
Lab1 vs Lab5	0.161*	0.145*	0.066*
Lab2 vs Lab3	0.053	0.205*	0.077*
Lab2 vs Lab5	0.208*	0.306*	0.027
Lab3 vs Lab5	0.177*	0.183*	0.082*

* $p < 0.05$

Survival and genetic diversity

A positive significant correlation ($p < 0.02$; $r = 0.847$) was found between LT25 and the Shannon information index (I) of genetic variation in *pmt*, after false discovery correction. Also, significant correlations ($p < 0.05$) were found between LT25 values and the frequency of *pmt* alleles A2 ($r = 0.852$), C ($r = 0.778$) and D2 ($r = 0.802$). However, only the correlation with A2 remained significant ($p < 0.018$), when the Benjamini-Yekutieli correction was applied. In the PCA, the first two axes explained 78.6% of total variation (Figure 4.2). Axis 1 explained 60.4% and clearly separated field populations from lab populations. The field populations were associated with higher genetic diversity and increased survival time. Survival (LT25) appeared to be more associated with *pmt* diversity and specifically with the frequency of A2, C and D1 alleles. We can also observe that D2 frequency was more related with PLO population.

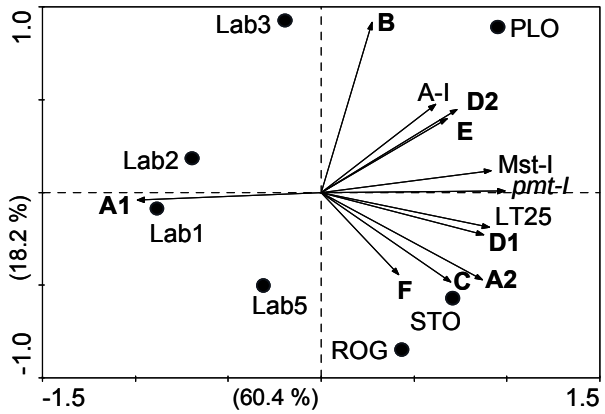

Figure 4.2 Principal Component Analysis (PCA) biplot based on the LT25 values and genetic diversity of all *Orchesella cincta* populations under study. PLO and STO are tolerant populations, ROG and Lab1 to Lab5 are reference populations. (A-I: AFLP Shannon information index; Mst-I: microsatellites Shannon information index; *pmt*-I: metallothionein promoter Shannon information index; A1, A2, B, C, D1, D2, E, F: metallothionein promoter alleles).

Metallothionein expression

Tolerant and reference populations

We compared *mt* expression in the two tolerant populations (PLO and STO) with *mt* expression in reference populations derived from the field (ROG) as well as derived from the lab (Lab3). Constitutive and induced *mt* expressions were significantly different ($p < 0.05$) for the PLO and ROG populations. When comparing *mt* expression among populations, a significant difference ($p < 0.05$) in constitutive expression, between tolerant (PLO and STO) and reference populations (ROG and Lab3), was found. No differences were obtained when comparing induced expression (Figure 4.3).

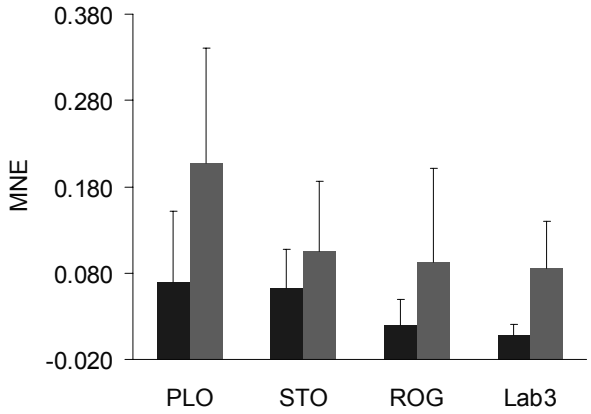

Figure 4.3 Mean normalized expression (MNE) of *Orchesella cincta mt* gene with standard deviation, of PLO and STO (tolerant populations) and ROG and Lab3 (reference populations). Constitutive expression – dark grey bars; Cd-induced expression – light grey bars.

