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**Geometric Morphometric and Genetic Diversity analyses
of two small mammal populations from heavy metal mines
in Portugal**

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À minha Avó

“Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.”

António Machado
“Proverbios y cantares” in *Campos de Castilla*, 1912

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Resumo

A indústria mineira tem tido ao longo dos últimos séculos um papel preponderante no desenvolvimento económico dos países. Contudo a sua intensa atividade tem deixado marcas profundas no ambiente tanto durante o seu período de exploração, como após o seu encerramento, sendo hoje em dia considerada uma das maiores fontes de poluição antropogénicas de metais. Durante os processos de britagem e moagem muitos materiais não são recuperados, sendo depositados em escombrelas. Estes locais sujeitos à ação dos elementos tornam-se fontes imprevisíveis de contaminação de água, solo, vegetação e atmosfera, representando sérios riscos ao nível biológico e ecológico. Esta exposição a metais, seja a metais pesados ou a elevadas concentrações de metais essenciais, tem efeitos tóxicos imediatos sobre os indivíduos ou a médio-longo prazo sobre as populações ou comunidades. Um dos efeitos deletério é a sua capacidade para aumentar a formação de espécies reativas de oxigénio induzindo *stress* oxidativo nos animais. Este efeito pode ter implicações nas células somáticas, podendo originar doenças degenerativas, processos carcinogénicos e mutagénicos (com possíveis consequências para a fitness das populações naturais) ou ao nível das células germinativas, prejudicando a geração seguinte. Em ambos os casos estas alterações poderão ter resultados demográficos na população. O *stress* ambiental causado pelos metais pesados pode afetar a composição das populações de maneiras distintas: através do aumento da taxa de mutação e/ou alteração da taxa de migração (*gene flow*) ou através de eventos de *bottleneck* (deriva genética) e/ou seleção de génotipos tolerantes (seleção natural). Esta alteração do padrão genético poderá ter consequências tanto ao nível fisiológico como morfológico. O uso de sentinelas é fundamental para este tipo de estudos toxicológicos, permitindo uma visão holística do impacto negativo que os metais têm no ambiente e na saúde humana. Devido à importância que têm na cadeia alimentar introduzem a componente espacial e temporal na análise, algo que não é possível através de uma análise química direta no ambiente. O uso de biomarcadores permite detetar e quantificar o efeito da exposição aos metais ao longo dos diferentes níveis de organização biológica.

Embora se saiba que os metais pesados têm efeitos negativos nos indivíduos, poucos estudos têm sido realizados em pequenos mamíferos a níveis de organização biológica superiores, ainda sendo limitado o conhecimento que o efeito crónico à exposição dos metais pesados pode ter nas populações. Neste contexto a presente tese pretendeu entender qual o impacto que os metais têm sobre a biodiversidade, através da análise das alterações ao nível morfológico e genético. Este tipo de estudos permite analisar de uma maneira indirecta o risco para a saúde humana.

No presente trabalho, foram estudadas duas espécies de pequenos mamíferos *Crocidura russula* (Hermann, 1780) e *Mus spretus* (Lataste, 1883), já anteriormente usados em estudos de biomonitorização. Estas duas espécies têm um tempo geracional muito curto, o que faz por exemplo, com que pressões ambientais possam gerar rápidas mudanças na história evolutiva destas espécies, fazendo delas bons biomonitores de estudos de ecotoxicologia evolutiva. Os animais do presente estudo foram capturados entre 2002 e 2003 em duas minas localizadas a sudeste de Portugal, a mina de Aljustrel e a mina da Preguiça, e numa zona de referência para fins comparativos (Moura). A mina de Aljustrel é uma mina que se localiza na Faixa Piritosa Ibérica. Esta operou intensamente entre 1867 a 1996 (ano em que cessa atividade), extraindo cobre, zinco, chumbo e prata. É uma área que apresenta uma profunda alteração e degradação da paisagem, reflexo dos longos anos de exploração. A mina da Preguiça é uma mina localizada na Zona da Ossa Morena, tendo estado ativa entre 1911 e 1964 e extraído essencialmente zinco e chumbo. Este local não aparenta grandes sinais de degradação ambiental tendo a vegetação invadido a mina e escondido escórias e resíduos existentes. Estudos anteriores realizados nestes locais mostraram que comparativamente com área de referência ambas as minas apresentaram elevadas concentrações de zinco, arsénio e chumbo nos solos, juntamente com zinco e chumbo nas

plantas. Alterações bioquímicas (níveis de metalotioninas, atividade enzimática antioxidante), histológicas (rins e fígado), fisiológicas (parâmetros morfológicos e hematológicos) e um aumento da frequência de micronúcleos foram observadas nestas mesmas minas em *Crocidura russula* e *Mus spretus*.

Partindo deste conhecimento, o principal objetivo da presente tese foi avaliar qual o efeito dos metais pesados a longo-médio prazo em duas populações de pequenos mamíferos que vivem em minas abandonadas, recorrendo a biomarcadores genéticos e análise de morfometria geométrica. A estrutura escolhida para a análise morfológica foi a mandíbula devido a ser uma estrutura amplamente usada em diversos estudos, nomeadamente em estudos toxicológicos. É uma estrutura composta só por um osso que apresenta uma estrutura plana, permitindo a aplicação de uma análise de morfometria geométrica em 2D. Esta análise foi realizada unicamente em *Mus spretus* devido ao limitado número de exemplares de *Crocidura russula* existentes na zona de referência. Um total de 124 indivíduos adultos foram analisados (mandíbula esquerda e direita), tendo sido todas as mandíbulas limpas através do método de água fervente. Posteriormente foram digitalizadas e colocado 19 *landmarks* sobre cada mandíbula usando software específico. Para analisar a forma os *landmarks* foram decompostos em *size* e *shape*, tendo sido a variação na *shape* analisada na mandíbula como um todo e em cada módulo funcional separado (ramo ascendente e a região alveolar). O tamanho da mandíbula foi obtido pelo *centroid size*. Para explorar as diferenças da *shape* e avaliar as distâncias morfológicas entre populações foi realizado uma análise da variante canónica para a componente simétrica e assimétrica. Por fim como biomarcador da instabilidade do desenvolvimento, foi calculado a assimetria flutuante para os três locais, tendo sido realizado um teste estatístico. As evidências para as mudanças genéticas populacionais nas duas espécies foram investigadas usando parâmetros de diversidade de dois marcadores mitocondriais sujeitos a pressões seletivas diferentes, o gene citocromo b (*Cyt b*) e a região controlo. O ADN genómico foi extraído de 63 *Crocidura russula* e 75 *Mus spretus*, tendo sido os fragmentos de ADN mitocondrial amplificados através de reações em cadeia da polimerase, utilizando *primers* específicos. Os produtos obtidos foram purificados e sequenciados. As sequências obtidas foram editadas, alinhadas e comparadas com as sequências existentes no GenBank. No caso de *Crocidura russula*, uma vez que o comprimento da região controlo é muito variável mesmo dentro de um indivíduo (heteroplasmia de tamanho), a região controlo foi aqui usada como “marcador genético neutro”. Como parâmetros de diversidade genética foram analisados: a diversidade haplóidica, nucleótídica, número de haplótipos, substituições sinónimas e não sinónimas, *variable sites* e *mismatch distribution*, enquanto a análise da estrutura populacional foi avaliada através da variação genética entre e dentro das populações por uma análise da variância molecular e pela relação entre haplótipos estabelecida pela rede de haplótipos.

Os resultados deste estudo confirmaram que para além de existirem alterações a nível individual, tanto as populações de *Crocidura russula* como *Mus spretus* nas duas minas estudadas estão a sofrer impactos negativos a outro nível de organização biológica. As análises morfológicas em *Mus spretus* revelaram diferenças entre as três populações e proximidade morfológica entre locais contaminados, embora se tenha verificado uma aproximação genética maior entre local de Referência e a Mina da Preguiça. Estas mudanças morfológicas sugerem que a baixa qualidade ambiental está a atuar de uma forma direta e indireta, através da disponibilidade de recursos entre os diferentes locais. Ao nível da assimetria flutuante não foram encontradas diferenças significativas entre as populações, o que pode estar relacionado com a sensibilidade da mandíbula a perturbações ambientais ou à sazonalidade da biodisponibilidade dos metais. Baseado no gene *Cytb*, ambas as espécies na mina de Aljustrel apresentaram altos valores de diversidade genética possivelmente devido ao *gene flow* das populações circundantes, atuando este lugar como um *ecological sink*. Os baixos valores registados em *Cytb* juntamente com os “marcadores genéticos neutros” da região controlo sugeriram que a população da

mina de Preguiça, possivelmente num passado recente sofreu um *bottleneck*, tendo sido recolonizada por indivíduos de populações próximas como a zona de referência. Esta diminuição populacional possivelmente deveu-se a um aumento da taxa mutacional nesta população, o qual é sugerido pelo aumento da diversidade genética na região controlo.

Os nossos resultados confirmam o impacto potencial que as minas abandonadas têm ao nível individual e ao nível das populações, permanecendo activos os efeitos nocivos da actividade mineira. Estes resultados reforçam a importância de planos de recuperação ou ações de remediação nas minas abandonadas, de modo a mitigar os efeitos adversos dos metais pesados. Uma vez que a resposta populacional nas duas espécies foi coincidente, conclui-se que as diferenças registadas são devido às diferentes características das minas, reforçando a importância das distintas variáveis que podem influenciar as respostas da população e que fazem os estudos de toxicologia evolutiva complexos. Por fim, este estudo corroborou a sustentabilidade das duas espécies usadas como bons biomonitores da qualidade ambiental, assim como o uso de biomarcadores genéticos e morfométricos para a identificação dos efeitos toxicológicos ao nível da população.

Palavras-chave: *Mus spretus*, *Crocidura russula*, Pequenos mamíferos, metais, biomarcadores

Abstract

Portugal has a long history of mining. Nonetheless, a decline in this activity has been observed over the last decades, with consequent abandonment of mining areas without recovery plans. Abandoned mines constitute one major environmental problem since they are unpredictable sources of metal pollution. Although environmental pollution may have effects at all levels of biological organization, few studies have been performed on high organizational levels, like the population level, so that the chronic effect of metal toxicity remains largely unknown. To understand the medium-long term impact of metals on biodiversity, in this study it was performed geometric morphometric and genetic analyses of the populations of two mammalian sentinel species (*Mus spretus* and *Crocidura russula*) living in two heavy metal polluted mines in southern Portugal (Aljustrel mine, deactivated since 1996, and Preguiça mine, deactivated since 1964), that were previously shown to harbour changes at the biochemical, histological and physiological levels. We observed that the morphological analysis in *Mus spretus* revealed mandibular morphological differences in the three populations and a greater morphological similarity between the animals from mines. These morphological changes may be associated with the low environmental quality in mines. In the Aljustrel mine, both species showed higher genetic diversity in the *Cytochrome b* gene, while in the populations from Preguiça, the diversity of the *Cytochrome b* gene and of the mitochondrial control region changed in opposite directions, high genetic diversity in the latter, and low diversity in *Cytb*. These results suggest that Aljustrel may have functioned as an ecological sink and that in Preguiça a bottleneck may have occurred in the recent past, possibly due to an increase in the mutational rate. Our results confirmed the potential environmental impact of mines at the individual level and showed that within a relatively short time, pollution by heavy metals had altered normal homeostatic pathways and the genetic structure of natural populations. Since the effects on the populations of both species were concordant, it may be concluded that the different responses are due to the different characteristics of each mine. This study also corroborates the sustainability of these two species as biomonitors of environmental quality and the use of genetic and morphological biomarkers in identifying toxicological effects at a population level. Ecotoxicological studies such as this have the potential to assess the impact of anthropogenic stress on the evolutionary history of natural populations, as well as to reinforce the importance of requalification plans or remediation actions in abandoned mines.

Keywords: *Mus spretus*, *Crocidura russula*, Small mammals, metals, Biomarker

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List of Abbreviations

- AMD – acid mine drainage
- AMOVA – analysis of molecular variance
- ANOVA – analysis of variance
- AS – antisymmetry
- CA – chromosome aberrations
- CR – control region
- CSB – conserved sequence blocks
- CV – canonical vector
- CVA – canonical variates analysis
- CVS – volcano-sedimentary complex
- Cytb* - cytochrome b
- DNA - deoxyribonucleic acid
- DS – directional asymmetry
- ETAS – extended termination associated sequences
- FA – fluctuating asymmetry
- h – number of haplotypes
- HSD – honestly significant difference
- IPB – Iberian Pyrite Belt
- LMS – landmarks
- MN – micronucleated cells
- mtDNA – mitochondrial deoxyribonucleic acid
- OMZ – Ossa Morena Zone
- PSZ – Portuguese Southern Zone
- RNA - ribonucleic acid
- ROS – reactive oxygen species
- SCE – sister chromatic exchange

1. INTRODUCTION

1. Introduction

1.1. Mining industry as a source of heavy metal pollution

The mining industry has contributed significantly to the economic, social, cultural and technological development of the societies in which they operate. However, its exploitation has left deep marks on the environment, degenerating and degrading natural resources and endangering the human health (Figure 1). Today, the mining industry is seen as one of the anthropogenic activities that produce the deepest impacts on the environment. It is responsible for the release of potentially toxic substances suspected of causing adverse effects at different levels of the biological organization, not only during its activity but after its unscheduled shutdown. Irrespective of whether the results vary according to site conditions and the type of exploration, it can be considered that there are three major problems:

1. Change in landscape;
2. Changes in hydrological conditions;
3. Tailing

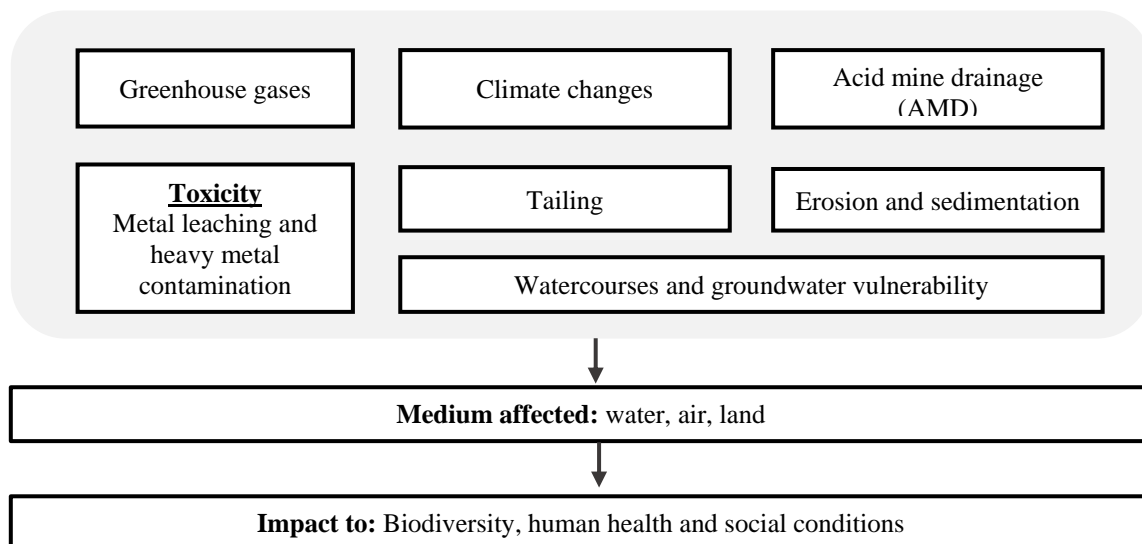


Figure 1 Mining issues with a negative effect on the environment and human health (adapted from Hatch, 2013).

In the case of the alteration of the landscape, this is visible from the beginning of the mining cycle, having different types of impact in the lithosphere as landscape change, landlessness, unused pits, and shafts (Zyl et al., 2002). These changes not only preclude the use of land for other purposes, (such as for agriculture), but also it is a loss of biodiversity through the degradation of vegetation.

One of the biggest problems in the mining industry is the amount of waste from excavation operations. For many years tailings were dumped into nearby watercourses, resulting in extreme conditions, for example water color change. Nowadays, these wastes are in a tailing disposal facility, which is, piles of mined materials that cannot be recovered during mill operations. These piles of waste are exposed to erosion (Henriques and Fernandes, 1991) mechanical dispersion, leaching, and oxidation by the oxygen present in pluvial waters (Santos Oliveira et al., 2002), releasing into the environment high concentrations of heavy metals. The mineral composition of these wastes will significantly

influence the quality of surface and groundwater of these sites. The effluents from mine drainage water or the simple rainwater on these residues can carry heavy metals to long distances in the form of leachates, through infiltration and percolation processes into groundwater (Santos Oliveira et al., 2002). The redox conditions between oxic water and anoxic sediments can have profound influences on the speciation and bioavailability of many trace metals (Morse and Luther, 1999).

The major environmental problems in mining are the acid mine drainage (AMD), i.e., the oxidation of sulphide in the presence of water and oxygen originating ferric hydroxides and sulphuric acid. While any mineral deposit containing sulphide is a potential source of AMD, pyrite (FeS_2) is the primary mineral associated with the generation of AMD (Gray, 1997). As water becomes more acidic, its capacity to leach other elements increases, producing a percolate rich in dissolved metals (Hofmann and Schuwirth, 2008). This water acidification is one of the effects of AMD, characterized by high metal content at low pH. The water quality is degraded due to pH reduction and increased solubility of soluble metals, transporting the metals from mine to water body. This degradation of water quality will have direct and indirect effects, which can compromise the ecological structure through biological and physical changes, such as increasing the concentration of heavy metals in sediments, facilitating the entry into the food chain (Gray, 1997). In many cases, mine areas show high levels of heavy metals in surface waters, groundwater and soils, aggravated by the long-term persistence in the environment (Hofmann and Schuwirth, 2008). It is hard to predict the impact of AMD due to the variability of the discharge, variation in composition that varies seasonally, the effect of surface runoff from exposed areas during heavy rains and the effect of discharge characteristics which affect the dilution and the concentration of organic matter (Gray, 1997).

1.1.1. Abandoned mines

An abandoned mine is a mine that has no activity, no management, no recovery plan and no one responsible for its rehabilitation or remediation (Mhlongo and Amponsah-Dacosta, 2015).

Despite the fact that Portugal has a long mining history dating since the Pre-Roman ages, over the last decades Portugal has witnessed a continuous decrease in mining. This reduction resulted in a high number of abandoned mining areas, mostly without an environmental recovery plan which represents a serious environmental problem. Between 1991 and 2001, an environmental report about the main degraded mining areas of Portugal indicated the existence of more than 85 abandoned mines (Santos Oliveira et al., 2002). Nowadays, about 175 mines are abandoned, 114 of polymetallic sulfides and 61 of radioactive ore (Ameixa, 2017). This growing abandonment resulted from an exploration based on economic criteria as a consequence of economic conditions, resource depletion or a combination of both (Santos Oliveira et al., 2002; EDM, 2011). Many mines underwent cyclical exploration processes interrupted with new re-openings due to fluctuations in the price of metals on the market, which led to a rapid expansion of the mining area, followed by a closure without a recovery plan.

