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COPPER TOLERANCE AND GENETIC DIVERSITY OF *PORCELLIONIDES SEXFASCIATUS* (ISOPODA) IN A HIGHLY CONTAMINATED MINE HABITAT

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Abstract—Organisms inhabiting metal-contaminated areas may develop metal tolerance, with either phenotypic adjustments or genetic changes (adaptation) or with both. In the present 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 sublethal levels of copper, through comparison of survival, avoidance, and feeding. The effects of metal contamination on genetic diversity were considered using random amplified polymorphic DNA (RAPD) markers. No evidence of increased metal tolerance of the population inhabiting the contaminated site was found. There was no correlation between metal exposure and within-population genetic variance, but the three populations were clearly separated from each other. In conclusion, the populations of *P. sexfasciatus* in the mine landscape live rather isolated from each other and show no differential tolerance to Cu or indications of genetic erosion. Their phenotypic plasticity provides a means to survive despite exposure to extremely high metal concentrations in the soil. Environ. Toxicol. Chem. 2013;32:884–888. © 2013 SETAC

Keywords—Isopods Metal contamination Tolerance Population genetics

INTRODUCTION

Mining practices mobilize several metals such as Cd, Pb, Cu, and Zn into the environment, where they act as potential toxic threats, impairing reproduction, growth, and survival of exposed organisms [1]. Individuals inhabiting metal-contaminated areas may actively avoid or limit the exposure to toxicants [2,3]. If they fail to avoid exposure, they may develop metal tolerance. The occurrence of metal-tolerant field populations, as a result of contaminant exposure, has been reported for various organisms, including aquatic species [4], plants [5], and soil invertebrates such as earthworms [6], collembolans [7], and isopods [8]. Populations may develop tolerance phenotypically (acclimation, maternal effects, and phenotypic plasticity) or genetically (adaptation), or both. Through adaptation, the most sensitive genotypes are eliminated, and this may cause a decrease of population genetic diversity [9]. Elimination of sensitive genotypes may occur due to reduced survival, diminished reproduction capacities, and also through migration out of the contaminated area (avoidance).

Soil-dwelling invertebrates may be directly affected by metal contamination because soil is a major sink for metals [10]. 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 [11]. Any change in their feeding rates affects the decomposition process and consequently organic matter and energy cycling through ecosystems [11]. Thus, food consumption is a relevant endpoint for studying the ecological effects of contaminants in the ecological functions of isopods. Essential metals such as Cu may have deleterious effects when present in high concentrations. In terrestrial isopods, Cu is known to be essential for respiration and immune responses and to promote digestive processes; however, at high concentrations it can decrease survival and reproductive success [12].

The present study sought to verify whether, as expected, a historically exposed population of a soil-living organism was more tolerant to lethal and sublethal levels of Cu than reference populations through the comparison of survival, avoidance, and feeding in laboratory exposures of the isopod *Porcellionides sexfasciatus*. Furthermore, the hypothesis of a reduction in genetic diversity resulting from metal exposure was tested by using neutral markers. Copper was chosen because previous chemical analysis revealed that it is present in elevated amounts (over one order of magnitude) in a historically contaminated area relatively to other nearby sites. Genetic diversity was estimated by random amplified polymorphic DNA (RAPD) [13]; RAPD markers have been used in several ecotoxicological studies [14,15] to assess the effects of anthropogenic contaminants on population genetic diversity. This method does not require previous DNA sequence information and so is adequate when few genetic data are available, such as for the terrestrial isopod *P. sexfasciatus*. Metal tolerance at the study area has already been found in plants [16] and aquatic invertebrates [4]; however, until now, no metal-tolerance studies have been performed with soil invertebrates.

MATERIALS AND METHODS

Study site

The present study was conducted at an abandoned cupric-pyrite mine, Mina de São Domingos, located in southeastern Portugal (37°40′N, 7°29′W). This region is part of the Iberian pyrite belt (IPB), which has an extension of 250 km length and 30 to 60 km width (an area of 12,500 km^2). It comprises the regions of Alentejo, Portugal, and Andalusia, Spain [17]. Along