When survival was compared to *mt* transcriptional activity no significant correlation between LT25 and *mt* expression was found. Nevertheless, *mt* constitutive expression was positively correlated ($p < 0.05$) with neutral marker diversity (microsatellites: expected heterozygosity, $r = 0.998$ and Shannon

information index, r = 0.989), as was shown for survival data. This correlation remained significant after the Benjamini-Yekutieli correction (p < 0.015). Furthermore, a positive significant correlation (r = 0.992) was found when comparing *mt* induced expression and D2 allele frequency, whereas a negative significant correlation (r = -0.958) was observed between *mt* constitutive expression and A1 allele frequency. However, after p value correction only the correlation between *pmt* induced expression and D2 frequency remained significant (p < 0.015). The first two PCA axes explained 85.7% of total variation (Figure 4.4). Axis 1 explained 58.9% and clearly separated Lab3, with higher AFLP diversity but less diversity concerning the remaining markers, from the other populations. Constitutive *mt* expression and LT25 were associated with microsatellite and *pmt* loci diversity. Induced *mt* expression was linked to the frequency of the D2 allele.

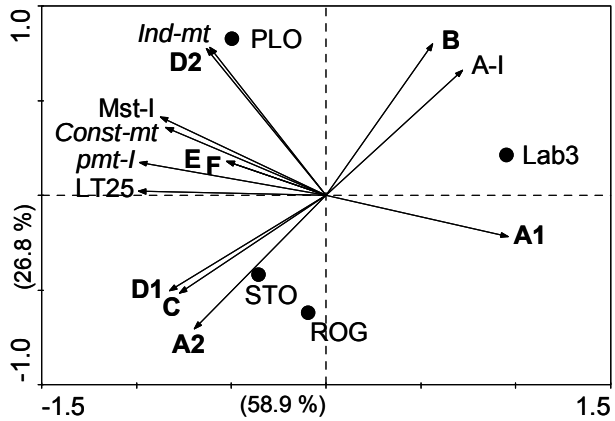

Figure 4.4 PCA biplot based on LT25 values, *mt* gene expression and genetic diversity for tolerant (PLO and STO) and reference (ROG and Lab3) *Orchesella cincta* populations. (Const-*mt*: constitutive *mt* expression; Ind-*mt*: Cd-induced *mt* expression; A-I: AFLP Shannon information index; Mst-I: microsatellites Shannon information index; *pmt*-I: metallothionein promoter Shannon information index; A1, A2, B, C, D1, D2, E, F: metallothionein promoter alleles).

Lab subpopulations

In order to have more insight about the influence of genetic background on transcriptional activity of specific *pmt* alleles we studied *mt* expression in subpopulations from Lab3 and Lab5. Six specific genotypes were retrieved from Lab3 (A1/A1, A1/D1, A1/B, B/B, D1/D1 and B/D1) and one genotype (A1/A1) from Lab5. The comparison of expression of the identical *pmt* genotype A1/A1 in different genetic backgrounds (Lab3 and Lab5) allowed us to study the influence of *trans*-regulation. To specifically address the contribution of *cis*-regulation to *mt* transcriptional activity, *mt* expression of individuals with different genotypes (A1/A1, A1/D1, A1/B, B/B, D1/D1 and B/D1) in an identical genetic background (Lab3) were studied. Constitutive and induced *mt* expressions were significantly different ($p < 0.05$) within all subpopulations except for the Lab3 B/B. When comparing *mt* expression between A1/A1 genotypes from the two populations (Lab3 and Lab5), no significant differences were found, either in constitutive or in induced expression of *mt* (Figure 4.5).