Due to various European pressures, there has been a growing increase in legislation over the last two decades, reinforcing the importance of a sustainable policy of environmental concern, aware of the legacy that the mining has left over. In Portugal, the first survey of environmental inventories and characterization of abandoned mines took place in 1994. This study concluded that abandoned mines could be a risk factor for public health and safety, increasing with the time of abandonment (Santos Oliveira et al., 2002). Although the environmental recovery in abandoned mining areas was already covered in 1987 (Lei de Bases do Ambiente - Decreto de Lei n.º 11/87, of April 7) only in 2001, was

defined the regulation of concession of degraded mining areas environmental recovery (Decreto-Lei n.º 198-A/2001 of July 6).

The delay in the rehabilitation of abandoned mines is due in large part to the lack of clearly attributed responsibilities, the absence of recovery criteria and standards for these mines, and the high cost of rehabilitation which are applied to the mining companies (UNEP and COCHILCO, 2001). Still, many abandoned mines no longer have owner or property rights, ending the responsibility on governments to inventory and evaluate mine sites. Portugal has already invested more than 139 million euros for remediation (Carvalho, 2017). Though efforts are being made to rehabilitation and reduce environmental impacts, abandoned mines have been sites of metal persistence with unpredictable consequences for living organisms including risks to human health.

1.2. Biological impact of heavy metals

Metals have been identified as toxic elements for almost all living organisms (WHO, 2007). Some of them are elements that occur naturally in the earth's crust. However, anthropogenic activities like the mining industry have contributed to the increased introduction of these metals into the environment.

Heavy metals constitute a very heterogeneous group of elements that vary widely in their chemical properties as well as in biological functions (Raikwar et al., 2008). Many metals, such as iron (Fe), copper (Cu), zinc (Zn), cobalt (Co), molybdenum (Mo) and manganese (Mn), are essential to living organisms (Valavanidis and Vlachogianni, 2010). They play a critical role in the physiological functions of plants and animals, in the constitution of some enzymes and various reactions of oxidation-reduction (WHO, 1996). Despite this undeniable importance, they can be toxic when in high amounts. On the other side, other metals are not required for metabolic activities (e.g., mercury (Hg), lead (Pb), nickel (Ni), chromium (Cr), cadmium (Cd) and arsenic (As)) and even at low concentrations are toxic. These metals may replace essential metals and interfere with the function of the associated enzymes and cofactors (Valavanidis and Vlachogianni, 2010). These metals are persistent and non-metabolized in other intermediate compounds, which leads to their accumulation in the food chain through uptake at the primary producer level and consumption at the consumer level (Raikwar et al., 2008).

The heavy metals bioavailability in different terrestrial compartments depends on many factors: total concentration and speciation of metals, mineralogy, pH, redox potential, temperature, total organic content and suspended particle content, as well as their transport and removal (John and Leventhal, 1995). This bioavailability facilitates the entrance into the food chain, increasing their bioaccumulation. The bioaccumulation will depend on the available amount of each metal in the environment and the way of entry. The accumulation along the trophic chain may give rise to magnification.

The use of a biomonitor allows a holistic view of the negative impact that metals have on the environment, allowing an analysis under real conditions, including a temporal and spatial component given by the accumulation of environmental impacts throughout the life of the organism, something that is not possible through direct chemical analysis of the environment. Exposure to toxicants in mining areas induces morphological alterations, namely, fluctuating asymmetries, lower weight and less mass in some internal organs in local animals (Nunes et al., 2001a, 2001b; Sánchez-Chardi et al., 2007). These changes reflect the efficiency of the mechanisms in the control of the organism stability, possible due to environmental disturbances (Nunes et al., 2001a, 2001b). Often, organisms exposed to high concentrations of metals use different defense mechanisms, such as reducing consumption, increasing

excretion and/or sequestration through the storage of metal-bound proteins, such as metallothionines (Valavanidis and Vlachogianni, 2010).

One of the deleterious effects of metals is their ability to increase the formation of reactive oxygen species (ROS) by inducing oxidative stress in animals (Sies, 1993). This imbalance caused by the production of ROS (Prá et al., 2008), can at a molecular level, lead to situations of oxidative stress (Belyaeva et al., 2012). The production of ROS can cause, for example, oxidative modification of proteins, DNA damage repair inhibition like adducts forming on the nucleotide basis of the DNA (Shugart et al., 2003) with following modification in tissue structure and chromosomal alterations (Cooke et al., 2003). The occurrence of mutations may lead to degenerative diseases (Andersen, 2004) carcinogenic and mutagenic processes (Raikwar et al., 2008), which may affect the fitness of natural populations (Sánchez-Chardi et al., 2008). Namely, genotoxic insults can alter somatic or germ cells, having direct implications to an individual or his offspring (Bickham, 2011). In both cases, these cell changes may have demographic effects on the population, which may reduce the individuals' longevity, differentiation of sexual proportions, reproductive success reduction, low viability or even infertility (Bickham et al., 2000). In the environment, the elements often appear mixed, being necessary not only to take into account their action individually but also the interaction of the elements.

However, not all chemical contaminants have a mutagenic effect; these are called non-genotoxicants and are the focus of interest in Evolutionary Toxicology, since they do not interfere directly with chromosome structures or alter DNA, but may have a genetic impact on populations. Non-genotoxicants can modify the population demographics, leading to selection for particular traits that promote survival and reproduction in contaminated environments (Bickham, 2011) alteration in migration rates or bottlenecks (Berckmoes et al., 2005) leading to changes in genetic diversity. Environmental pollution has, therefore, the potential to rapidly change the evolutionary history of wild populations of small mammals.

Regarding human health, studies in populations near to abandoned mines have shown that there may be adverse effects on people's health as loss of smell (anosmia), high levels of metals in blood, increased respiratory problems and cases of cancer (Coelho et al., 2007; Kim et al., 2008). Abandoned mines with no remediation plans can cause serious problems in the long term and may sometimes have more significant impacts than active mines since it exposes populations to unpredictably heavy metal concentrations over long periods. Studies developed in communities living near abandoned mines have shown that these populations may be subject to high concentrations of heavy metals through feed or water consumption (Ji et al., 2013; Kim et al., 2008). Although consumption may not occur directly from contaminated sites, a magnification may take place along the trophic chain and may affect local human populations, for example through hunting.

1.3. Small mammals as sentinels of heavy metal pollution

The usage of animals to assess and predict the effects of contaminants on the human population is dated for more than a century ago. A classic example is the miner's canary used to warn the presence of potentially lethal carbon monoxide concentrations in coal mines. In 1914, one of the first articles that mention the use of birds and mice as good indicators of the existence of poisonous gas was published in the *Journal of Industrial and Engineering Chemistry* (Reif, 2011). As these two examples illustrate, it was soon realized that using animals as biological monitoring tools is advantageous. These biological monitoring tools allow us: to know anthropogenic changes in nature; understand dynamic balance in

ecosystems; provide early warning of significant environmental changes; check the effectiveness of established regulatory mechanisms; define critical pathways of pollutants for humans in the different compartments of the earth; and integrate the physical and chemical effects in a biological context (review by Wren, 1986).

The concept of sentinel can be easily confused with two other concepts: biomonitor and bioindicator. According to O'Brien et al. (1993) bioindicators are organisms whose characters are used to indicate the absence or presence of a particular contaminant in the environment; on the other hand, biomonitor is when it is possible to measure the extent of this contamination to know health implications of other species or the environment. Sentinel concept distances itself from this latter by the simple question of being applied to man, since it allows evaluating the implications for human health and gives warnings of these implications. Not all species fall into these categories. The sentinel animals have more selection criteria, such as physiological characteristics similar to man's (so that the biological and pathological effects after exposure can be compared), as well as the same pathways (route of toxic exposure), through ingestion and inhalation of contaminants (O'Brien et al., 1993). However, some criteria are common among biomonitors and sentinels, such as:

- to provide measurable responses (ideally including accumulation of tissue residues);
- to reflect the population response;
- to give a proportional response to the degree of contamination or degradation (through structural changes appearance or physicochemical composition) (Holt and Miller, 2010);
- to be common in the analysed areas (so that it is abundant and easy to capture);
- to tolerate a certain level of contamination (Zakrzewska et al., 2010);
- its ecology and life history has to be well known (Holt and Miller, 2010);
- to have a long enough life span to demonstrate the effects of exposure over time and;
- to have a good position in the food chain (O'Brien et al., 1993).

In this context, the majority of species used in terrestrial biomonitoring studies have been small mammals. They are useful sentinels because they are in close contact with soil, water, and air pollutants, their analysis represent a realistic approach of mixtures and concentrations of metal exposure (Tovar-Sánchez et al., 2012), play a significant role in food chains (considered intermediates for metal transfer to higher trophic levels) (Levengood and Heske, 2008), and since they are relatively common, they are easily captured and distributed in both contaminated and uncontaminated reference sites (Talmage and Walton, 1991).

Several studies have been carried out with small mammals documenting the accumulation of heavy metals within individuals (Marques et al., 2008; Mussali-Galante et al., 2013b; Sánchez-Chardi et al., 2013; Tovar-Sánchez et al., 2012). These studies provide information on availability within an individual and to some extent the population, which may affect the entire community (Kendall et al., 2001). The exposure to a particular contaminant can alter the composition of the community by, for example, reducing the abundance of a species (Kendall et al., 2001). An adequate way of monitoring the environmental quality is through the use of multiple species, mainly species occupying different positions in the food chain. In Portugal and Spain, Algerian mouse (*Mus spretus*) and the greater white-toothed shrew (*Crocidura russula*) (Figure 2) have been widely used as sentinels or biomonitors (Lopes et al., 2002; Marques et al., 2008; Nunes et al., 2001a; Sánchez-Chardi et al., 2013, 2008, 2007; Tapisso et al., 2009; Viegas-Crespo et al., 2003).

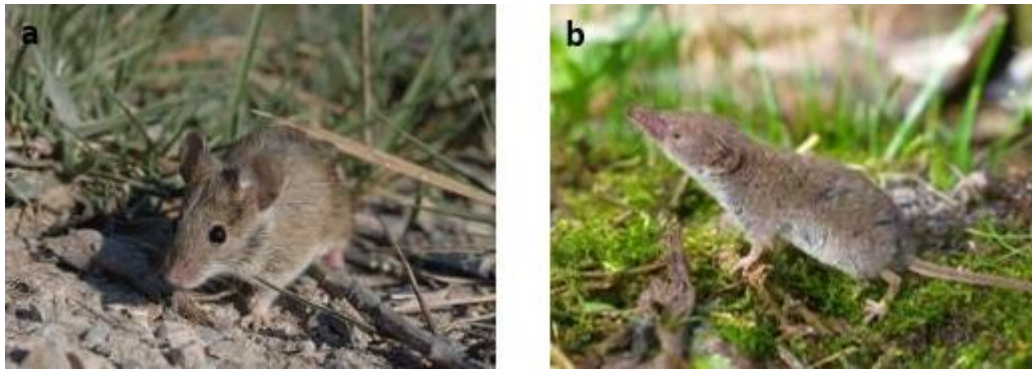


Figure 2 Small mammals commonly used as sentinels: a) *Mus spretus* and b) *Crocidura russula*.

These two species differ in some ecological characteristics. *Crocidura russula* is a small insectivore of the Soricidae family and is distributed throughout southern and western Europe and North Africa, with remarkable genetic uniformity (Ramalhinho et al., 1999). This species lives in very diverse environments, including lands adjacent to rivers, places with abundant undergrowth to open habitats. The shrews have a peculiar breeding system of monogamy and female dispersal contrarily to polygamy, and male dispersal observed in mammals (Balloux et al., 1998) and have about four litters from March to September (Cantoni and Vogel, 1989). The spatial dispersion is small, presenting a significant correlation between genetics and geographic distance (Balloux et al., 1998).

Mus spretus is an endemic rodent from the Mediterranean region belonging to the Muridae family. It occurs in south-eastern Europe and North Africa, being found in pastures and cereal fields and avoiding open areas. It shows two phases marked in the reproductive cycle, a period of sexual inactivity in winter (November-January), and two phases of maximal activity in April-May and August-September (Palomo et al., 2009). On average, there are about five pups, ranging from two to ten (Palomo et al., 2009). Concerning spatial dispersion of males, it appears to be strongly related to the presence of other species, with a mean range of 343 ± 95 m² (Gray et al., 1998).

These two species occupy distinguished positions in the food chain. *Mus spretus* represents an intermediate stage between trophic levels since this species is important in the diet of carnivorous birds and mammals. *Crocidura russula* accounts for a high position in the food chain and has a high metabolic rate, accumulating larger amounts of toxic pollutants (Talmage and Walton, 1991). Environmental pressures can generate rapid changes in the evolutionary history of these species since it has short generation times, which makes them good bioindicators of evolutionary toxicology studies.

1.4. Contaminant effects on natural populations.

Environmental contaminants can affect genetic systems at a variety of organizational levels. Nevertheless, understanding the contaminant effects on natural population's presents a high complexity since different mechanisms can have similar answers and similar mechanisms can give different answers (Van Straalen and Timmermans, 2002).

As described in the sub-chapter *Biological impact of heavy metals*, contaminants can be mutagenic, called genotoxicants (causing direct damage to the DNA in somatic cells and potential heritable effects), or non-genotoxicants. These latter, although not having a direct effect on the structures, can have an indirect genetic impact in the population (Rose and Anderson, 2005). Heavy metals can affect

populations through somatic cell changes or heredity. In the latest case, this can occur by induced mutations in the germ cells passing to offspring. This mutation may remain in the population even after the removal of the contaminant, by selection or another process at the population level, increasing its permanence due to the stress caused by the contaminated environment. Alterations at the somatic cells level, can lead to change in the structural age of the population (Theodorakis et al., 2001); reduction of longevity of individuals (Agarwal and Sohal, 1994; Harshman and Haberer, 2000); modification of the size of individual (which may have implications for survival; behavior or fecundity) (Siegel et al., 1992); or alteration of the population sex ratio (McDonald et al., 2014; Scheirs et al., 2006). These changes may have ecological effects, resulting as in demographic changes (Bickham et al., 2000) (Figure 3). These population changes are correlated with genetic and morphological variations in the population (Frankham, 1996). It is expected that a decrease in population will have negative impacts on genetic and morphological variation in the population. All these changes in the population structure cause indirect genetic effects. Bickham (2011) based on concepts presented by Van Straalen and Timmermans (2002) considers that there are four categories of population genetic responses to contaminant exposure: a) genome-wide changes in diversity, b) changes in allelic or genotypic frequencies due to contaminant-mediated selective pressure, c) changes in gene flow between populations, and (d) increased mutation rates.

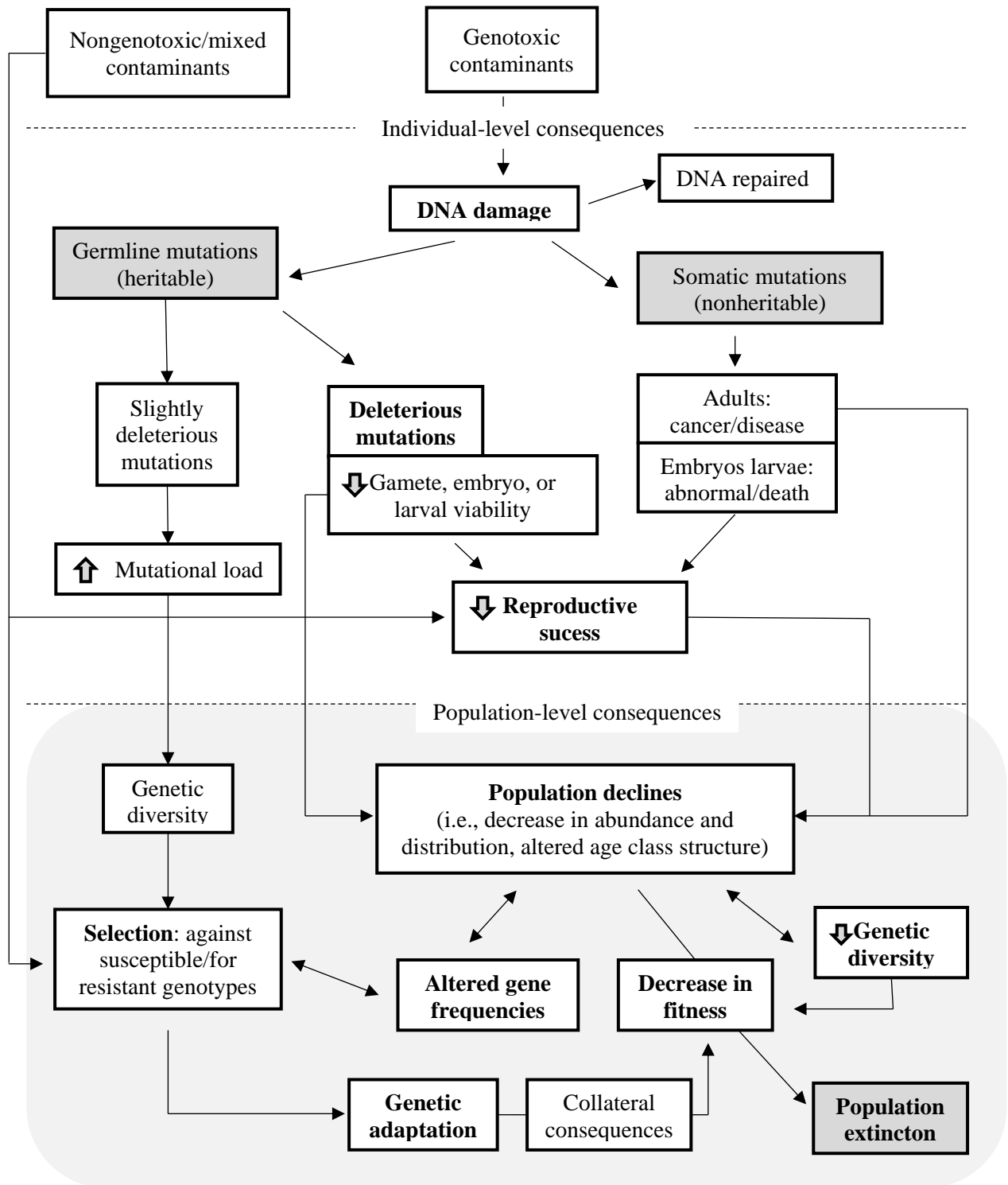


Figure 3 Summary of how genotoxic and non-genotoxic contaminants may lead to population-level consequences. The up and down arrows within the boxes suggest increase and decrease, respectively (adapted from Rose and Anderson, 2005).