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with massive amounts of pyrite are deposits of Mn and Fe and veins of Cu, Sb, Pb, and Ba [18]. Natural vegetation in the study area is dominated by *Quercus ilex* and *Eucalyptus* spp. trees and by *Lavandula stoechas* and *Genista hirsuta* shrubs [16]. Three sampling sites were defined in the mine area: one contaminated site, Santana de Cambas (37°37′56″N, 7°31′06″W), and two references, Tronco (37°40′55″N, 7°30′54″W) and Corte do Pinto (37°42′10″N, 7°27′31″W; Fig. 1). The study sites have notably high metal concentrations both in soil and in litter because of their location in the IPB area. Soil is naturally metal enriched across the whole mine area [17,19]. Sites closer to the open pit have metal concentrations above background levels, from contamination occurring during treatment and transport of the metal ore and also from wind deposition (e.g., Santana de Cambas). Soil and litter samples were collected and total metal concentrations measured after aqua regia digestion, according to Natal-da-Luz et al. [20].

Model organism and sampling

The present study was performed with *Porcellionides sexfasciatus* (Koch), the only species common to all three sampling sites. Animals were collected by hand in spring and taken to the laboratory where they were kept in soil from their sampling site at 20 ± 2°C with a photoperiod of 16 h light:8 h dark.

Ecotoxicity tests

One lethal and two sublethal (avoidance and feeding) ecotoxicity tests were performed with copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$ from Fluka, 99% purity), mixed into soil or leaves. Preliminary tests were made to establish the Cu concentrations to be used in these experiments.

For the survival experiment, approximately 100 animals from each site were placed individually in test containers with contaminated Organisation for Economic Co-operation and Development (OECD) soil (5% organic matter) [21] and were frequently observed (every hour during the first 12 h, and then frequency was gradually reduced along the test). The nominal Cu concentration used was 10 mg Cu/g soil dry weight. The test was performed until all animals had died. Isopods were preserved in ethanol for later DNA extraction.

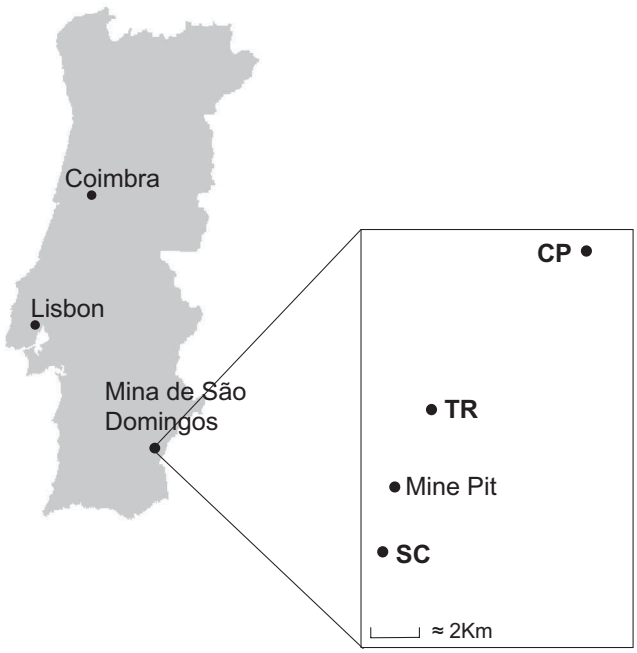

Fig. 1. Schematic representation of the sampling sites position. CP = Corte do Pinto; TR = Tronco; SC = Santana de Cambas.

Approximately 30 animals per site were used to test the avoidance response. The dual avoidance tests were performed using a two-chamber test box [3,22]. Isopods were individually exposed to a clean and a contaminated (nominal concentration of 100 μg Cu/g soil dry wt) OECD soil (5% organic matter) for 24 h; the position, on clean or contaminated soil, of the animal was observed after 6 and 24 h.

For the feeding experiment, isopods were individually placed in test containers with a bottom of plaster of Paris. Alder leaves (*Alnus glutinosa*) contaminated with 500 μg Cu/g leaf dry weight (nominal concentration) were used to measure weight increase, food consumption, and food assimilation efficiency over a 28-d exposure [8] of 15 isopods from each population. Leaves were cut into small discs (≈12 mm), and dry weight was recorded. Leaf discs were contaminated with the Cu solution and dried overnight at room temperature. Dry, contaminated leaves were weighed, rehydrated, and given to the isopods. Every week, fecal 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