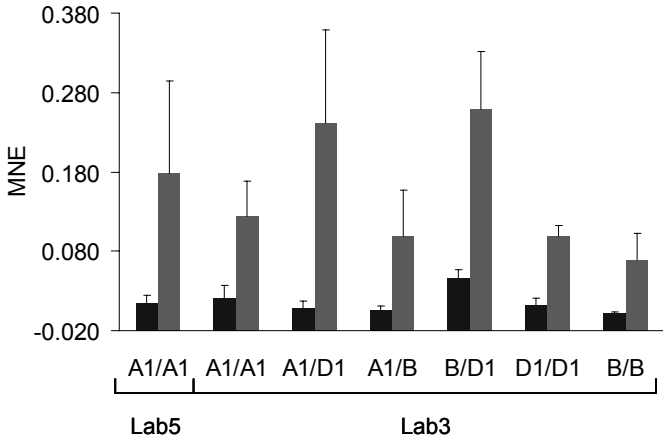

Figure 4.5 Mean normalized expression (MNE) of *Orchesella cincta mt* gene with standard deviation, of specific genotypes from Lab3 and Lab5 populations (reference populations). Constitutive expression – dark grey bars; Cd-induced expression – light grey bars. (A1/A1, A1/D1, A1/B, B/D1, D1/D1, B/B: metallothionein promoter genotypes).

To compare the different genotypes from population Lab3, two separate analyses were made (group 1: A1/A1, A1/D1, A1/B and group 2: B/B, D1/D1, B/D1) due to the different number of replicates in each group. In group 1 A1/A1 constitutive *mt* expression was significantly different from A1/B. Genotype A1/D1 showed significantly differential Cd-induced *mt* transcription when compared to the two remaining genotypes (A1/A1 and A1/B). In Group 2 B/D1 genotypes showed differential constitutive as well as Cd-induced *mt* transcription when compared to B/B and D1/D1 genotypes (Figure 4.5). The first two PCA axes explained 72.4% of the total variation (Figure 4.6). Axis 1 clearly separated genotypes with the D1 allele from those with A1 and B alleles. Apparently, the D1 allele seemed to be more associated with both constitutive and induced *mt* expression.

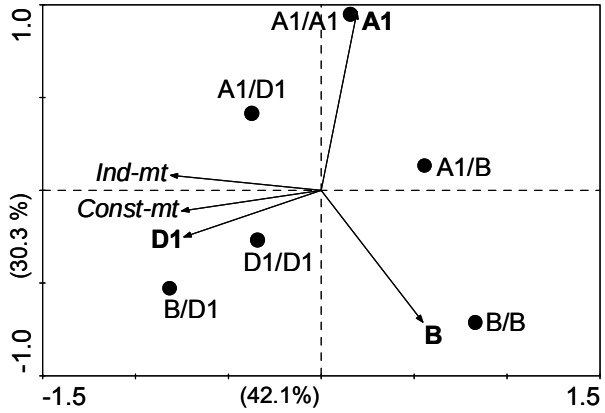

Figure 4.6 PCA biplot based on *pmt* genotypes and *mt* gene expression of *Orchesella cincta* Lab3 subpopulations (Const-*mt*: constitutive *mt* expression; Ind-*mt*: Cd-induced *mt* expression; A1, B, D1: metallothionein promoter alleles; A1/A1, A1/B, A1/D1, B/B, D1/D1, B/D1: metallothionein promoter genotypes).

Discussion

Despite our expectation that selection against sensitive genotypes may cause a decrease in genetic variation (Van Straalen and Timmermans, 2002) no evidence of a toxicant-related decrease in genetic diversity of *O. cincta* populations from