These four categories are called the four cornerstones of Evolutionary Toxicology and are used to explain the differences among populations driven by anthropogenic contaminants in the different studies that have been performed (Figure 4).

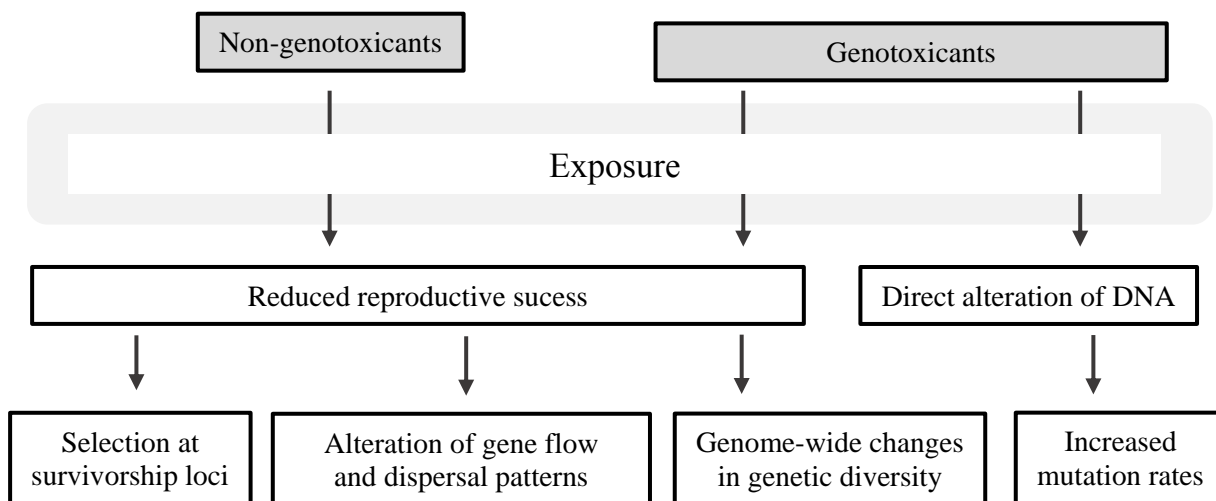


Figure 4 The four cornerstones of Evolutionary Toxicology (adapted from Bickham, 2011).

The first cornerstone focuses on the genetic diversity conservation because this is proportional to extinction probability (Bickham, 2011). Populations initially exposed to contaminants may suffer a decrease in population size and may lead to loss of genetic variability through genetic drift (Bickham, 2011). This decrease will depend not only on the initial population size but also on the response time a population will take to recover (Medina et al., 2007). This loss of genetic diversity due to anthropogenic effects is called *genetic erosion* (Van Straalen and Timmermans, 2002). An increase in genetic variability also may occur (Eeva et al., 2006) resulting from new mutations, migration or mixed populations and diversifying selection (Theodorakis et al., 1997).

In the case of migration of several populations (gene flow between populations), these may contribute to an increase of genetic variation, masking the genotoxic effect that may exist on the site (Baker et al., 2001; Matson et al., 2006, 2000; Theodorakis et al., 2001). This phenomenon occurs mainly in sites where the polluted area appears to have all the habitable conditions, but the animals that lived there fail to reproduce or survive. Migrants maintain the number of inhabitants from other places (Matson et al., 2006) i.e., the increase of genetic diversity results from the increase of gene flow from neighboring populations. These areas are called *ecological sinks*.

The immigrants from neighboring areas play an important role in maintaining the population gene pool, especially after a bottleneck. The emergent population will result not only from immigrant individuals but also from individuals that survived polluted environments due to favorable genotypes in degraded areas. This selective pressure mediated by contaminants conduces to changes in the genetic or allelic frequency of populations (Bickham and Smolen, 1994).

Populations continuously exposed to contaminants can, in turn, develop a certain tolerance. There has been an inherited genetic selection that leads to evolutionary processes on a small scale, called *micro-evolution*. This micro-evolution occur rapidly in a few years or a few generations, involving a set of morphological and physiological characteristics that may have implications for the populations, their ecological response capacity and the global participation of the ecosystem, thus having an associated

ecological cost (Medina et al., 2007). In some cases, stress-induced variation may play a crucial role in the divergence of species (Badyaev and Foresman, 2000).

In addition to changes in genetic diversity with phenotypic implications, stress environments can cause development instability during the ontogeny of the individual (Eeva et al., 2000; Nunes et al., 2001a; Parsons, 1990; Sánchez-Chardi et al., 2013), giving rise to small deviations of the symmetry. These asymmetries may have an indirect effect on the population, for example, through mate choice decisions (females assess the genetic quality of potential mates by symmetry, opting for symmetric individuals) (Moller and Pomiankowski, 1993; Morris, 1998) or mating success (the asymmetry can lead to its infeasibility) (Blanckenhorn et al., 1998).

The studies of the effects of contaminants on natural populations are complex, and although several studies have been conducted on wild populations, the understanding of evolutionary responses in wildlife due to exposure is limited (Bickham, 2011).

This complexity is due to the numerous factors associated with the contaminants (such as species susceptibility, the degree of disturbance or ecosystem), as well as the possibility of the change of the genetic pattern due to natural processes. The interpretation of results from evolutionary toxicology studies should be viewed very carefully, to exclude the possibility of the historical event having caused the observed genetic patterns (Meeks et al., 2009). Therefore, it is important to consider a set of biomarkers at different organizational levels to have a holistic view of the problem.

1.5. Biomarkers

1.5.1. Genetic biomarkers

Several biomarkers allow us to characterize the health of individuals in toxicological terms, knowing their exposure, effect, and susceptibility to the presence of heavy metals in abandoned mines. Genetic alteration resulting from genotoxic exposure may lead to the formation of adducts (modified base pair), inversions, deletions or additions, excess micronuclei and mitotic aberrations, DNA strand rupture, and chromosomal rearrangement (Medina et al., 2007). However, these biomarkers are focused on physiological and genetically mediated changes in tolerance at the individual level only (Figure 5) (Medina et al., 2007). In the case of a population or community, the point of interest increases with chronic exposures at low concentrations to a mixture of chemical agents (Depledge, 1994).

This change leads to the need of using specific biomarkers at each level of biological organization. Specific biomarkers become increasingly complex as we move up in the organization (e.g., from population to the ecosystem), increasing the time scale and ecological importance (Mussali-Galante et al., 2013a). These should be chosen to reflect the changes in the fitness of an organism (e.g., premature death, ability to mate, fecundity and the viability of offspring) since there are changes that affect the population (Evdenden and Depledge, 1997). In this context there are two reasons why the study of DNA repair and damage are important: firstly because DNA is the fundamental unit of heredity and reproduction, and therefore disturbances in structure and functions can lead to changes in population dynamics or demographics; and secondly because the effects may be more refractory than at the individual level, since due to genetic diversity the return to the pre-contamination point may take several generations (Bickham and Smolen, 1994). Mitochondrial DNA is more vulnerable than nuclear DNA in some genotoxic environments. This feature has been explained by its physical location since the mtDNA

nucleoids are anchored to the side of the internal membrane matrix, which is close to the respiratory chain (electron transport chain) that generates a significant amount of ROS and due to its reduced DNA repair capacity (Meyer et al., 2013). The mtDNA is a target for oxidative damage which, once damaged, can amplify oxidative stress by increasing the expression of proteins essential for the respiratory chain, becoming a vicious cycle (Van Houten et al., 2006). In studies of phylogenetic relationships, mtDNA is often used in detriment of nuclear DNA because it does not undergo meiotic recombination (not being "shuffled" during the production of the gametes as are nuclear genes), have a slower degradation than nuclear DNA (advantageous in case of degraded or old samples), and presents a higher rate of mutation compared to nuclear DNA (Arif and Khan, 2009). Also, mtDNA allows a view of the regional patterning since its maternal, haploid mode of inheritance exposes it to only a one-quarter of the gene flow (Bickham et al., 2000).

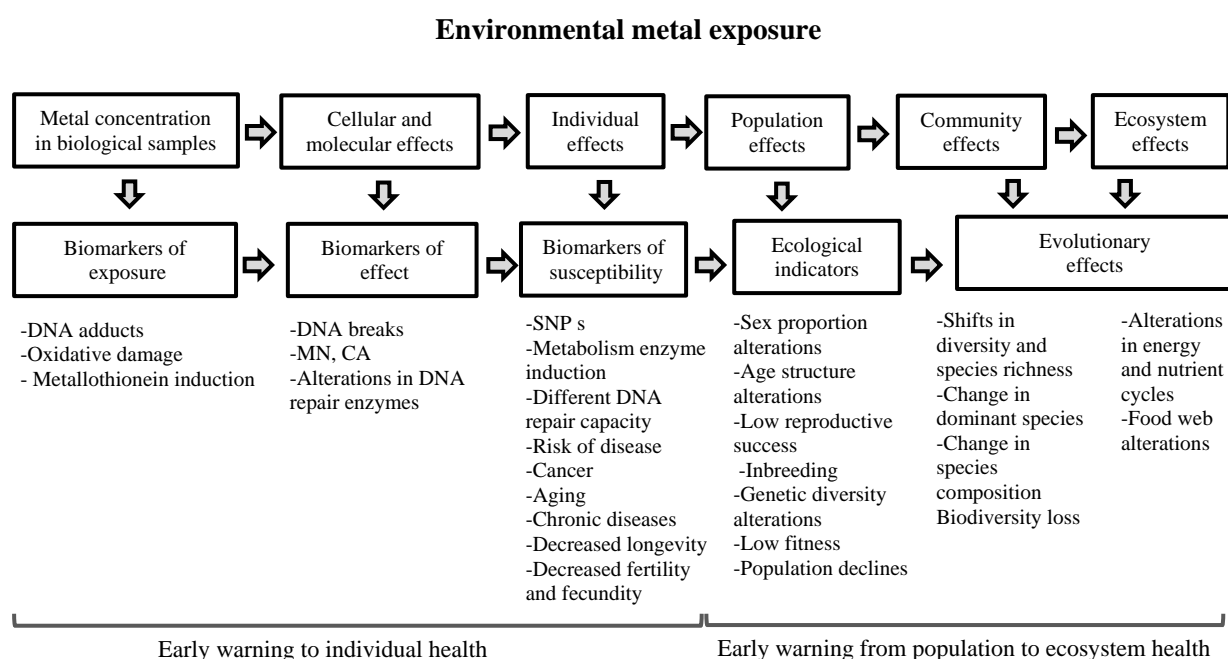


Figure 5 Effect of environmental pollutants on all biological organization levels and the most used biomarkers at each organizational level (adapted from Mussali-Galante et al., 2013a).

The choice of the markers is an important factor in the analysis of the genetic diversity of the populations since not all markers are subject to the same selective pressure.

1.5.1.1. Cytochrome b and the Control Region

Taking into account genetic ecotoxicology studies already performed (Baker et al., 2001; Eeva et al., 2006; Giska et al., 2015; Matson et al., 2006; Theodorakis et al., 2001), two mitochondrial markers were chosen for the present study, Cytochrome b (*Cytb*) and the Control Region (CR).

Cytochrome b is an enzyme component of respiratory chain complex III, and, within this complex, it is the only component encoded from the mitochondrial genome (Dumoulin et al., 1996; Irwin et al.,

1991). It is responsible for the transmembrane electron transfer by which redox energy is converted into a proton motive force (Degli Esposti et al., 1993). Beyond playing an important role in the electron transport chain, its molecular evolution is well understood in mammals (Irwin et al., 1991) and it is a protein coding gene which evolves at an intermediate rate when compared to the Control region (Baker et al., 1999). In the case of the Control region, it exhibits high levels of variation compared to a coding protein, because of the reduced functional constraints and the relaxed selection pressure, with an extremely rapid rate of evolution. Functioning as a neutral locus, it is expected to be sensitive primarily to historical factors such as population bottlenecks (Bickham and Smolen, 1994). In terms of structure, it has a similar structure in all organisms, divided into three domains: a central conserved domain (implicated in formation of D-loop and the regulation of H-strand replication) surrounded by two variable domains, left domain adjacent to the Pro-tRNA gene (R1) which contain ETAS (extended termination-associated sequences) and a right domain adjacent to the Phe-tRNA gene (R2) which contain CSBs (conserved sequence blocks that play the major role in the regulation of the replication process of DNA) (Fumagalli et al., 1996). A study performed by Sbisà et al. (1997) with ten different mammalian orders identified that in all the organisms considered in the study, events of repeats sequences occur in one of the domains (ETAS or CBS domain). In *Crocidura russula* these tandemly repeated sequences can occur in both domains, which makes the mtDNA control region size variable, even between individuals (Fumagalli et al., 1996).

1.5.2. Morphological variation

Morphological variations are changes that can occur directly from genetic changes or epigenetic effects in the process of development or growth of the organism (considering epigenetic effects developmental interactions among cells, tissues and their environments) (Young and Badyaev, 2007). A variety of biological processes can produce differences in form between individuals or their parts, whether by disease, adaptation to local geographic factors, long-term evolutionary diversification or different responses to the same selective pressures (or differences in the selective pressures themselves) (Zelditch et al., 2004)

These morphological variations are characterized and quantified by a biological discipline called Morphometry which has undergone a revolution over the last decades. Between the 60's and 80's, the data were obtained through sets of direct measurements of traits, so-called Classical Morphometry. Through the implementation of several statistical tools for shape analysis, Morphometry was reinvented by appearing the Geometric Morphometry.

In the geometric morphometric analysis, the form of an organism and the morphological structure is represented by landmarks, discrete anatomical points that are arguably homologous in all individuals in the analysis (Zelditch et al., 2004). The shape variation is analysed by the displacement of these landmarks which can be represented in both 2D and 3D, and it is classified into different categories according to anatomical and geometric criteria (Bookstein, 1991). The form is the combination of shape and size, in which the size is the structure scale and the shape the geometric information which allows recognizing of the structure (Bookstein, 1991) i.e., geometric properties except for its size, position, and orientation (Klingenberg, 2010).

Although shape and size are analysed separately, this does not mean that they cannot depend on each other. The dependence of the shape on size is called allometry (Klingenberg, 2016), and is an

important relation to take into account in the analysis of the shape since the changes observed in shape can be only due to size.

Most animals are symmetric and bilateral, i.e., when divided into two sides of a plane or axes they present the two parts with an equivalent property of size and shape, in which the left and the right sides are the mirror image of each other. This type of symmetry is so-called matching symmetry (an example is the mandible of a rodent) (Klingenberg, 2015). The other type of symmetry is called object symmetry and is when the object is symmetric in itself, the axis or plane of symmetry runs through the structure, such as the cranium. In symmetrical structures, it is to be expected that both sides of the structure are identical. However, this is not always the case and deviations from perfect symmetry may arise. These deviations may be measured as variances (or related measures of dispersion) of linear dimensions, shape variation involving landmarks, or as continuous symmetry measures (Graham et al., 2010). Three types of asymmetry have been distinguished according to the distribution of left–right differences in a population (Klingenberg, 2015; Palmer and Strobeck, 1986; Van Valen, 1962): directional asymmetry (DS), fluctuating asymmetry (FA), and antisymmetry (AS) (Figure 6).

Directional asymmetry is a tendency for a trait to be consistently different between the left and right side and can be quantified by the difference between left and right averages differing from zero (Graham et al., 2010; Klingenberg, 2015). This type of asymmetry is observed in the internal organs of most bilateral animals and is defined under genetic control. Robust statistical methods are needed to identify this asymmetry.

Antisymmetry is a pattern of asymmetry where most individuals are “left-sided” or “right-sided”, which may result in a bimodal or platykurtic distribution due to the mixture of random left- and right individuals (Graham et al., 2010; Klingenberg, 2015). The biological interpretation of AS has been debated, attributed both to hereditary characteristics (Graham et al., 1993) and as a response to stress (Graham et al., 2010). Nevertheless, a work by McKenzie and Clarke (1988) observed antisymmetry in *Lucilia cuprina*, during the evolution of the flies' resistance to Diazanone, leading some authors to consider that AS may appear as an evolutionary manifestation (Graham et al., 1993; Palmer, 1996). Some registered AS are morphological manifestations of a particular type of behavior, such as in *Octopus vulgaris* (Byrne et al., 2004) and *Sepia lycidas* (Lucky et al., 2012) in which the way they attack their prey shows a differentiation between individuals in the lateral asymmetry of the eye.

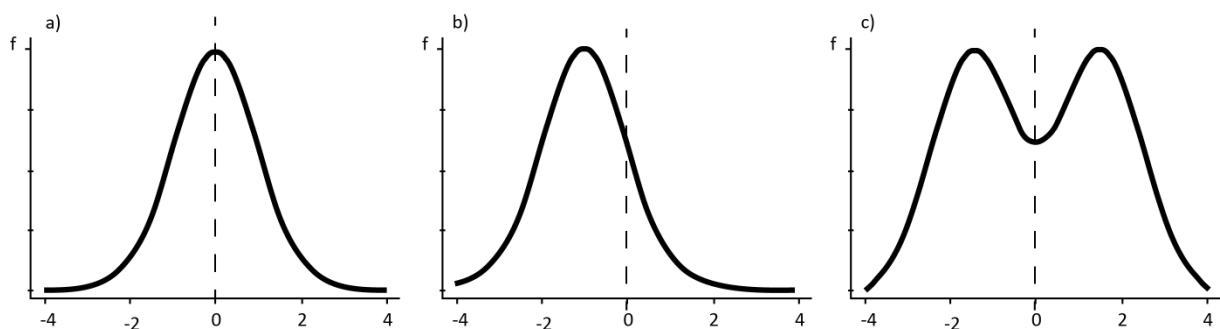


Figure 6 Frequency distribution (f) of left - right differences for three types of asymmetry a) fluctuating asymmetry, b) directional asymmetry and c) antisymmetry (modified from Klingenberg, 2015). In the X-axis, “0” means perfect symmetry.