The DNA was extracted from isopod muscles, gonads, and nervous tissue, according to Kocher et al. [23]. The RAPD amplification [13] was performed with two primers (R2: 5′-TGCCGAGCTG-3′ and R12: 5′-TCGGCGATAG-3′), in two mixes containing 4 μl Taq buffer, 1 U Taq, 0.01 nmol R2 or R12 and dNTPs (2.15 mM each). Amplification was performed with an initial step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 1.5 min, and a final step at 72°C for 5 min. Amplification products were visualized in a 2.5% agarose gel. Bands were scored semiautomatically (manual adjustments were made whenever necessary) with GelAnalyzer2010a software (available from www.gelanalyzer.com). To avoid unbiased estimates of heterozygosity, only markers for which the frequency of null alleles (band absence) was higher than $3/N$ (N = total sample size) were included in the analysis [24]. To ensure reproducibility of banding patterns, positive and negative control samples were included.

Data analysis

Copper survival data were fitted to a logistic model to estimate the median lethal time (LT50) according to the equation $survival = (maximum/[1 + (time/LT50) \times slope])$. Survival curves were compared using the likelihood ratio test. Avoidance behavior at 6 and 24 h was analyzed via a χ^2 test, considering that the numbers of isopods located on clean and contaminated soil were similar among the three populations. Results from the feeding experiment 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. Weekly food consumption was measured as the difference in the initial and final weights of the leaf discs; food assimilation was calculated as the food consumption minus the feces production. Results for the four weeks were summed, and total consumption and total food assimilation efficiency ($summed\ food\ assimilation/summed\ food\ consumption \times 100$) were determined. Feeding parameters (isopod wt increase, total food consumption, and total food assimilation efficiency) were

compared via one-way analysis of variance (ANOVA). Prior to analyses, 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, χ^2 tests, and ANOVA were performed in Statistica 7.0 software (StatSoft).

The RAPD markers were considered to be in Hardy – Weinberg equilibrium and were scored as presence (1) or absence (0). The DNA fingerprints were converted to a binary matrix that was used to assess population genetic diversity, through estimation of Shannon information index (I) and expected heterozygosity (He); significant differences were tested with the Kruskal – Wallis test. Total genetic variance was partitioned among and within populations with an analysis of molecular variance (AMOVA); significance was determined by using a permutation test (999 permutations). Pairwise Φ_{PT} values (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 [25]. Isolation by distance was tested by plotting pairwise $\Phi_{PT}/(1-\Phi_{PT})$ and Nei's genetic distances against ln-transformed geographic distance; significance was tested with Mantel tests (999 permutations). Furthermore, a principal component analysis (PCA) was performed to analyze genetic distance patterns more effectively. Population genetic analyses were performed with Genalex 6.4 software [26]. To confirm that the studied RAPD loci behaved as neutral markers, Ewens – Watterson test for neutrality was performed in Popgene 1.32 software [27].

To determine the association among LT50, genetic diversity, and soil and litter Cu concentrations, Pearson correlations were used. Normality was tested with the Kolmogorov – Smirnov test. Correlation analysis was performed in Statistica 7.0.

RESULTS

Soil and litter metal content

As expected, all selected areas presented considerably high metal concentrations, both in soil and in litter (Table 1). The highest metal concentrations, mainly Cu, Fe, Zn, Cd, and Pb, were found in the Santana de Cambas site.

Ecotoxicity tests

The highest and the lowest LT50 values were found at the least contaminated sites, Corte do Pinto and Tronco, respectively (Table 2). Survival curve (Fig. 2) comparisons revealed significant differences ($p < 0.05$) in LT50 values among all populations. Avoidance behavior was significantly different ($p < 0.05$) between Tronco and Santana de Cambas animals when considering the responses after 6 h. When considering just the response after 24 h, no differences were found among populations (Fig. 3). Most isopods from Tronco (75%) and Corte do Pinto (94%) had an increased weight after the 28-d exposure to contaminated food, but only 53% of Santana de Cambas animals gained weight. Significant differences ($p < 0.05$) were found between Corte do Pinto and Santana de Cambas isopods weight increase (Table 3). When 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).