Plombières and Stolberg was observed. Also, Frati *et al.* (1992) and Timmermans (2005) analysed genetic diversity, both in neutral and selectable markers, of springtails from the same sites and did not observe a metal-driven decrease in genetic variation. The capacity of neutral markers to detect contamination-induced environmental changes appears to be limited, due to the fact that a decrease in genetic variation will only occur when population size drops severely and gene flow is restricted (Hoffmann and Willi, 2008). In *O. cincta* a decline of genetic variability is prevented by the very large effective population size, since this springtail is regularly found in densities of more than several thousand individuals per squared meter (Van Straalen *et al.*, 1989) and by high gene flow between populations over a distance of several km (Timmermans, 2005). Other studies have also found no evidence of decreased genetic diversity caused by toxicant exposure, as for example, Berckmoes *et al.* (2005) using microsatellites to measure genetic diversity of wood mice (*Apodemus sylvaticus)* exposed to metal contamination and Theodorakis *et al.* (2001) assessing genetic diversity of kangaroo rats (*Dipodomys merriami*) exposed to radionuclide contamination. These authors also suggested that gene flow could mask the effects of toxicant exposure. In the present study, tolerant populations generally revealed a slightly higher genetic diversity both in neutral and in selectable markers. Also, Theodorakis *et al.* (2006) found higher genetic diversity in populations of redbreast sunfish (*Lepomis auritus*) exposed to pulp mill effluent. They suggested that increased mutation rate and gene flow caused this increase. In the present study, mutagenic properties of Cd (Bertin and Averbeck, 2006) can be a source of the increased genetic diversity observed in Plombières and Stolberg populations. An increased genetic diversity in microsatellites might be caused by mutagenic contaminants that can enhance the already high mutation rates associated with this kind of neutral markers (Van Straalen and Timmermans, 2002). Increased mutation rates were observed, for example, by Ellegren *et al.* (1997) who analysed microsatellite loci in barn swallows (*Hirundo rustica*) from Chernobyl. However, it should be mentioned that the comparison of the genetic diversity among field populations is limited due to the existence of only one reference population.

It is still unknown which molecular entities are targeted by the selective pressure of environmental Cd contamination. The cadmium tolerance mechanism

in *O. cincta* is assumed to act mainly through altered *mt* expression (Sterenborg and Roelofs, 2003). As expected, *mt* gene expression was induced upon cadmium exposure in all populations. Like before, a higher basal *mt* expression was observed for tolerant (PLO and STO) compared to reference populations (ROG and Lab3). Timmermans *et al.* (2005a) suggested that constitutive *mt* expression acts in primary cadmium protection by preventing cellular damage and increasing fitness of individuals that encounter cadmium regularly in their habitats. Despite not being significantly different, induced expression in Plombières was higher than in the other tolerant population, Stolberg. This is in agreement with Sterenborg (2003) who found higher cadmium concentrations in protein homogenates of animals from Plombières than from Stolberg, suggesting that tolerance mechanisms, other than changes in the metallothionein gene, may be involved. A recent microarray study (Roelofs *et al.*, 2009) showed that the molecular mechanism of cadmium-induced metal tolerance is more complex than initially proposed. Differential transcriptional regulation of many additional genes was confirmed in *O. cincta* populations from Roggebotzand and Plombières (Roelofs *et al.*, 2007, 2009). In addition to metallothionein, genes involved in the stress-activated protein kinase pathway (SAPK) were shown to be altered in transcriptional activity in tolerant animals, suggesting that this signalling pathway is an important modulator of metal detoxification and target of stress-adapted evolution.

Our data do not support a strong link between survival time and *mt* expression, suggesting that Cd selection pressure may not directly target *mt* transcription. Nevertheless, both phenotypes (increased survival and higher constitutive *mt* expression) were associated with higher population genetic diversity. Also, induced *mt* expression and LT25 were specifically correlated with the frequency of the *pmt* D2 allele, confirming that this allele contributes to survival by enhancing cadmium-induced *mt* expression, as suggested earlier by Janssens *et al.* (2008). Constitutive *mt* expression was found to be negatively correlated with the frequency of the A1 allele. This could be explained by a replacement of the (wild type) A1 allele in tolerant populations, where constitutive *mt* expression is higher (Janssens *et al.*, 2008).