The last type of asymmetry is FA. Fluctuating asymmetry refers to small differences between the left and right sides due to random imprecisions in developmental processes. These perturbations are a consequence of the stochastic nature of cellular processes (Klingenberg, 2003). This type of

asymmetry usually is associated with a bell-shaped distribution of left–right differences, commonly accepted as a normal distribution. The study of normality distribution and kurtosis test is usually done to distinguish this type of asymmetry from the others. Several studies have used fluctuating asymmetry as a biomarker of instability in development (*Crocidura russula* –(Sánchez-Chardi et al., 2013); *Apodemus agrarius* –(Velickovic, 2007, 2004); *Apodemus flavicollis* –(Blagojević and Vujošević, 2004); *Mus spretus*– (Nunes et al., 2001a; Yalkovskaya et al., 2016)), since the left, and right sides of the same organism share the same genome (Graham et al., 2010; Klingenberg, 2015). These studies correlated these small variations with contaminated or stress environments, sometimes associated with the negative fitness of an individual (Badyaev et al., 2000; Møller, 1997). Even though the relationship between environmental stress and FA is not yet a clear process, it is possible that it may be associated with the amount of energy available for buffering or regulation of developmental processes (Graham et al., 1993; Mitton, 1993). The increase of FA is sometimes associated with bone reduction, which is consistent with the energetic explication between stress and FA (Nunes et al., 2001a; Siegel et al., 1992). Nonetheless, although results may differ with species, combinations of specific stressors, level and duration of pollution, and morphological characters, FA is considered a suitable indicator of environmental-induced developmental instability (Beasley et al., 2013). Fluctuating asymmetry has been used as an individual marker of exposure to stress induced by contaminants during the developmental period (Badyaev et al., 2000).

1.5.2.1. The mouse mandible

The mandible of *Mus spretus* was chosen in the present study to assess the morphological effects of heavy metal contaminants. The mouse mandible is a complex structure, composed by multiples parts that have different origins, rates of differentiation, composition, and function. It is composed of four morphogenetic regions, the ramus and three processes (condylar, angular and coronoid) (Figure 7) (Atchley and Hall, 1991; Klingenberg and Navarro, 2012). Usually, it is grouped into two main modules (alveolar region and ascending ramus) (Atchley et al., 1985; Cheverud et al., 1997, 1991; Klingenberg, 2003; Klingenberg et al., 2004). The alveolar region bears the teeth (that include the molar and incisor alveolar), and the ascending ramus includes the three processes and the part that articulates with the skull and which participates in the attachments of muscles. The mandible is composed of one bone and has already been used previously in toxicology studies (Sánchez-Chardi et al., 2013; Yalkovskaya et al., 2015). Because the mandible is a plane structure, it allows a 2D geometric morphometric analysis.

The mandible is a structure that presents plasticity, shaping as an active response to the various interactions and feedbacks with its functional environment. This adaptation is due in large part to the muscular activity that leads to an adjustment of the bone to its function (Renaud and Auffray, 2010; Tanaka et al., 2007). Several studies have demonstrated that the consistency of the food can lead to different mandible morphologies (Luca et al., 2003; Mavropoulos et al., 2005, 2004; Michaux et al., 2007; Tanaka et al., 2007; Yamada and Kimmel, 1991). Morphological changes caused by the environment can lead to adaptive functional changes that increase the biomechanical efficiency of food processing in the result of a larger request (Anderson et al., 2014).

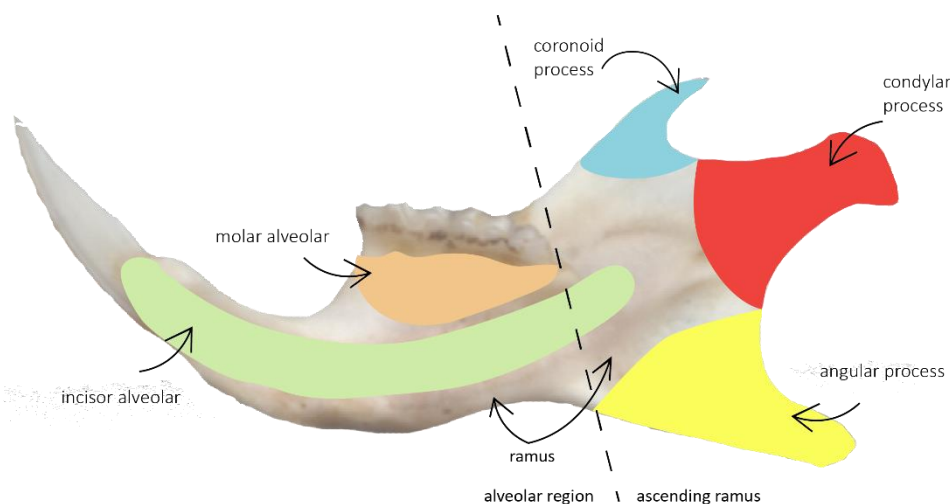


Figure 7 Mouse mandible with the four components: ramus and three processes. Two main modules: alveolar region and ascending ramus (adapted from Atchley et al., 1985).

Since genetic variation is the basis of diversity and evolution (Medina et al., 2007), and that morphological changes may be a biomechanical, ecological or genetic response to the degradation of environmental quality, both types of biomarkers – genetic and morphometric- are useful tools to study population responses to contaminants.

1.6. Case study - Aljustrel and Preguiça mines

The Aljustrel Mine is located near the village of Aljustrel, in the district of Beja (Figure 8). It is located in the Iberian Pyrite Belt (IPB), known for its richness in deposits of volcanogenic solid sulfides (pyrites). These sulfide deposits of the IPB are associated with a volcano-sedimentary complex (CVS) geological formation between the Devonian and the Carbonic (CMA, 2017). The IPB form the most of the Portuguese Southern Zone (PSZ) of the Iberian segment of the Variscan belt (Carvalho et al., 1971). This area as an extension of 250 km long and 30 to 60 km wide, covering part of Alentejo, Algarve, and Andalusia (CMA, 2017, Carvalho et al., 1971).

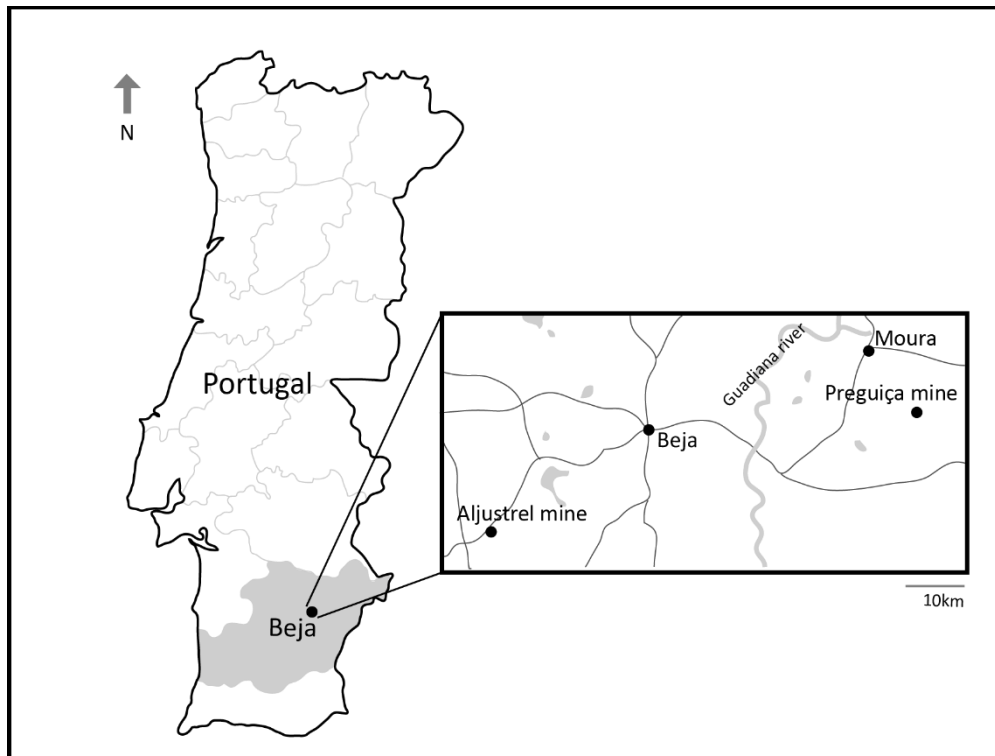


Figure 8 Map of Portugal showing the location of the Aljustrel and Preguiça mines.

The mining area presents six pyrite deposits arranged along the CVS structure with about 6km, distributed in two main alignments: Fetais, Estação e Algares, Moinho, São João and Gavião (Carvalho et al., 1971).

The mines of the IPB have a long history, dating from the time of the Tartessos, Carthaginians and later by the Romans (Carvalho et al., 1971). These mines are an example of environmental impact due to long extension and duration of intense mining. Their continuous work for more than 3000 years that has modified the landscape and caused increasing pollution (Tornos, 2006). Throughout its history, it has presented some instability, much associated with the value of the pyrite in the market. In 1993 it entered a period of suspension, having resumed activity in 2006, due to the concession change. In 2002 and 2003, Aljustrel mine presented a high degradation of the landscape with oxidized mine structures, water with a reddish color and tons of mining tails, as showed in Figure 9A.

AMD is a particularly important problem in the Aljustrel mine because the ore exploited is mostly sulfides, producing very acidic waters and the release of potentially toxic metals. This process is a cyclical process that extends well beyond the life of mine exploration, silently acting in the environment.

The Preguiça mine is located in Serra da Preguiça, 17km from the City of Moura, belonging to the district of Beja (Figure 8). It is located in Ossa-Morena Zone (OMZ), in Magnetitic – Zinciferous Belt, contacting in the south with PSZ. OMZ is characterized by a marked paleogeographic heterogeneity with pre-Cambrian, Cambrian, and Silurian formations ending with a late Devonian period (Martins, 2013a).

The first miners registered in this area date from 1852 with emphasis on the concession of Umbria Preguiça, which was recorded as a manifesto for iron, lead and other minerals. In 1911, four concessions were registered and operated until 1915, in which, after the beginning of the First World War, the work was suspended and abandoned in 1926. In 1964 and 1966 the mine was in full operation with a production of 13,000t/year, the last year in which it was in operation, and is currently abandoned (Martins, 2013b). Over the last decades, the vegetation has taken over the mine hiding scaring and tailing existing in the mine (Figure 9B).



Figure 9 General appearance of the Preguiça and Aljustrel mine at 2002-2003. A) Aljustrel mine and B) Preguiça mine (Marques, 2008).

Studies in the bodies of small mammals (*Mus spretus* and *Crocidura russula*) sampled in the area showed a significant increase of metals in these animals in both the liver and kidneys when compared to nearby reference sites (Marques, 2008; Sánchez-Chardi et al., 2007). In these sites, a vascular endothelial damage, fibrosis, and cell infiltration marks in *Mus spretus* has also been observed (Marques, 2008) as well as an increase in the number of pathological alterations with severity (necrosis, apoptosis and cytoplasmic vacuolization in hepatic tissues) in *Crocidura russula* from Aljustrel mine (Marques, 2008; Sánchez-Chardi et al., 2008).

In the case of genotoxicity tests, in *Crocidura russula* a correlation was found between the frequency of micronuclei and the concentration of heavy metals, which may indicate a clastogenic effect in hepatic tissues (Sánchez-Chardi et al., 2008). Also a GST activity decreased compared to the reference site was observed (Marques, 2008; Sánchez-Chardi et al., 2008). The combination of these two results may suggest that increased exposure to heavy metals over several generations will have given rise to selective pressure leading to the selection of more metal-tolerant individuals adapted to low-quality environments (Viegas-Crespo et al., 2003).

Though previous studies have warned of the environmental problem of abandoned mines, these studies are at the individual level. Only knowledge at the level of higher biological organizations, such as at the level of populations, allows a deeper insight into the real impact that heavy metal mines can have on the health and quality of life of natural populations.

2. THESIS CONTEXT AND OBJECTIVES

2. Thesis context and objectives

This study follows previous works (Marques, 2008; Marques et al., 2008, 2007, Sánchez-Chardi et al., 2008, 2007), performed in two distinct sentinels (*Mus spretus* and *Crocidura russula*) which were living in two mines in southern Portugal, Preguiça and Aljutrel mine, in comparison with a reference population (Moura). Since previous work have shown high concentrations of heavy metals in the environment, animal tissues and changes at the molecular level, the present work intends to understand the medium-long term effects that heavy metals can have on the individual and population levels, through the study of genetic and morphological variations of populations. In addition to this main objective, the present study intends:

- To contribute to the growing of the number of studies on the long-medium term effects of environmental pollution on wild populations of the terrestrial ecosystem, since the number is limited.
- To contribute to an understanding of the potential permanent effects that chronic exposure to heavy metals may have on humans.
- To be the first population study in genetic diversity of *Mus spretus* and *Crocidura russula* living in a contaminated environment, as well as the first geometric morphometric study of the mandible of *Mus spretus*.

3. PAPER

Paper Quina, A.S., Durão, A.F., Mathias, M.L. - Geometric Morphometric and Genetic Diversity analyses of two small mammal populations from heavy metal mines in Portugal (manuscript *in preparation*)

Geometric Morphometric and Genetic Diversity analyses of two small mammal populations from heavy metal mines in Portugal

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ABSTRACT

The mining industry is one of the largest sources of anthropogenic environmental pollution. The use of biomarkers and biomonitors is essential to detect, quantify and assess the effects of contaminants on natural populations across different levels of biological organization. Few studies have addressed high organizational levels, like the population level, such that the medium-long term impact of metal pollution remains largely unknown. We addressed this question through a geometric morphometric and genetic analyses of the populations of two sentinel mammalian species (*Mus spretus* and *Crocidura russula*) living in two heavy metal polluted mines in Portugal (Aljustrel and Preguiça mines), that were previously shown to harbour changes at the biochemical, histological and physiological levels. We observe that both species have acquired alterations in the shape of the mandible and in the genetic diversity of the gene *Cytb* and the mitochondrial control region. The mandibular shape from the animals of the mines was morphologically more alike. In the Aljustrel mine, both species showed higher genetic diversity in *Cytb*, while in the populations from Preguiça, the diversity of the two markers changed in opposite directions, high genetic diversity in the control region, and low diversity in *Cytb*. These results suggest that Aljustrel may have functioned as an ecological sink, and that in Preguiça a bottleneck may have occurred in the recent past, possibly due to an increase in the mutational rate. We show that within a relatively short time, pollution by heavy metals has altered normal homeostatic pathways and the genetic structure of natural populations. Ecotoxicological studies such as this have the potential to assess the impact of anthropogenic stress on the evolutionary history of natural populations, as well as to warn of the importance in recovering abandoned mines.

INTRODUCTION

Heavy metal mining is one of the most pollutant human activities to our natural environment. During extraction and mineral processing, heavy metals are exposed at the earth surface and become bioavailable to wild organisms. By generating acid or alkaline drainage, they pollute the neighbouring water, soil, sediment, and organic materials (vegetation), and as metals are assimilated, all trophic levels in a community become contaminated. At high internal concentrations essential metals become toxic, whereas other metals, like mercury, lead, nickel, chromium, cadmium, and arsenic, are already toxic at low concentrations. To organisms living in the surroundings of the polluted sites toxic effects can be immediate, but the whole community and ecosystem functioning can change if the level and duration of pollution is sustained. Alterations in a short period of time may involve physiological and morphological traits as well as genetic changes. “Biomarkers” or “early-warning” signals may be used to detect and quantify exposure to metals across different levels of biological organization (Mussali-Galante et al., 2013a). For instance, animal behavior or growth parameters may be studied to measure individual susceptibility to toxics, and used as evidence of the presence of alterations at lower biological levels (e.g. cellular and physiological) (Clements, 2000). At higher levels of biological organization, such as the population level, biomarkers measure shifts in population parameters like genetic diversity and structure, or demographic declines (Mussali-Galante et al., 2013a).

Terrestrial vertebrates living in contaminated areas have been shown to bioaccumulate heavy metals in their tissues (e.g. (Marques et al., 2008; Pereira et al., 2006; Sanchez-Chardi et al., 2007; Sanchez-Chardi et al., 2009a; Viegas-Crespo et al., 2003)). The measure of toxicity levels in these sentinel species provide a valuable insight into the health status of the entire ecosystem, and are thus very helpful for human and environmental risk assessments. Because of their key importance in the food chain (diversified food habits like herbivory, omnivory or insectivory, and serve as prey to other animals), small mammals are particularly suitable as sentinels of ecosystem quality (da Silva et al., 2017; Talmage and Walton, 1991). We have previously studied two of such small mammals, the Algerian Mice (*Mus spretus*) and the Greater white-toothed shrew (*Crocidura russula*), living in the surroundings of two heavy metal mines in Portugal. Compared to a nearby reference population, these animals carried alterations at the biochemical (antioxidant enzyme activities, metallothionein levels), histological (liver and kidneys) and physiological (morphological and haematological parameters) levels, including an increase in micronuclei frequency (Marques, 2008; Marques et al., 2008; Marques et al., 2007; Sanchez-Chardi et al., 2008; Sanchez-Chardi et al., 2007). Few studies have addressed small mammals at higher levels of biological organization particularly in what concerns heavy metal pollution (Baker et al., 2001; Berckmoes et al., 2005; Mikowska et al., 2014; Mussali-Galante et al., 2013b; Rakitin et al., 2016; Sanchez-Chardi et al., 2013; Theodorakis et al., 2001; Wickliffe et al., 2002; Wickliffe et al., 2006), such that the extent of the negative impacts of metal toxicity on small mammal populations remains largely unknown. In theory, population persistence in a polluted site will depend on the individual physiological resilience. In species with short generation times like *Mus spretus* and *Crocidura russula* sensitive individuals will die or reproduce less efficiently resulting in population declines and contaminant-induced selection of tolerant phenotypes. Environmental pollution has therefore the potential to quickly change the evolutionary history of wild populations of small mammals.

To better understand the chronic effects of environmental pollution by heavy metals, we here analysed at the population level the two previously studied sentinels *Mus spretus* and *Crocidura russula* living in two heavy metal mines in Southern Portugal, the Aljustrel and the Preguiça mines. In doing so, we intended to get a comprehensive portrayal, from biochemical to genetic, molecular to population, of the impact of heavy metal pollution on biodiversity, using the same samples as before. The Aljustrel

mine is located in the Iberian Pyrite Belt. This region contains large polymetallic sulphide deposits that have been exploited since 3000 BC, by Phoenicians and Romans, and formally operational from 1867 to 1996. The Preguiça mine is located in the Magnetite-Zinc Belt of the Ossa-Morena zone in the Iberian Peninsula, and laboured between 1911 and 1964, with main extractions of zinc and lead ores. Compared with the nearby reference region, the natural environment within both mines is highly polluted, with increased levels of zinc, arsenic and lead in the soil, and zinc and lead in plants (Marques, 2008). High concentrations of manganese and copper, and of manganese, iron, and arsenic were also found, respectively, in the soil and vegetation of Aljustrel (Marques, 2008; Marques et al., 2008). Since we observed alterations in *Mus spretus* and *Crocidura russula* from these mines using biomarkers at lower levels of biological organization (biochemical, physiological, individual), we now used biometric and genetic markers to evaluate associated population effects. We searched for form perturbations in the mandible of adult *Mus spretus* with a geometric morphometrics analysis. Population genetic changes in *Mus spretus* and *Crocidura russula* were investigated using diversity parameters of two mitochondrial markers that are under different selective pressures, the gene cytochrome b (*Cytb*) and the control region (CR). Mitochondria are cellular organelles particularly vulnerable to the toxic effects of heavy metals (Meyer et al., 2013), and mitochondrial markers have been used in genetic ecotoxicology studies before (Andre et al., 2010; Baker et al., 2001; Eeva et al., 2006; Giska et al., 2015; Kim et al., 2003; Matson et al., 2006; Theodorakis et al., 2001; Wickliffe et al., 2002; Wickliffe et al., 2006). With this study, we provide, to our knowledge, the first population study on the genetic diversity of *Mus spretus* and *Crocidura russula* living in heavy metal contaminated locations, as well as the first geometric morphometric study of the mandible of *Mus spretus*.