Table 1. Soil and litter metal concentrations (mg/kg) and pH (measured in H_2O) from all sampled areas

	Soil			Litter		
Metals	TR	CP	SC	TR	CP	SC
Cu	34	66	933	22	31	302
Fe	54,663	53,013	94,413	31,068	23,568	50,935
Mn	979	1,530	179	1,500	1,331	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	3,276	<45	<45	1,192
Co	54	58	50	29	34	37
Ni	50	59	28	39	34	33

TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

Table 2. Median lethal time (LT50) values (h) with corresponding standard error (SE) and 95% confidence intervals (CI) for the survival of different *Porcellionides sexfasciatus* populations exposed to 10 mg Cu/kg soil dry weight, estimated with a logistic model

Populations	LT50	SE	CI
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

TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

Population genetic analysis

In total, 57 loci were analyzed, of which 74% were polymorphic. The isopod population at the most contaminated site had the lowest values for the two indices of genetic diversity, but the differences from the two other sites were not significant ($p = 0.12$, Santana de Cambas: $I = 0.262 \pm 0.031$; $He = 0.162 \pm 0.022$, Tronco: $I = 0.345 \pm 0.032$; $He = 0.219 \pm 0.023$; and Corte do Pinto: $I = 0.276 \pm 0.033$; $He = 0.174 \pm 0.023$). The AMOVA

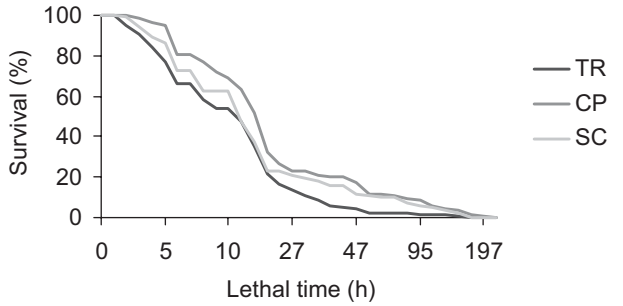

Fig. 2. Survival along time (h) of different *Porcellionides sexfasciatus* populations exposed to 10 mg Cu/kg soil dry weight. TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

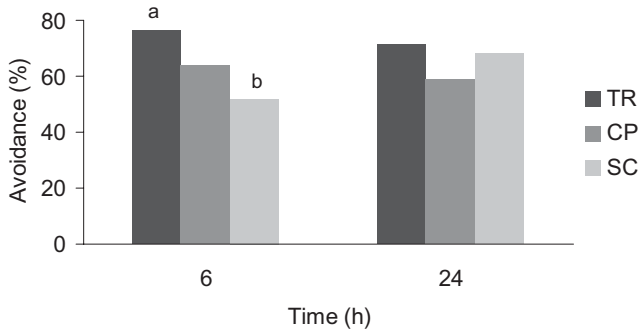

Fig. 3. Avoidance response (percentage of isopods that avoided the contaminated soil) of different *Porcellionides sexfasciatus* populations exposed to 100 μg Cu/g soil dry weight. Observations were made after 6 and 24 h exposure (a, b significant differences between populations). TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

Table 3. Feeding experiment parameters (mean ± standard deviation) of different *Porcellionides sexfasciatus* populations exposed to 500 μg Cu/g leaf dry weight

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

[a,b] Significant differences between populations.
WI = isopod wt increase [mg]; FCt = food consumption after 28 d [mg]; FAEt = food consumption assimilation efficiency [%]; TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

Table 4. Nei's genetic and geographic distances (km), Φ_{PT}, and Nm (number of migrants) among all *Porcellionides sexfasciatus* populations

Pairwise comparisons	Nei's genetic distance	Geographic distance	Φ_{PT}	Nm
TR vs CP	0.06	6.66	0.195[a]	1.03
TR vs SC	0.05	5.47	0.212[b]	0.929
CP vs SC	0.07	10.2	0.263[c]	0.701

[a,b,c] Significant differences between populations.
TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

results showed that 78% of total genetic variance was explained by variation within populations, whereas 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 4). Gene flow (Nm) varied between 1.03 and 0.701 (Table 4). Mantel tests showed no correlations between genetic and geographic distances. In the PCA plot, axis 1 (explaining 30.1% of total variance) separated the most polluted site (Santana de Cambas) from Corte do Pinto and to a lesser extent from Tronco. Axis 2 (explaining 22.9% of total variance) separated Corte do Pinto from Tronco populations (Fig. 4). 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%).