Cis/trans regulation

At the transcriptional level adaptation can be either *cis*- or *trans*-regulated, depending on whether is caused by polymorphisms in binding site structure for transcriptional factors, or in the structure or amount of these factors (Janssens *et al.*, 2009). The use of reference lab populations, with reduced number of *pmt* alleles, allowed us to tackle this question. No differences in *mt* expression in individuals with identical genotypes but different genetic background were found. This suggests a minor influence of *trans* factors (Wittkopp *et al.*, 2004). In contrast, differences in animals from a highly similar genetic background but having different *mt* genotypes were revealed. These results indicate that metallothionein gene expression is mostly influenced by its *cis*-regulatory promoter genotype. Other studies have reported a *cis*-regulatory effect on tolerance evolution, as in insecticide resistance of *Drosophila melanogaster* (Daborn *et al.*, 2002) and in metal hyperaccumulation capacity of *Arabidopsis halleri* (Hanikenne *et al.*, 2008). Janssens *et al.* (2007) showed that D1 luciferase *pmt* constructs had a higher basal expression when compared with A1. In the present study, a higher constitutive *mt* expression was more associated with *pmt* genotypes that have the D1 allele than to those with A1. Janssens *et al.* (2007) also showed that the D2 allele conferred high expression of metallothionein in a cell assay, which is in accordance with our observation that *O. cincta* carrying D2 had a higher induced *mt* expression. Our data confirm that high *mt* expression in tolerant populations involves specific private genotypes, including the D2 allele (Janssens *et al.*, 2008). However, it is puzzling why tolerant populations are not fixed for the D2 allele. Despite the obvious importance of *cis*-regulation in metal tolerance, we cannot exclude additional *trans*-regulatory effects. Janssens (2008) and Van Straalen *et al.* (2011) suggested that combined *cis/trans*-regulatory mechanisms would take place, particularly in tolerant populations, where *cis*-regulation would be more related with induced *mt* expression and *trans*-regulation more associated with constitutive *mt* expression. All populations, whatever the *pmt* alleles/genotypes present, were able to increase *mt* expression when exposed to cadmium. Thus, the lower survival observed in reference populations may be caused by the inability to maintain high induced *mt* expression, possibly because of associated energy costs or because energy allocated to other important functions was reduced (Brulle *et al.*,

2007).

To conclude this study showed that tolerance, evolved through adaptive phenotypes, does not involve a decrease of genetic diversity in natural populations. This suggests that tolerant populations still have the capacity to adapt to future disturbances. Metal-tolerant phenotypes seem to be shaped by *cis*-regulatory evolution of the metallothionein promoter. However, more studies should be performed to clearly unravel the influence of *cis/trans*-regulatory action on tolerant populations. Currently we are inbreeding tolerant as well as sensitive populations. Quantitative Trait Loci mapping of F2 strains from a tolerant x sensitive cross will reveal genome regions associated with the Cd-tolerant phenotype and as such shed more light on the intriguing process of stress-adaptive evolution.

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Chapter 5

Discussion

Soil-dwelling invertebrates, such as isopods and collembolans may be directly affected by metal contamination, since soil is a major sink of metals (Martin and Coughtrey, 1981). These animals may cope with metal contamination by avoiding or limiting exposure. Isopods from less contaminated areas showed a quicker avoidance response when compared with animals from a highly contaminated site (Chapter 3). They may also develop tolerance, namely by improving survival despite metal exposure. Both isopods and collembolans from highly contaminated areas showed longer survival time, when compared to reference populations (Chapter 3 and 4).

Metal contamination can also affect soil-living populations at the genetic level. Population genetic structure results from an interplay between evolutionary processes, gene flow, genetic drift, mutation and selection on the one hand, and the historical genetic background on the other hand. In many cases, genetic structure of soil invertebrate populations still reflect the historical events that have occurred at large geographical scales, such as glacial periods and geological rearrangements (Chapter 2). Environmental stressors, e.g. metal contamination, may affect the evolutionary processes, changing the genetic structure. Metal contamination, acting as a selective pressure, might have contributed to isopod population genetic differentiation in Mina de São Domingos area (Chapter 3). Also, the influence of genetic drift should be considered. In fact, metal contamination appears to have only a limited influence on population genetic structure. The genetic differentiation observed in several studies may be the result of other not metal-related processes (Chapter 2). A metal-driven decrease of genetic diversity was observed neither in isopods nor in collembolans (Chapter 3 and 4). The capacity of neutral markers to detect pollution-induced environmental changes is limited to population size reduction and gene flow restriction circumstances (Hoffmann and Willi, 2008), and thus the lack of decreased genetic variation may have been caused by a high population size and by the existence of a sufficient gene flow, particularly in the case of *O. cincta* (Chapter 4). In general, soil collembolans and isopods are considered to have limited dispersal capacities. However, the existence of both short and long distance migration through passive dispersal has been considered an important factor in the maintenance of genetic homogeneity among populations (Chapter 2). No relationship between increased