MATERIAL AND METHODS

1. Samples and Studied sites

All specimens of *Mus spretus* and *Crocidura russula* used in this study were sampled during 2002 and 2003, in the vicinity of two abandoned mines (Aljustrel and Preguiça) and of a reference location (Moura), in Southern Portugal (Fig. 1). Previous studies had already shown that animals from these mine sites had high body burdens of heavy metals (Marques et al., 2007; Sánchez-Chardi et al., 2007, leading to alterations in enzymatic activities (Marques et al., 2008; Sanchez-Chardi et al., 2008), and in haematological, histological and genotoxic parameters (Marques, 2008; Sánchez-Chardi et al., 2007).

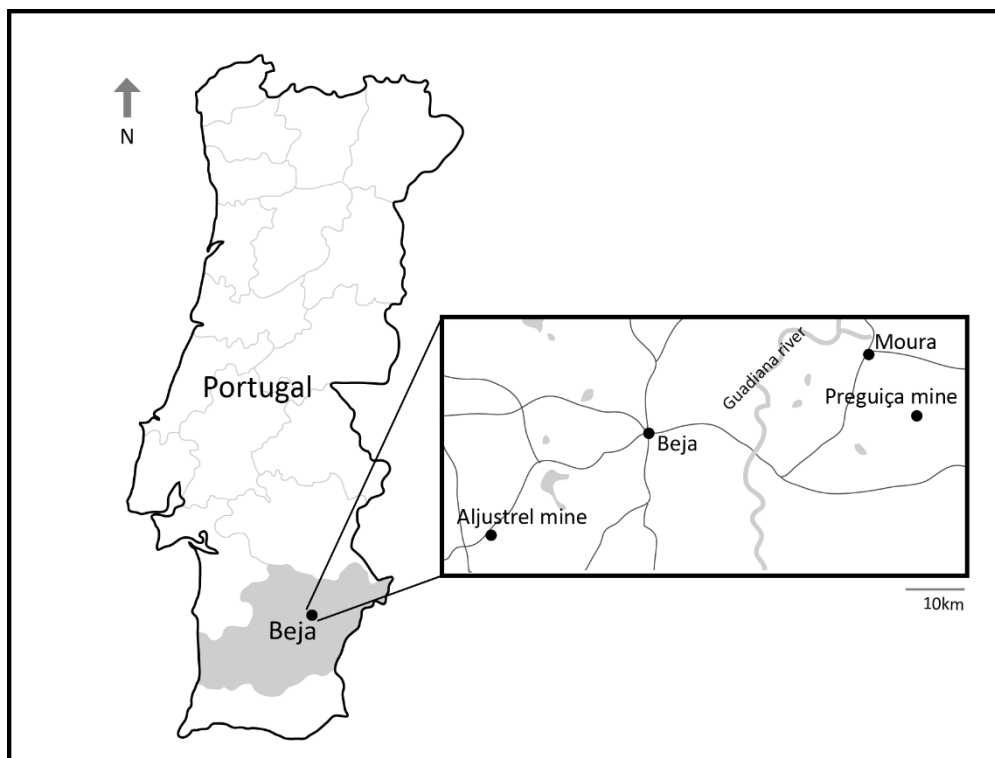


Fig. 1. Location of sampling sites: Aljustrel and Preguiça mines and the Reference site (Moura).

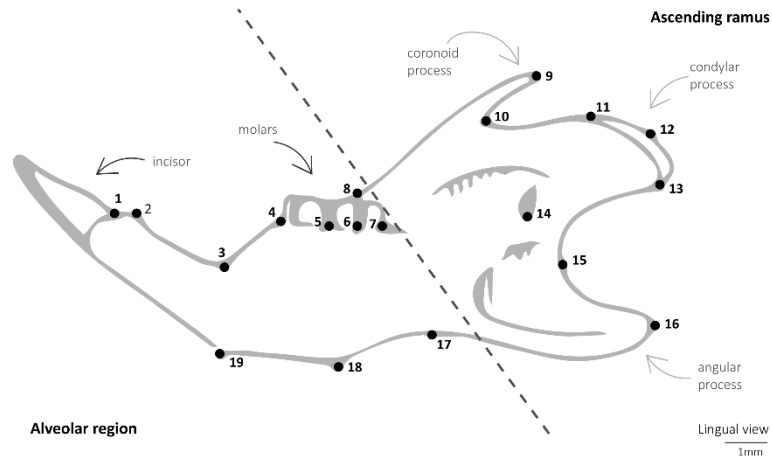
In 2002-2003, both Preguiça and Aljustrel mines were inactive. The Preguiça mine is located approximately 20 km southeast from the reference location (see below), in Preguiça Mountain ($38^{\circ}02'15''\text{N}$; $07^{\circ}17'01''\text{W}$) in the Iberian Magnetic-Zinciferous Belt. This area was dominated by trees, shrubs and herbaceous species at the time of sample collection. The Aljustrel mine is located approximately 72 km southwest ($37^{\circ}53'08''\text{N}$; $08^{\circ}08'32''\text{W}$) of the Reference site in the Iberian pyrite belt, known for its pyrite deposits. Sampling was performed along “Água Forte” riverside, which is characterized by its red color due to stored acidic effluents discharged during rainy periods. For comparative purposes, a site with similar features to the mining surroundings, in terms of climate and vegetation, and without known exogenous sources of heavy metals was selected, and referred hereafter as the Reference site (Moura) ($38^{\circ}11'13''\text{N}$; $07^{\circ}24'34''\text{W}$).

2. Morphometric analyses

In the individual morphometric analysis only *Mus spretus* specimens were used since the short number of available samples of *Crocidura russula* was inadequate to allow inter-site comparisons. The right and left side of the mandible of 124 specimens of *Mus spretus* were analyzed: 48 from Aljustrel, 49 from Preguiça, and 25 from the Reference site. To reduce a source of bias from growth patterns, only adult mice were used, classified according to the criteria established by Mira and Mathias (1996): weight > 8g, body length >70mm, and with a brownish pelage. All specimens were cleaned by the simmering method. The lingual views of right and left mandible were scanned with a scale in a HP Scanjet 4470c Scanner (2400px, 24bit color) in the same conditions. Nineteen two-dimensional landmarks (LMS) were digitized three times by the same person using tpsDig2 software v. 2.26 (Rohlf, 2010). To analyze the form of the hemi-mandible, each set of landmarks was decomposed into size and shape, which were studied separately. Size of hemi-mandibles were estimated from their centroid size, calculated as the square root of the sum of squared distances of each landmark from their centroid (Bookstein, 1991). The landmarks of the left hemi-mandible were mirrored, superimposed using generalized Procrustes fit and projected onto the shape tangent space (Goodall, 1991; Klingenberg and McIntyre, 1998). In this way the variation of size, position and orientation is removed, and the landmark coordinates of the superimposed configurations (called Procrustes coordinates) were used for further multivariate analyses (Klingenberg et al., 2003).

2.1. Analyses of source of size and shape variation

Procrustes coordinates and centroid sizes were analyzed by means of multivariate statistics methods: a Procrustes analysis of variance (ANOVA) and two-factorial ANOVA, respectively. Individual and side were the random and fixed main effects and the Procrustes distance the dependent variable (Palmer and Strobeck, 1986). The individual factor stands for individual variation (symmetric component of variation), while the latter characterizes directional asymmetry (i.e. the average difference between the left and right side of the mandible in the whole sample). The interaction between these two factors (individual and side) represents the non-directional asymmetry (fluctuating asymmetry and antisymmetry - asymmetric component of variation, i.e. the variation of differences between the left and right mandible among individuals) (Klingenberg and McIntyre, 1998; Palmer, 1994). Measurement error was quantified from residual variance between replicate components. Site and sex were additional main effects in Procrustes ANOVAs. Since no statistically significant value was found for sex, all data were pooled. Differences in size and shape were tested between sites for all mandibles. To study the total shape variation, Procrustes ANOVAs were partitioned into symmetric (variation among individuals) and asymmetric (variation within individuals) components (Klingenberg et al., 2002; Klingenberg et al., 2003). To assess variations in shape in each functional module, the hemi-mandible was divided into two subsets corresponding each to a mandibular module, the alveolar region and the ascending ramus, composed of eleven and eight landmarks, respectively (Fig. 2).



Landmark	Description
1	Anterior most point on incisor alveolar (point of symphysis)
2	Posterior most point of symphysis
3	Minimum of depression of incisor ramus
4	Anterior most point of first-molar
5	Anterior most point of second-molar
6	Anterior most point of third-molar
7	Posterior most point of third-molar
8	Intersection of molar alveolar and base of coronoid process.
9	Dorsal-most point of coronoid process
10	Minimum of depression posterior of coronoid process – Mandibular notch
11	Anterior most point of condylar process at junction with the articular disc
12	Central most point of condylar process at junction with the articular disc (middle of articular disc)
13	Posterior most point of condylar process at junction with the articular disc
14	Anterior-most point along anterior edge of the mandibular foramen
15	Intermedium point between condylar and angular process
16	Posterior most point of angular process
17	Minimum of depression formed by angular process and incisor ramus
18	Posterior most tuberosity of insertion site of mandibular transverse muscle
19	Anterior most tuberosity of insertion site of mandibular transverse muscle

Fig. 2 Location and description of the 19 landmarks used in the geometric morphometric analysis in the right hemi-mandible of *Mus spretus* (lingual view). The dash line divides the alveolar module of the ascending module.

2.2. Analyses of size and Allometry

Allometry is the dependence of shape on size and can potentially bias the analyses of shape. Allometry was analysed through multivariate regression of both components, using the symmetric and asymmetric component of shape onto symmetric and asymmetric log centroid size, respectively. Statistical significance of regression was obtained through permutation tests with 10000 iterations ($p < 0.01$) under the null hypothesis of independence between size and shape (Klingenberg, 2011). Mean centroid size and standard deviation were calculated among specimens of sites, for the entire mandible and the two mandibular modules separately.

2.3. Analyses of shape variation and Morphological distances

To explore mandible shape difference and assess morphological distance (in Mahalanobis distance), a canonical variates analysis (CVA) for symmetric and asymmetric components was performed. This analysis is intended to capture the amount of variation in one set of variables that is accounted by the other set of variables along one or more axes. Mahalanobis distances were calculated from size-corrected raw data between sites. Statistical difference was calculated by a permutation test (10000 permutation rounds; $p < 0.01$) (Klingenberg, 2011).

2.4. Fluctuating Asymmetry

When analyzing fluctuating asymmetry (FA), it is important to perform a preliminary analysis to detect features that may confound the analysis of FA (Palmer, 1994; Palmer and Strobeck, 1986), such as directional asymmetry (DA; a side of a bilateral character that is consistently larger than the other) and antisymmetry (AS; a systematic deviation from symmetry) (Klingenberg, 2015; Palmer, 1994). To check if variation among individuals (FA and DA) was significant, parametric F-tests were performed in Procrustes and two-factor ANOVAs. These three types of asymmetry can be distinguished according to the distribution of left-right individual means of population (Graham et al., 2010; Klingenberg, 2015). In fluctuating asymmetry, differences between left and right trait values (L-R) in a population are randomly clustered around a mean of zero that represents perfect symmetry (Palmer, 1994). In directional asymmetry, the mean difference between traits values is not zero, and in antisymmetry, a systematic deviation from symmetry results in a bimodal or platikurtic distribution (large dispersion of data along the X axis, with thinner tails in comparison with normal distribution) with a mean of zero (Graham et al., 2010). To check for the existence of antisymmetry, asymmetric components of shape variation were inspected for signs of deviations from normal distribution using the Kolmogorov-Smirnov test and kurtosis test. All sites revealed a bimodal distribution and significantly negative values in kurtosis (see results), suggesting the existence of antisymmetry. Considering that one of the factors that can influence the pattern and amount of FA is allometry (Klingenberg, 2015), it was investigated if this antisymmetry came from the relation between size and shape. Since the allometric results in the asymmetric component of shape revealed a great dependence on size (see results), a distribution of the sum of the absolute residual values across the landmarks of the asymmetric components was performed. Normality was tested with a Kolmogorov-Smirnov and a kurtosis test. Since a normal distribution was found (see results), and the asymmetric direction was negligible (see results), residual values were considered to correspond to shape FA. To detect shape FA divergences among sites a one-way ANOVA was performed, where FA values were the dependent variable and sites the fixed effects. For evaluating differences between sites, a Tukey's Honestly Significant Difference (HSD) was used in ANOVA procedures (Spjøtvoll and Stoline, 1973). The Kolmogorov-Smirnov test of normality, the kurtosis test and the Tukey's HSD were performed using SPSS v.23.0 software (IBM Corp, 2013). All the other analyzes were conducted with the geometric morphometric methods implemented in MorphoJ v.1.06d.

3. Genetic analyses

3.1. DNA extraction, PCR amplification and Sequencing

A total of 63 *Crocidura russula* (25 from Aljustrel, 22 from Preguiça, and 16 from the Reference site) and 75 *Mus spretus* (25 from each site) were studied. Total genomic DNA was extracted from the tail using a commercial DNA extraction kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Inc) following

the manufacturer's protocol. For *Mus spretus*, the mitochondrial *Cytb* gene was amplified using the primer pair L14723 (Lecompte et al., 2002) and H15915 (Irwin et al., 1991), and the control region using the primer pair L15774 (Shields and Kocher, 1991) and H2228 (Searle et al., 2009). The *Cytb* gene of *Crocidura russula* was amplified using the primer pair L14727-SP and H-15915-SP (Jaarola and Searle, 2002). All PCR reactions were performed with 12,5 μ l *Taq* DNA Polymerase Master Mix 2X (2mM MgCl₂) (VWR, Belgium), 0.1 μ M of each primer and 10-20ng of genomic DNA in a total volume of 25 μ l. Amplification conditions for *Mus spretus* were the following: pre-denaturation at 95°C for 5 min (one cycle), 30 cycles of denaturation at 95°C for 1 min, annealing temperature at 61°C (*Cytb*) or 64°C (control region) for 1 min, and extension at 72°C for 1 min, plus a final extension at 72°C for 10 min. Amplification of *Crocidura russula* *Cytb* was performed with the following conditions: 95°C for 5 min (one cycle), 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were purified by enzymatic clean-up (Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Termo Scientific, USA)), followed by sequencing in the forward direction by STABVIDA (Almada, Portugal), or in both directions whenever ambiguous positions were found. Sequences were aligned using the Clustal W algorithm (Thompson et al., 1994), revised and edit manually in Bioedit Sequence Alignment Editor 7.2.5 (Hall, 1999), and trimmed to a final length of 1038bp (*Cytb*) and 824bp (control region). To discard amplification of nuclear pseudogenes, the *Cytb* sequence was translated to protein using the vertebrate mitochondrial code in MEGA v.6 (Tamura et al., 2013), and checked for premature stop codons, as well as compared against published sequences from GenBank. Shared sequences were collapsed into haplotypes using the program DNACollapser (Villesen, 2007).

The control region of *Crocidura russula* is found to vary in size, even within one individual (length heteroplasmy), because of variation in the number of repeat motifs in tandem repeat sequences that are present in the left (R1) and right domains (R2) (Fumagalli et al., 1996). To count the number of heteroplasmic and homoplasmic individuals in each population, a region in the control region that includes the R1 domain was amplified using the primer L15774 (Shields and Kocher, 1991) and a designed reverse primer CR_REV (5'-AAC TAC TTC GGC CCC CAT AG-3'). Amplification was performed as follows: 95°C for 5 min (one cycle), 20 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, followed by 12 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, plus a final extension at 72°C for 10 min. Absence or presence of multiple-sized PCR products per individual (homoplasmic and heteroplasmic individuals, respectively) was checked on standard 1% agarose gel electrophoresis. Banding pattern per individual was confirmed by altering amplification conditions. Number of amplified bands varied from 1 to 5, with band modal size of approximately 1200bp. Target amplification was attested by sequencing a subset of PCR products in both directions, followed by comparison with GenBank published sequences.

3.2. Data analysis - Genetic diversity and Population structure

Nucleotide (π) and haplotype (H_d) diversities, numbers of haplotypes (h), variable sites and synonymous and nonsynonymous substitutions were determined with DnaSP v.5.10.01 (Librado and Rozas, 2009). The distribution of pairwise differences for each population (mismatch distributions) were calculated in Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010), and used to statistically test differences in nucleotide diversity values between populations with Mann-Whitney U Test (with continuity correction) using SPSS v.23.0. Differences in haplotype diversity values between populations were tested with a chi-square test in DnaSP v.5.10.01. For all tests, statistically significant values were considered for $p < 0.05$. Analysis of molecular variance (AMOVA) was used to assess genetic differentiation among (ϕ_{ST}) and between (pairwise ϕ_{ST}) populations in Arlequin v.3.5.2.2. Statistical

differences were tested with a permutation test (10000 permutations; $p < 0.05$) in the same software. Haplotype network trees were constructed in Network v.5.0.0.0 (Bandelt et al., 1999), using the median-joining method. Fasta files were converted into nexus format in Concatenator v.1.1.0 (Pina-Martins and Paulo, 2008). Frequencies of individuals with specific haplotypes and with heteroplasmy were compared between populations with a chi-square test ($p < 0.05$) using SPSS v.23.0.

RESULTS

1. Geometric Morphometric Analysis – *Mus spretus* mandible

1.1. Sources of size and shape variation and Analyses of size

As shown in Tables 1 and 2, Procrustes analysis of variance (ANOVA) and two-factors ANOVA conducted on the entire sample revealed a significant effect of the individual, side (directional asymmetry) and their interaction (non-directional asymmetry) in mandible size and shape. A significant difference between sites was found in mandible shape but not in size. Since variation between replicates was significantly exceeded by variation of non-directional asymmetry, the variation due to measurement error was taken as negligible. The centroid sizes (mean \pm standard deviation) of the whole mandible and modules from animals of each site are shown in Table 3.