Survival and genetic diversity

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

DISCUSSION

As pointed out by Morgan et al. [28], "the presence of a population of reproducing individuals of a given species at a chronically polluted site does not justify the immediate inference that it is adapted". Our expectation was that the population inhabiting the contaminated site, Santana de Cambas, would present increased tolerance compared with the reference sites, Tronco and Corte do Pinto; however, no evidence of increased tolerance was observed. This result contrasts with results obtained at other historically contaminated sites. Donker and Bogert [8], studying the terrestrial isopod *Porcellio scaber* from a Zn smelter area and a Pb mine site, and Posthuma [7], with the collembolan *Orchesella cincta* from various contaminated areas, found increased metal tolerance. Also, Langdon et al. [6] found that the terrestrial oligochaetes *Lumbricus rubellus* and *Dendrodrilus rubidus* from abandoned mining areas were resistant to arsenate and Cu. This lack of increased tolerance of the Santana de Cambas population may be due to the capacity of isopods to accumulate and regulate large amounts of Cu [29,30], which may be a mechanism that allows them to cope with the existing Cu concentrations in this area. In addition, the selective pressure acting on the isopod populations in this abandoned mining area was probably insufficient for an increased tolerance. As stated by Postuma and Van Straalen [31], "similarity of populations may simply indicate that exposure was low in comparison with the species' ability to regulate or tolerate heavy metals".

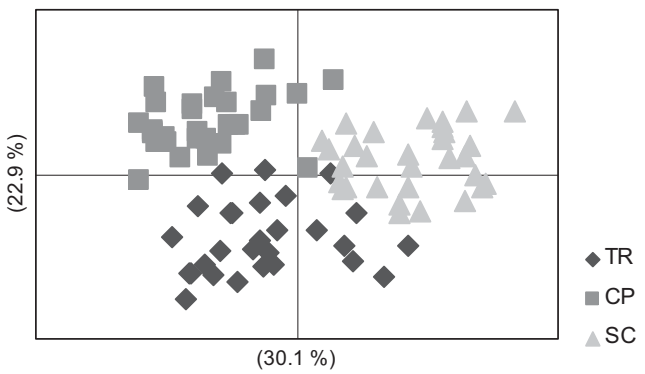

Fig. 4. Principal components analysis plot based on standardized genetic distance data for different *Porcellionides sexfasciatus* populations. TR = Tronco; CP = Corte do Pinto; SC = Santana de Cambas.

The reduced weight increase of the isopods inhabiting the contaminated site suggests the existence of physiological costs associated with metal contamination. Donker et al. [32,33] showed that isopods living in metal-contaminated sites had smaller bodies and increased reproductive allocation, indicating that energy is shifted towards reproduction.

No significant differences in genetic diversity using neutral markers were found among populations. This lack of reduced genetic diversity [9] was also observed in other studies using similar techniques. Martins et al. [34], studying *D. longispina* from the same abandoned mining area, did not find evidence for genetic erosion. Also, Timmermans [35], 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, because a decrease in genetic diversity will be detected only when population size is reduced and gene flow is restricted [36]. For instance, in a *D. longispina* case study, no decreased genetic variation was observed with AFLP loci [34]; although, when considering selectable traits, such as tolerance to lethal levels of Cu, genetic erosion was observed with the elimination of the most sensitive individuals from the contaminated populations [4,37].

According to Wright [38], populations in the present study showed moderate levels of genetic differentiation. Population differentiation may be caused by several factors, such as geographic isolation, habitat fragmentation, genetic drift, and local selective pressures, and may be counteracted by gene flow. The number of migrant individuals (Nm) between populations was low and, except between the two less contaminated populations, Tronco and Corte do Pinto, was less than 1 (it is theoretically considered that an Nm ≈ 1 is sufficient to maintain continuous gene flow among populations [25,39]). Isopods are considered to be inefficient active dispersers, because 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 or local selective pressures. It is generally considered that selection is a more powerful (and directional)

evolutionary force, because large populations are not very susceptible to genetic drift and tend to maintain their original degree of genetic variance [40]. Metal contamination may then be a sufficient selective pressure to justify population genetic differentiation. 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, which are slightly more distant. The population from the most contaminated site, Santana de Cambas, was genetically differentiated from the other two more than the other two were from each other, suggesting the existence of metal effects on population genetic structure.

CONCLUSIONS

The populations of the isopod *P. sexfasciatus* considered in the present study live rather isolated from each other. No evidence of increased Cu tolerance or indications of genetic erosion were found. Isopod phenotypic plasticity provides a means to survive despite exposure to extremely high metal concentrations in the soil. Additional studies, including more sampling sites and (potentially) more sensitive endpoints, such as reproduction, should be performed to confirm the subtle effects on population genetic structure of terrestrial isopods living in highly contaminated environments.

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