survival (tolerance) and genetic diversity of neutral markers was observed (Chapter 3 and 4), but when considering genetic diversity of a selectable locus (*O. cincta pmt*) a positive correlation was found (Chapter 4). As with neutral markers, no metal-related decreased of genetic diversity was observed also at this selectable locus (Chapter 4). The increased frequency of a high cadmium-inducible promoter allele (D2) and the replacement of the wild type allele (A1) in metal-tolerant populations (Chapter 4; Janssens *et al.*, 2008) were not sufficient to significantly affect *pmt* variation. Environmental contamination affects variation of selectable loci mainly if alleles at such loci have a large effect on the phenotype. Therefore, despite the contribution of the D2 *pmt* allele to survival by enhancing cadmium-induced *mt* expression this allele was not fixed in the tolerant populations (Chapter 4; Janssens *et al.*, 2008), suggesting that the tolerant phenotype is additionally influenced by other factors acting in *trans*, mainly in the regulation of constitutive *mt* expression (Janssens, 2008; Van Straalen *et al.*, 2011). Furthermore, also genes involved in the stress-activated protein kinase pathway (SAPK) were shown to be altered in transcriptional activity in tolerant animals (Roelofs *et al.*, 2007, 2009), indicating that the tolerant phenotype is not only determined by the regulation of the metallothionein gene. The tolerant phenotype is the result of the action of several genes in a complicated molecular network (Van Straalen *et al.*, 2011). Each of these genes on its own might have only a small effect on the phenotype; obvious tolerance is expressed only by the additive action of many genes. Therefore, the alleles at a selectable locus, such as *pmt* will possibly have a small but fundamental effect on the phenotype.

As previously mentioned (Chapter 1), the study of metal tolerance is of evolutionary and ecological importance. From an evolutionary point of view, no metal-related decrease of genetic diversity was observed, suggesting that populations inhabiting metal-contaminated sites may keep their capacity to adapt to novel environmental stressors. However, this conclusion is just applied to the species analysed, since different results may be obtained when considering several species from a metal-contaminated area (e.g. Ross *et al.*, 2002). Therefore, when studying the effects of metal contamination on an ecosystem, several species should be considered.

From an ecological point of view, increased survival (tolerance) was observed both in isopods and collembolans. Whether the observed tolerance was achieved through acclimation of individuals during long-term field exposure or adaptation through selection of tolerant populations in areas with a long history of pollution, it may influence the results obtained when performing ecological risk assessment (Eckwert and Kohler, 1997). If acceptable toxic environmental concentrations are derived from bioassays performed with tolerant populations, protection criteria may be biased (Reinecke *et al.*, 1999). Therefore, the existence of metal-tolerant populations should be considered when performing ecological risk assessment studies.

It is worth mentioning that most studies aiming to determine metal tolerance are performed using artificial substrates and/or food contaminated with a single metal. Despite the importance of such tests to assess the capacity of organisms to develop metal tolerance, and to isolate the effects of a particular toxic substance, this approach does not reflect the actual field situation. In the soil mixtures of metals in several chemical forms are usually found. These metals may compete for the same uptake route causing either antagonistic or synergistic effects (Peijnenburg, 2002). Also, the several chemical forms (others than those used for soil and/or food contamination) of a specific metal are known to have different toxicities (Peijnenburg, 2002; Calhôa *et al.*, in press). Moreover, experimental conditions such as uptake route (soil or food), selected contaminant, concentrations used and exposure time may lead to different results, when assessing metal tolerance. Therefore, future studies should, as much as possible, approach the *in situ* conditions.

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