Table 1 Procrustes ANOVA conducted on the entire sample to evaluate the influence of measurement error on shape data.

Effect	Shape						
	SS	df	Ms	F	P	Pillai tr	P
Individual	0.827	4046	2.045 x 10 ⁻⁴	2.77	<0.0001	25.29	<0.0001
Side	0.047	34	1.372 x 10 ⁻³	18.61	<0.0001	0.86	<0.0001
Individual x Side	0.303	4114	7.372 x 10 ⁻⁵	14.43	<0.0001	20.56	<0.0001
Site	0.044	68	6.430 x 10 ⁻⁴	3.15	<0.0001	1.21	<0.0001
Measurement error	0.085	16592	5.110 x 10 ⁻⁶				

SS, sum of squares; df, degree of freedom; MS, mean squares; F, F statistic; P-value; Pillai tr, Pillai's trace

Table 2 Two-factor ANOVA conducted on the entire sample to evaluate the influence of measurement error on size data.

Effect	Size				
	SS	df	Ms	F	P
Individual	330.846	119	2.780	9.12	<0.0001
Side	2.333	1	2.333	7.66	0.0065
Individual x Side	36.871	121	0.305	13.59	<0.0001
Site	8.956	2	4.478	1.65	0.204
Measurement error	10.94	488	0.022		

SS, sum of squares; df, degree of freedom; MS, mean squares; F, F statistic; p-value

Table 3 Symmetric centroid size (mean \pm standard deviation) of the whole mandible of *Mus spretus* and of the two mandibular modules, in each sampling site.

Sites	Centroid Size (mm)		
	Whole mandible	Alveolar region	Ascending ramus
Aljustrel	18.53 \pm 0.043	8.83 \pm 0.016	6.38 \pm 0.019
Preguiça	18.72 \pm 0.042	8.85 \pm 0.016	6.45 \pm 0.019
Reference site	18.79 \pm 0.059	8.95 \pm 0.022	6.50 \pm 0.026

1.2. Symmetric component

A significant dependence of the symmetric component of shape on size (allometry) was detected in all sites (6.93%; $p < 0.0001$), in which Aljustrel displayed the highest percentage of allometry (10.01%). The CVA performed in size-corrected data of the entire sample set showed a marked separation between mines sites (Aljustrel and Preguiça) and the Reference site along the axis of the first canonical vector (CV1), which explained 72.69% of total mandible shape variation (Fig. 3). This variation was mainly due to the shape differences in the ascending ramus, in the condylar process. Variation in the second canonical vector (CV2) showed a separation between mines mainly due to shape differences in the condylar and angular processes. The CVA performed on the two separate modules showed the same separation of sites along the CV1 and the CV2 axes (Fig. 4 and 5), with the CV1 axis explaining 72.46% and 57.05% of shape variation in the ascending ramus and alveolar region, respectively. Table 4 shows the values of the Mahalanobis distances between sites of the entire sample set. Significant Mahalanobis distances for the whole mandible and for each module were found ($p < 0.0001$), indicating that significant differences among individuals exist in mandible shape. The shortest distance was detected between Aljustrel and Preguiça, while the longest distance was obtained between Preguiça and the Reference site. These results show that animals from mine sites present a more similar shape variation between each other than between Preguiça and the Reference site, which although geographically closer, have more dissimilar populations in terms of variation in the mandibular shape.

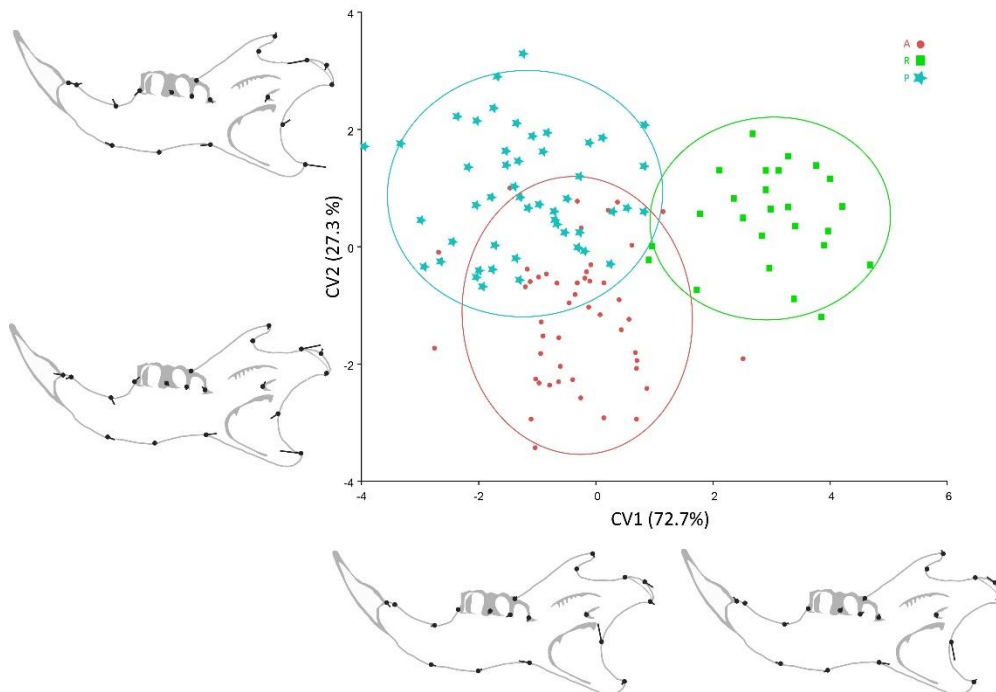


Fig. 3 Plot of the canonical variate 1 (CV1) and 2 (CV2) of symmetric component (A-Aljustrel, R-Reference site, P-Preguiça). Diagrams of shape changes associated with the extreme values of CV1 and CV2 are shown magnified three times.

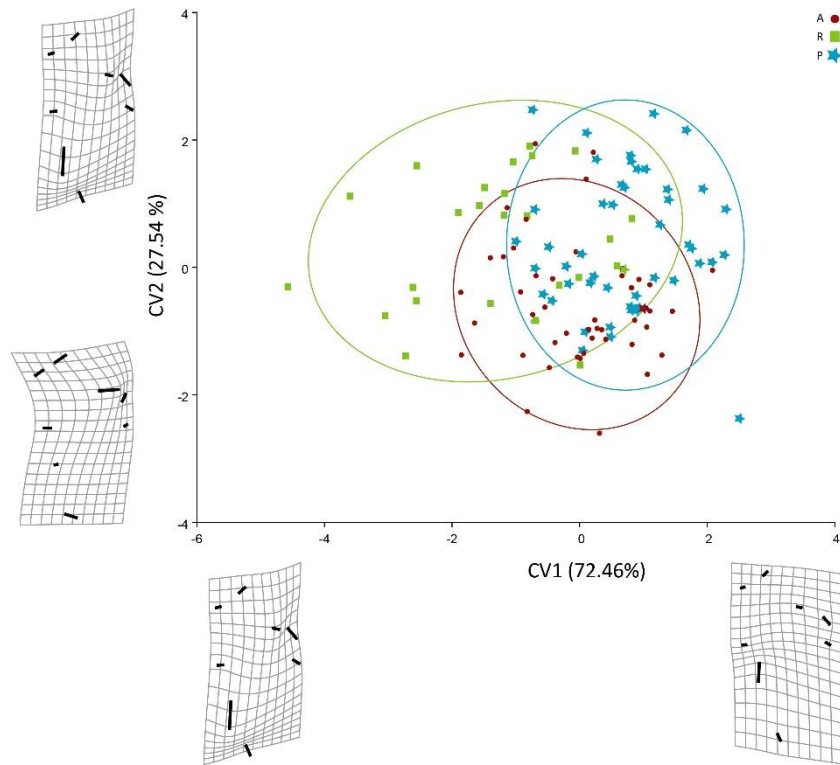


Fig. 4 Plot of the canonical variate 1 (CV1) and 2 (CV2) of symmetric component of ascending ramus (A-Aljustrel, R-Reference site, P-Preguiça). Diagrams of shape changes associated with the extreme values of CV1 and CV2 are shown magnified two times.

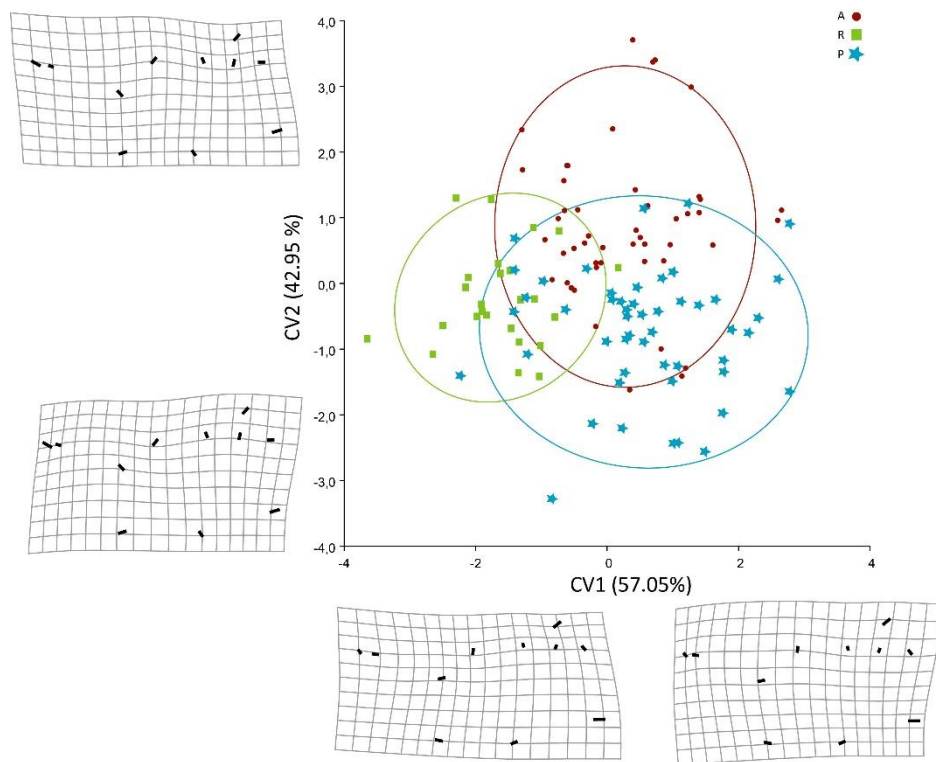


Fig. 5 Plot of the canonical variate 1 (CV1) and 2 (CV2) of symmetric component of alveolar region (A-Aljustrel, R-Reference site, P-Preguiça). Diagrams of shape changes associated with the extreme values of CV1 and CV2 are shown magnified two times.

Table 4 Mahalanobis distances between sites using size-corrected data obtained in the canonical variate analysis of symmetric and asymmetric components.

Sites	Mahalanobis distance					
	Whole mandible		Alveolar region		Ascending ramus	
	Symmetric	Asymmetric	Symmetric	Asymmetric	Symmetric	Asymmetric
Reference site vs Aljustrel	3.69*	2.29*	2.19*	1.65*	1.61*	0.78
Reference site vs Preguiça	4.21*	2.34*	2.24*	1.81*	2.04*	0.89
Aljustrel vs Preguiça	2.26*	1.54**	1.63*	1.07***	1.20*	0.71

*p < 0.0001; ** p < 0.001; ***p < 0.05

1.3. Asymmetric component

A significant dependence of the asymmetric component of shape on size was detected in all sites (43.27%; $p < 0.0001$), in which Preguiça registered the highest percentage of allometry (47.51%). The CVA conducted in the asymmetric component from the entire sample set revealed that the CV1 explained 62.2% of shape variation. As with the symmetric component, separation between mines and the Reference site was explained along the CV1 axis. This separation was also observed in the alveolar region (CV1: 67.81%). The CVA conducted in the ascending ramus did not show a clear distinction between sites (CV1: 58.5%). Significant Mahalanobis distances for the whole mandible were found ($p < 0.001$), indicating significant shape differences within individuals. The shortest distance was again detected between Aljustrel and Preguiça, and the longest distance between Preguiça and the Reference site. This indicates that mandibular shape varies in a more resembling way within individuals of the mines, a characteristic likely linked to environmental degradation or to the lower quality of food found in mines.

1.4. Antisymmetry and Fluctuating Asymmetry

All sites revealed a non-normal distribution ($0.227 < K-s-d < 0.294$; $p > 0.05$) and negative kurtosis values ($-1.285 < Kurtosis < -1.840$). This indicates that the mandible showed antisymmetry (suggested by the bimodal and platykurtic distribution), i.e. specimens of *Mus spretus* from all three sites had consistently the left mandible larger than the right mandible or vice versa. The distribution of the sum of the absolute residual values across the landmarks, performed to investigate if this antisymmetry came from allometry, showed no significant values of Kolmogorov-Smirnov for normality ($K-s-d = 0.108$; $p > 0.15$), as well as positive values of kurtosis (Kurtosis = 2.631; std.err.kurtosis = 0.435) with a mesokurtic distribution, suggesting that antisymmetry is due to size. Since the variation of non-directional asymmetry was much greater than the variation due to directional variation (Table 1 – Procrustes ANOVA), we considered that this was negligible. The Turkey's HSD test indicated that shape FA values did not differ significantly between sites (Fig. 6).

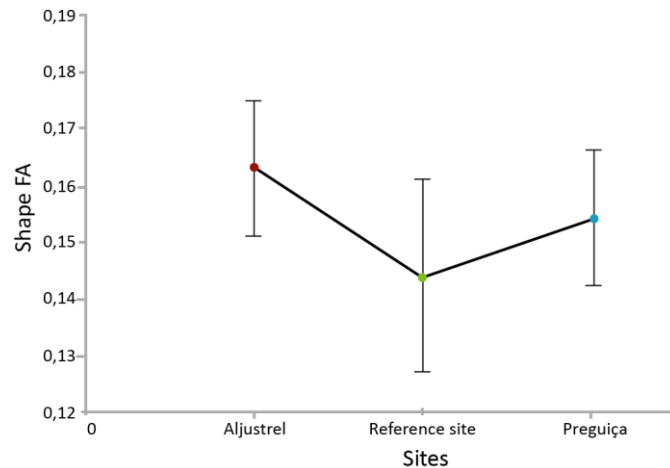


Fig. 6 Variation of Shape FA between sampling sites.

2. Population genetic analysis – *Mus spretus* and *Crocidura russula*

2.1. *Cytb* gene

The amplified *Cytb* sequence was 1038bp in size both in *Mus spretus* and *Crocidura russula* (sequence data have been deposited in GenBank with accession numbers XXXX-XXXX). A total of 26 *Cytb* haplotypes were found in *Mus spretus*: 14 in the Reference site, 12 in Aljustrel and 10 in Preguiça (Table 5). Three haplotypes were common to all sites: haplotypes 1, 2 and 3. The most frequent haplotype (haplotype 2) is identical to an already published haplotype (Accession number JX457726) and was found in individuals captured in northern Portugal and southern Spain (Barbosa et al., 2013). This haplotype is therefore apparently widespread in the Iberian Peninsula and may be an ancestral haplotype in this species. Most individuals in Preguiça (72%) had haplotypes shared with individuals from Aljustrel and the Reference site, while in these populations, sharing was observed in 60% and 56% individuals, respectively. The Reference site had the highest number of polymorphic sites (18), while Preguiça had the lowest (14) (Table 6). Among the three populations, Preguiça also had the lowest number of singleton and private sites (35% and 29% of the variable sites, respectively). A haplotype network using the median-joining approach revealed a star-like pattern for haplotypes from Preguiça and the Reference site, where most haplotypes are connected to haplotype 2 (Fig. 7A). In Aljustrel this pattern is not apparent, since haplotypes were connected to four major haplotypes (15, 1, 2, 18). Population diversity values are presented in Table 5 and Fig. 8. Both haplotype and nucleotide diversities were the lowest in Preguiça. In Aljustrel, nucleotide diversity was also lower when compared to the Reference site, but haplotype diversity was higher, reflecting the more heterogeneous distribution of individuals among haplotypes as already suggested by the network tree. The distribution of haplotypes among the three populations was statistically significantly different between Aljustrel and the other two populations (chi-squared test; $p < 0.05$). The distribution of the pairwise differences (mismatch distribution) in the population of the Reference site differed significantly from that of Preguiça (Mann-Whitney U test; $p < 0.05$) (Fig. 9). The analysis of molecular variance (AMOVA) revealed no genetic structure for *Mus spretus*, indicating that the global source of genetic variation is within and not among groups (Table 7). This analysis revealed little genetic differentiation among the three populations of *Mus spretus* ($\phi_{ST} = 0.0338$), likely reflecting the high dispersion capacity of this species (Gray et al., 1998) and the geographic proximity between locations. Nonetheless, pairwise ϕ_{ST} comparisons showed that the Aljustrel population was differentiated from Preguiça and the Reference site (Table 8), which were more genetically similar with each other in spite of the lower genetic variability found in Preguiça.

Table 5 Distribution of haplotypes, number of haplotypes (h) and Diversity indices (Hd and π) for the *Cytb* gene of *Mus spretus* from the three sampling sites.

Population	Haplotype																										h	Diversity indices		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		Hd \pm s.d.	π \pm s.d.	
Reference site	1	9	2	1	1	1	2	2	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	14	0.870 \pm 0.061	0.0024 \pm 0.0015
Aljustrel	4	3	1	-	-	-	-	-	-	-	-	-	-	-	5	2	1	3	1	2	1	1	1	-	-	-	12	0.920 \pm 0.029	0.0022 \pm 0.0014	
Preguiça	1	9	1	3	1	-	-	-	-	-	-	-	-	-	2	1	-	-	-	-	-	-	-	2	2	3	10	0.850 \pm 0.057	0.0021 \pm 0.0013	
Total	6	21	4	4	2	1	2	2	1	1	1	1	1	1	7	3	1	3	1	2	1	1	1	2	2	3				

Hd \pm s.d. haplotype diversity \pm standard deviation; π \pm s.d. nucleotide diversity \pm standard deviation

Table 6 Sequence diversity values for the *Cytb* gene and the Control region of *Mus spretus* and the *Cytb* gene of *Crocidura russula* from each sampling site. A - Aljustrel, P - Preguiça, R - Reference site.

Sequence diversity	<i>Mus spretus</i>						<i>Crocidura russula</i>		
	<i>Cytb</i>			Control region			<i>Cytb</i>		
	A	P	R	A	P	R	A	P	R
No. of variable sites	15	14	18	9	18	9	16	9	12
No. of singleton variable sites	8	5	9	5	10	5	7	3	6
No. of parsimony informative site	7	9	9	4	8	4	9	6	6
No. of private sites	8	4	10	1	10	0	14	4	7
No. of transitions	14	14	16	9	16	8	15	9	12
No. of transversions	1	0	2	0	3	1	1	0	0
No. of synonymous substitutions	13	13	16	-	-	-	12	7	8
No. of nonsynonymous substitutions	2	1	2	-	-	-	4	2	4

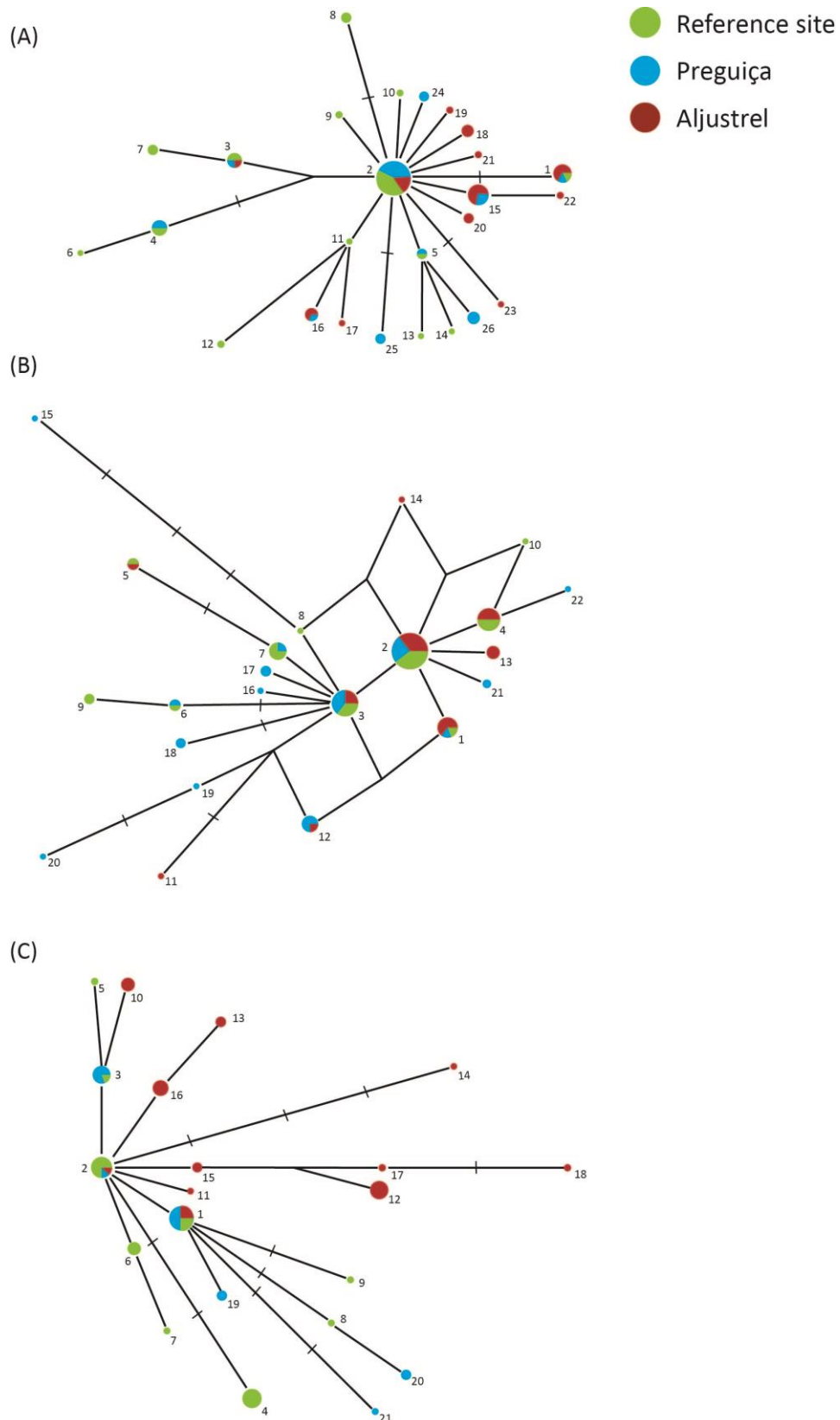


Fig. 7 Median-joining networks of the *Cytb* (A) and the Control region (B) haplotypes found in *Mus spretus*, and of the *Cytb* haplotypes from *Crocidura russula* (C). Sampling sites are coloured differently in a grey scale. Numbers of circles correspond to haplotypes as described in Tables 4, 5 and 6. Areas of the circles are proportional to the haplotype frequency. Each bar on the connecting lines separates two mutational events.

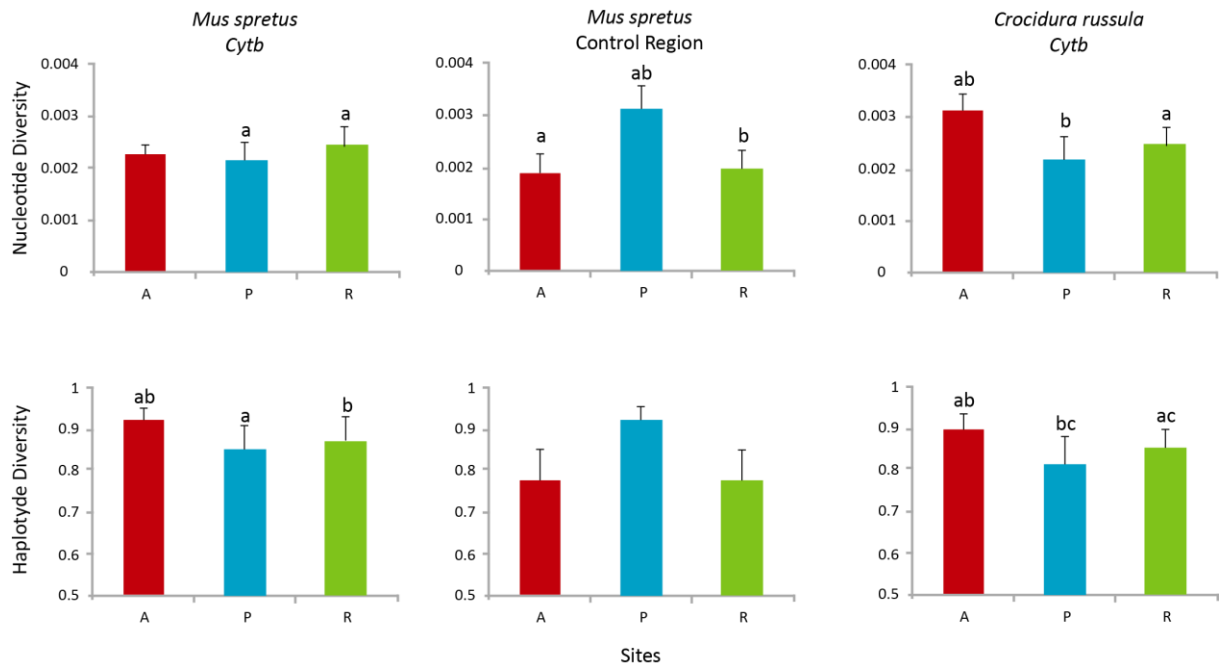


Fig. 8 Nucleotide and haplotype diversities for the *Cytb* gene and the Control region of *Mus spretus* and *Crocidura russula* at each sampling site. The same letter means that values are significantly different ($p < 0.05$). A - Aljustrel, P - Preguiça, R - Reference site. Error bars represent s.d.

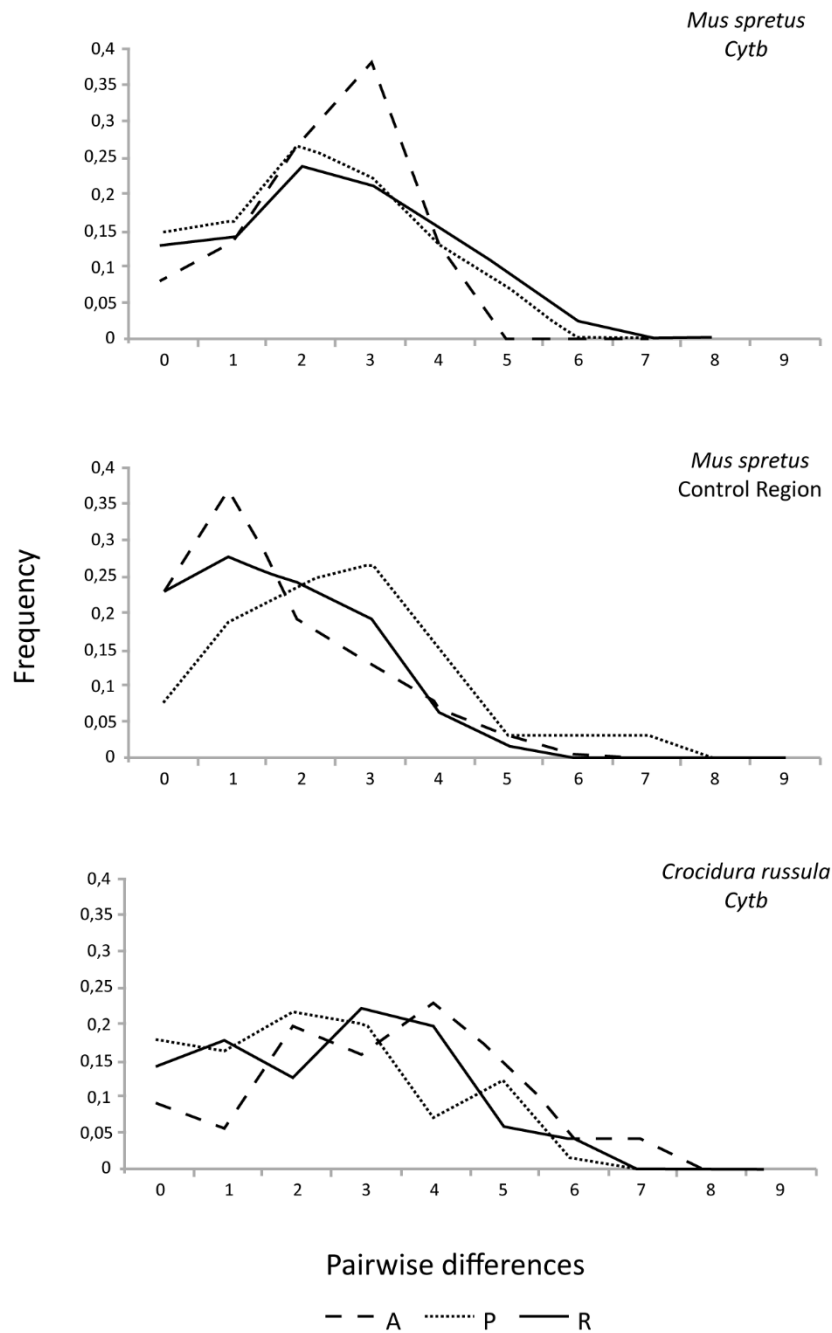


Fig. 9 Frequency distributions of the number of pairwise nucleotide differences (mismatch distributions) between haplotypes of the *Cytb* gene and the Control region in *Mus spretus* and *Crocidura russula* from each sampling site (A-Aljustrel, P-Preguiça, R-Reference site).

Table 7 Fixation index and sources of variation in the *Cytb* gene and the Control region of *Mus spretus* and in the *Cytb* gene of *Crocidura russula*. A - Aljustrel, P - Preguiça, R - Reference site.

Fixation index and source of variation	<i>Mus spretus</i>		<i>Crocidura russula</i>
	<i>Cytb</i>	Control region	<i>Cytb</i>
ϕ_{st}	0.0338	0.0458	0.1594
% among populations	3.38	4.58	15.94
% within populations	96.62	95.42	84.06

Table 8 Pairwise ϕ ST comparisons between populations from each sampling site of *Mus spretus* and *Crocidura russula*, for the *Cytb* gene and the Control region.

Pairwise ϕ ST	<i>Mus spretus</i>				<i>Crocidura russula</i>	
	<i>Cytb</i>		Control region		<i>Cytb</i>	
	Aljustrel	Preguiça	Aljustrel	Preguiça	Aljustrel	Preguiça
Preguiça	0.044*	-	0.065*	-	0.182*	-
Reference site	0.055*	0.0017	0.037	0.033*	0.159*	0.127*

* $p < 0.05$

A total of 21 haplotypes were found in the three populations of *Crocidura russula*: 9 in the Reference area, 11 in Aljustrel and 6 in Preguiça (Table 9). The three sites differed in the most frequent haplotypes: 1 and 3 in Preguiça, 2 and 4 in the Reference area, and haplotype 12 in Aljustrel. Most individuals in Preguiça (69%) had a shared haplotype, which contrasted with Aljustrel and the Reference site, where sharing was observed in 19% and 43% of individuals, respectively. Aljustrel had the highest number of polymorphic sites (16) (Table 6), while Preguiça had the lowest number of polymorphic sites (9), and of singleton and private sites (33% and 44% of the variable sites, respectively). The haplotype network shows that, in each population, haplotypes are not connected to a single haplotype but to the most common haplotypes (Fig. 7C), suggesting that none population is expanding. Haplotype and nucleotide diversity values were lower in Preguiça and higher in Aljustrel (Table 9 and Fig. 8). Haplotype diversity was statistically different among the three populations (chi-squared test; $p < 0.05$). Aljustrel had a mismatch distribution that differed from the Reference site and Preguiça (Mann-Whitney U test; $p < 0.05$). The analysis of molecular variance also revealed that the global source of genetic variation in *Crocidura russula* is mostly within and not among groups of populations (Table 7). All three populations were greatly differentiated among them (ϕ ST=0.1594), and between each other (pairwise ϕ ST; Table 8). This result is comparable to that found with microsatellite and D-loop markers, where significant differentiation was observed among populations located within small geographic distances (Balloux et al., 1998; Ehinger et al., 2002).

A smaller fragment of *Cytb* with 904bp was used to allow haplotype comparisons with known *Crocidura russula* *Cytb* sequences. The smaller versions of haplotypes 1 and 3, the two most common in Preguiça, were identical, respectively, to published haplotypes H1 (Accession number AY918373) and Eur6 (Accession number KT167331). These haplotypes are apparently widely spread throughout the distribution range of this species in Europe: haplotype H1 (=H8 or EUR1) was found in northern France, southeast Switzerland, southern Portugal and central Iberian Peninsula (Brandli et al., 2005; Fontanillas et al., 2005; Gargan et al., 2016), and haplotype EUR6 in northern Spain (Gargan et al., 2016).

Table 9 Distribution of haplotypes, number of haplotypes (h) and Diversity indices (Hd and π) for the *Cytb* gene of *Crocidura russula* from the three sampling sites.

Population	Haplotype																					h	Diversity indices	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		Hd \pm s.d.	π \pm s.d.
Reference site	3	6	1	6	1	3	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	9	0.858 \pm 0.044	0.0025 \pm 0,0015
Aljustrel	3	1	-	-	-	-	-	-	-	3	1	6	2	1	2	4	1	1	-	-	-	11	0.903 \pm 0.034	0.0031 \pm 0,0018
Preguiça	5	1	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	1	6	0.817 \pm 0.063	0.0022 \pm 0,0014
Total	11	8	6	6	1	3	1	1	1	3	1	6	2	1	2	4	1	1	2	2	1			

Hd \pm s.d. haplotype diversity \pm standard deviation; π \pm s.d. nucleotide diversity \pm standard deviation

Table 10 Number of animals of *Crocidura russula* with length heteroplasmy in the Control region.

Frequency	Site		
	Aljustrel	Preguiça	Reference site
Homoplasmic	14 (56%)	3 (19%)	13 (57%)
Heteroplasmic	11 (44%)	13 (81%)	10 (43%)

Table 11 Distribution of haplotypes, number of haplotypes (h) and Diversity indices (Hd and π) for the control region of *Mus spretus* from the three sampling sites.

Population	Haplotype																						h	Diversity indices	
	1	2	3	3	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		Hd \pm s.d.	π \pm s.d.
Reference site	1	7	4	4	1	1	3	1	2	1	-	-	-	-	-	-	-	-	-	-	-	10	0.877 \pm 0.040	0.0025 \pm 0.0016	
Aljustrel	4	7	3	4	1	-	-	-	-	-	1	1	3	1	-	-	-	-	-	-	-	9	0.870 \pm 0.039	0.0023 \pm 0.0015	
Preguiça	1	6	4	-	-	1	1	-	-	-	3	-	-	-	1	1	2	2	1	1	2	1	14	0.923 \pm 0.031	0.0033 \pm 0.0020
Total	6	20	11	8	2	2	4	1	2	1	4	1	3	1	1	1	2	2	1	1	2	1			

Hd \pm s.d. haplotype diversity \pm standard deviation; π \pm s.d. nucleotide diversity \pm standard deviation

2.2. Control region

In *Crocidura russula*, the frequency of individuals with length heteroplasmy in the control region was statistically different between sites (chi-squared test; $p < 0.05$). The population from Preguiça was mostly heteroplasmic (81% individuals), contrasting to the Reference site and Aljustrel where, respectively, 43% and 44% individuals had heteroplasmy (Table 10).

The amplified sequence in the control region of *Mus spretus* was 825bp in size (deposited in GenBank with accession numbers XXXX-XXXX). We found a total of 22 haplotypes: 10 in the Reference site, 9 in Aljustrel and 14 in Preguiça (Table 11). Haplotype 2 was the most frequent haplotype among the three populations. Haplotype 3 is also a common haplotype and is identical to the haplotype found in three specimens caught near Madrid, Spain (Accession numbers DQ266070-72). Contrasting with that found with the *Cytb* gene, nearly 40% of the Preguiça population carried exclusive haplotypes. In Aljustrel and in the Reference site 80% and 84% of the individuals had shared haplotypes, again in an opposite pattern to that found with the *Cytb* gene. As shown in Table 6, Preguiça had the highest number of polymorphic sites (18), and of singleton and private sites (55% of the variable sites). Most variable sites in Preguiça were transitions of each type (C/T and A/G), while in Aljustrel and in the Reference site variation were mostly C/T transitions (67% and 63%, respectively). An indel was found in position 674. The majority of the individuals had haplotypes with deletion: 80%, 72% and 92% in Aljustrel, the Reference site and Preguiça, respectively (Table 12). This deletion is also present in the above-mentioned three specimens from Madrid but absent in an individual sampled near Cádiz, Spain (Accession number U47539), and therefore its presence/absence seems to be polymorphic in *Mus spretus*. Haplotype network revealed an intricate pattern of relationships among haplotypes from all the populations, largely reflecting the sharing of variable sites between haplotypes (Fig. 7B). This indicates that most variation occurs in preferential sites (“hotspots”) along the control region and that the observed haplotypes are different combinations of these variable sites. This is particularly noticeable in the populations of Aljustrel and the Reference site, while in the population of Preguiça the majority of variation is observed in private sites. In an opposing pattern to that observed with *Cytb*, both haplotype and nucleotide diversities in the control region were the highest in Preguiça and the lowest in Aljustrel (Table 12 and Fig. 8). The distribution of haplotypes did not differ between populations (chi-squared test), but Preguiça differed significantly from the Reference site and Aljustrel in the distribution of pairwise differences (Mann-Whitney U test; $p < 0.05$) (Fig. 8). The analysis of molecular variance revealed no genetic structuring in *Mus spretus* (Table 8). Genetic differentiation was low among the three populations ($\phi_{ST} = 0.0458$), however, pairwise ϕ_{ST} estimates showed that the population from Preguiça was genetically differentiated from those of the Reference site and Aljustrel (Table 9), again contrasting to that found with the *Cytb* gene.

Table 12 Number of animals of *Mus spretus* with an insertion or deletion at position 674 of the Control region.

Frequency	Site		
	Aljustrel	Preguiça	Reference site
w/ insertion	5 (20%)	2 (8%)	7 (28%)
w/ deletion	20 (80%)	23 (92%)	18 (72%)

DISCUSSION

In this study, the same populations of *Mus spretus* and *Crocidura russula* living in heavy metal polluted mines, that were previously found to harbour changes at the biochemical, histological and physiological levels (Marques, 2008; Marques et al., 2008; Marques et al., 2007; Sanchez-Chardi et al., 2008; Sanchez-Chardi et al., 2007), were shown here to have acquired alterations at two other levels of biological organization as well, the morphometric and the genetic levels.

The individual morphometric geometric analysis of the mandible of adult *Mus spretus* revealed that animals from the three sites differed in the shape of the mandible. Also, mandibular shape from the animals of the polluted sites was morphologically more alike, as measured by the Mahalanobis distances of the shape, than the mandibles from animals of the Reference site. The highest difference between the mandible shape of animals from the polluted sites and of the Reference site was found in the ascending ramus, particularly in the condylar process, whereas between animals of the two mine sites shape variations were located in both the angular and condylar processes. The alveolar module holds the teeth and is involved in the cutting and chewing of food or other material, while the ascending ramus articulates with the skull and participates in the attachments of masticatory muscles. In the evolution of Murinae, changes in the shape of the mandible were shown to be related to the functioning of the mandible, namely with the type of consumed food (Michaux et al., 2007). *Mus spretus* is omnivorous and slight preferences in diet could explain the separation of the groups as a result of local adaptation to the available food type (Homsí and Aulagnier, 2010; Luca et al., 2003; Maki et al., 2002). The landscape from Aljustrel mine was particularly degraded and mine tailings were un-vegetated at the time of sampling; in contrast, Preguiça was covered by vegetation that hid all the tailings and scoria produced by mining. Because bone growth is a dynamic process, with formation and resorption of bone that responds to the mechanical forces experienced (Renaud et al., 2010; Vecchione et al., 2010), mandibles may have been shaped differently in young mice from both sites during their post-natal development (Martinez-Vargas et al., 2017). Nonetheless, vegetation and general landscape at the Reference sampling site were very similar to that of Preguiça. Assuming a similar genetic background in these two populations of *Mus spretus* (as suggested by the diversity of *Cytb* gene), the observed differences in mandibular morphology could have been induced by distinct internal stressors that disturb metabolic pathways and gene expression of developmental and bone remodelling genes. Exposure to environmental pollutants *in utero* is known to affect fetal skeleton development and growth (Allen and Leamy, 2001; Sholts et al., 2015). Interestingly, animals from Preguiça and the Reference site had the most dissimilar mandible shapes among all sites (higher Mahalanobis distances between them), despite genetic proximity, with mandibular shape varying in a more resembling way within individuals of the mines. This is highly suggestive for a role of environmental quality in defining mandibular morphological plasticity in *Mus spretus*. For instance, higher hepatic concentrations of selenium were found in *Mus spretus* from both mines, and deficit or excess of selenium has been shown to influence the biomechanical strength of bones (Turan et al., 2000).

One phenotypic manifestation of variability in development induced by toxics is the presence of deviations from perfect symmetry of paired-structures in the body of exposed organisms. Fluctuating asymmetry has been used as an individual marker of exposure to stress induced by contaminants during the developmental period (Badyaev et al., 2000). This type of asymmetry has been reported in various studies of small mammals living in polluted sites (Nunes et al., 2001a; Sanchez-Chardi et al., 2013; Velickovic, 2004), but there are also examples where it failed to be observed (Allen and Leamy, 2001; Owen and Mcbee, 1990). Nonetheless, although results may differ with species, combinations of specific stressors, level and duration of pollution, and morphological characters, fluctuating asymmetry

is considered a suitable indicator of environmental-induced developmental instability (Beasley et al., 2013). In spite of the presence of contaminants in the soil and vegetation of Aljustrel and Preguiça relative to the Reference site and the evidence of physiologic alterations in the animals from these mines, we did not detect significant differences in mandibular shape fluctuating asymmetry between sites. One possible reason is that this species may have acquired an ability to efficiently buffer any environmental disturbance so that no major disruption of the normal mandibular developmental processes, in terms of symmetry, occurs during the prenatal and early post-natal periods. Fluctuating asymmetry of dental traits size has been reported in *Mus spretus* living in a riparian heavy metal-polluted area in Central Portugal (Nunes et al., 2001a), but susceptibility to pollutants may differ between jaw and dental characters due to their distinct differentiation process (Swiderski and Zelditch, 2013). Also, non-integrated traits, such as teeth traits, seem to be particularly sensitive to environmental perturbations associated with stress (Badyaev and Foresman, 2000). One other possibility could be related with a non-coincident seasonality between heavy metal bioavailability and the reproductive cycle of *Mus spretus* (Lopes et al., 2002). This species typically reproduces in spring and summer (Palomo et al., 2009), and seasonal variation in metallothionein induction (highest in wet season) and in antioxidant enzyme activities (highest in dry season) was in fact observed in adult animals captured in Aljustrel (Marques et al., 2008). Such variation could influence the physiological condition of pregnant females and the consequent induction of offspring asymmetry. Finally, and unexpectedly, *Mus spretus* was found to be antisymmetric in mandible shape, i.e. most individuals were found to be either “left-sided” or “right-sided” asymmetric, a finding not yet reported for this species (not observed in dental characters (Nunes et al., 2001a)), or even described in other small mammals (Palmer, 1996). We are uncertain if this type of asymmetry has confounded our estimates of FA in the mandible of *Mus spretus*, although we tried to reduce this effect by using residual values of shape FA. Antisymmetry in morphological characters in other animal groups has been linked to behavioral laterality (cuttlefish *Sepia lycidas* (Lucky et al., 2012)) or sexual dimorphism (larger claw in male fiddler crabs of the genus *Uca*). *Mus spretus* shows a behavioral pattern in the use of the mouth not reported in other rodents, with both males and females picking up feces in their mouths or rolling them forwards or sideways with the tip of the snout, often repeatedly, in distances of several centimeters (Hurst and Smith, 1995). Whether this behavior is associated with evolution of mandibular antisymmetry in *Mus spretus* is purely hypothetical. Moreover, to ascertain that this peculiarity is truly species-specific and not particular to this region of the Iberian Peninsula (due to phylogeography or a local adaptation), a larger sampling effort covering the whole distribution range of *Mus spretus* should be accomplished in the future.

The genetic composition of populations can be affected by environmental pollutants in four main ways: by increasing mutation rates, selecting tolerant genotypes, causing bottleneck events, or altering migration (gene flow) (van Straalen and Timmermans, 2002). The study of the genetic variability of natural populations living in polluted sites may thus reveal a higher, lower variability or no altered patterns relative to a reference area [reviewed in (Mussali-Galante et al., 2014)]. The *Cytb* gene revealed an overall genetic similarity among the three populations of *Mus spretus* ($\phi_{st}=0.03384$; little genetic differentiation). This finding is not unexpected given the high dispersive capacity of this species (Gray et al., 1998). Nonetheless, the Aljustrel population was genetically differentiated from the populations of Preguiça and the Reference site, and had higher haplotype diversity than the latter. This could be explained by several processes: phylogeography, reduction in effective population size in the past followed by genetic drift and population expansion, or admixture of different source populations. The scarcity of phylogeographic studies of *Mus spretus* in the Iberian Peninsula precludes us from confirming or excluding this hypothesis. As a polluted and unsuitable habitat, Aljustrel could have had its population number diminished in a not recent past, with animals failing to adequately survive and reproduce there, being now constituted by a stable population already adapted to that environment; or it

may have been populated by migrant individuals coming from surrounding populations, in which case Aljustrel would have functioned as an ecological sink (Matson et al., 2006). The results from *Crocidura russula*, a species that has a lower dispersive capacity than *Mus spretus* (Balloux et al., 1998), may support the latter scenario. Phylogeographic studies of *Crocidura russula* in the Iberian Peninsula show that this species can be highly variable in the *Cytb* gene even between close localities (Gargan et al., 2016), and indeed our three populations were genetically highly differentiated ($\phi_{st}=0.15936$). Still, the population from Aljustrel had higher genetic diversity values than in the Reference site, with 84% of the animals carrying unique haplotypes (contrasting to 57% and 31% in the Reference site and Preguiça, respectively). Moreover, haplotypes in Aljustrel were more polymorphic than in the other sites, differing on average in 3.24 bases, while in the Reference site and Preguiça average nucleotide differences between haplotypes were 2.55 and 2.26, respectively. The presence of more polymorphic haplotypes in Aljustrel could result from immigration of individuals originating in different neighbouring populations.

The Preguiça mine, on the other hand, seems to have relatively homogeneous populations of *Mus spretus* and *Crocidura russula* as regards *Cytb* diversity: lower nucleotide and haplotype diversities relative to the reference population and high level of haplotype sharing with the Reference site and Aljustrel. Populations living in contaminated environments can undergo a decrease in genetic variation or changes in allele frequencies because of demographic bottlenecks that result from lower fitness and/or reproductive success of the individuals and selection of more tolerant genotypes. This loss of genetic diversity in populations subjected to anthropogenic stress has been labeled “genetic erosion” (van Straalen and Timmermans, 2002). It is therefore conceivable that the populations of both species in Preguiça may have suffered a bottleneck in the recent past, with loss of the rarer haplotypes, and arose from founder populations most probably originating in the Reference site (or its surroundings) given the genetic similarity and geographic proximity between these two sites (20 km apart and no major physical obstacle in-between). The presence/absence of structural variations in the control region of *Mus spretus* and *Crocidura russula* also sustained this scenario. We used these variations as “neutral genetic markers” to measure shifts in population number. The frequency of individuals carrying such structural features in the control region (an indel in position 674 in *Mus spretus* and length heteroplasmy in *Crocidura russula*) was compared between sites. In *Crocidura russula*, length heteroplasmy in the control region is constitutive (Fumagalli et al., 1996), and indeed we found that 43% of shrews in the Reference site were heteroplasmic. A similar frequency was found in Aljustrel (44%), but contrasts with the significantly higher number of heteroplasmic animals found in Preguiça (81%). The results from *Cytb* point to the plausibility of a genetic drift bottleneck being at the origin of this shift in heteroplasmy frequency in Preguiça, but we cannot reject that an elevated instability in repeat length could also have contributed to this increased frequency (Rinner et al., 2011; Yauk and Quinn, 1996), for instance, through loss of replication accuracy of the mitochondrial DNA (increase in replication slippage), deficient repair, or increased mutations in sites near repeats (forming secondary structures). In *Mus spretus*, the indel in position 674 of the control region is also apparently polymorphic in this species. For instance, a deletion is present in three specimens sampled near Madrid, Spain, but absent in an individual sampled near Cádiz, Spain. In our populations, deletion was found in 72% and 80% of the individuals from the Reference site and Aljustrel, respectively, but present in the majority of the individuals from Preguiça (92%). These frequencies, though not statistically different, are again consistent with the lower diversity found in *Cytb* and suggestive that, as for *Crocidura russula*, a decrease in genetic diversity in *Mus spretus* may have occurred in the past in Preguiça.

A change on the diversity parameters of any two mitochondrial regions should coincide in direction due to genetic linkage. Remarkably, in Preguiça, we observed changes in opposite directions in the two markers used in *Mus spretus*. Nucleotide diversity in the control region was significantly

higher in Preguiça than in the Reference site or Aljustrel (these two sites did not differ in the genetic diversity of the control region). Nearly 40% of the individuals had unique haplotypes, contrasting with that found with the *Cytb* gene where most individuals (78%) had shared haplotypes. Preguiça had the highest number of polymorphic and private sites (55% of the variable sites). An increase in genetic variation in mitochondrial DNA may arise from gene flow or new mutations. The former possibility is unlikely since the results from *Cytb* show a relatively low diversified population in Preguiça. Instead, exposure to heavy metals may have increased the genetic diversity of populations through increased mutation rates (Eeva et al., 2006). Many chemicals and heavy metals are known to be mutagenic and genotoxic to *Mus spretus* (da Silva et al., 2013; Ieradi et al., 1998; Marques et al., 2006; Mateos et al., 2008; Tanzarella et al., 2001; Tapisso et al., 2009). Presumably, both mitochondrial regions are equally susceptible to the genotoxic effects by heavy metals but given the involvement of the *Cytb* gene in protein complexes of the OXPHOS pathway, deleterious variants in this gene might be purged from the population (purifying selection). On the other hand, the control region, although functionally important to mitochondrial replication and transcription, is a very variable region and, for this reason, frequently used in intraspecific phylogenetic studies. The intricate pattern of haplotype relationships observed in the network tree might indicate that in *Mus spretus* variation in the control region was concentrated in preferential sites (“hotspots”), especially in the ETAS and CSB domains. The ETAS domain is the most variable domain of the control region in rodents (Larizza et al., 2002). In Preguiça, variable sites were mainly observed in this domain but mutations in conserved regions of the CSB domain were also found and only in this population. The CSB domain in *Mus spretus* is constituted by three functionally important conserved sequence blocks (CSB1-3) (Larizza et al., 2002). Intriguingly, one of the mutations found in Preguiça was located in CSB1, a region that contains the RNA/DNA transition sites, the MRP cleavage sites, and the 3’ end of short RNA primers. These new mutations might only be slightly deleterious, contrasting with *Cytb* mutations. It is possible that conservation of secondary structuring in the control region might act as a selective force that retains novel mutations in this mitochondrial region (Pereira et al., 2008). Moreover, because these mutations were not found in a heteroplasmic form (mutation heteroplasmy), sufficient time must have passed to allow their fixation in the population (Bickham, 2011).

In summary, we have found that populations of two small mammalian species at different trophic levels in the ecosystem, both living in sites polluted with heavy metals, present alterations at their morphology and genetic composition in comparison with a nearby reference population. These alterations are both present in Aljustrel and Preguiça but differ between mines, which could be associated with their distinct metal bioavailability, intensity of metal extraction, landscape, and length of mining (i.e. duration of metal exposure) (Marques, 2008). Our data underlines the notion that metal pollution impacts the immediate natural community at various interconnected levels of biological organization. Animals living in the surroundings of mines or polluted sites display changes at the histologic, enzymatic, transcriptomic and proteomic levels, reflecting the toxic effects of internal contaminants (Abril et al., 2015; Abril et al., 2011; Bonilla-Valverde et al., 2004; Garcia-Sevillano et al., 2014; Marques et al., 2008; Marques et al., 2007; Montes-Nieto et al., 2007; Nunes et al., 2001b; Ruiz-Laguna et al., 2016; Sanchez-Chardi et al., 2009a; Sanchez-Chardi et al., 2009b; Viegas-Crespo et al., 2003). These changes may potentially modulate developmental pathways resulting in morphological variations (Sanchez-Chardi et al., 2013). Even so, animals seem to survive and thrive in these stressful environments (Biser et al., 2004; Levengood and Heske, 2008; Phelps and Mcbee, 2010). Within a relatively short time, the population genetic structure may be altered, and individuals from mines become selected to resist to pollutants, a process already referred as “micro-evolution due to pollution” (Medina et al., 2007). Studies of genetic ecotoxicology such as this have the potential to evaluate the impact of anthropogenic stress in the evolutionary history of natural populations (Banks et al., 2013;

Bickham, 2011). Because the ecological costs of any genetic change are largely unpredictable (Medina et al., 2007), sustainable metal extraction and processing are needed, as well as environmental recovery of abandoned mines to mitigate this impact (Hughes et al., 2008).

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4. FINAL CONSIDERATIONS AND CONCLUDING REMARKS

4. Final considerations and concluding remarks

4.1. Final considerations

Several works have warned of the risks and hazards associated with abandoned mining areas due to the presence of heavy metals (Marques, 2008, Marques et al., 2008, 2007, Sánchez-Chardi et al., 2008, 2007). These metals come from mine tailings and scorias, becoming unpredictable sources of contamination. Portugal, despite having a long mining history, has verified a decline in this activity over the last decades, with consequent abandonment of mining areas without recovery plans. The two mines studied in this work, the Preguiça and the Aljustrel mines, are good examples of how these fluctuations influenced exploration, leading to cyclical mining processes interrupted with new reopening and rapid expansion of mining areas, followed by closure without planned recovery.

Although the regulation and concession of the recovery of abandoned mines date from 2001 (Decreto-Lei n.º 198-A/2001 of July 6), until then this practice of abandonment without a recovery plan was common, leaving a heavy legacy. Currently, a strategic plan for the rehabilitation of abandoned mining areas is in force, starting in 2014 with the concession deadline of 2020, the year in which it is expected to have acted on the main degraded mining areas. Portugal, although it has made some efforts to address this environmental problem, with serious risks to the health and safety of the population, still has a long way to go, regarding the recovery of these areas.

The present work reinforces and once again warns of the importance of abandoned mines recovery planes, and of the relevance of the rehabilitation of areas that have long been deactivated. As mentioned by Santos Oliveira (2002), these areas could be a risk factor for human health and safety, increasing with the time of abandonment. With the present work, we add that mines without rehabilitation are continued sources of pollution, in which the harmful effects of mining remain active and may present high costs to ecological communities.

The use of small mammals as environmental biomonitors allows assessing the effects of medium-long term contaminants in real-time with a realistic measure of toxicity on wildlife populations. The two species used in the present study had already been widely used as sentinels or biomonitors in other studies. However, the present study strengthens not only the relevance and usefulness of *Crocidura russula* and *Mus spretus* in toxicological studies but also its relevance in the field of evolutionary toxicology studies, in particular in the assessment of toxicological effects at the population level. The results of this study confirm that in addition to individual level changes, *Crocidura russula* and *Mus spretus* populations of Aljustrel and Preguiça mine acquired alterations in other organizational levels, having been observed variations in the genetic patterns of these populations and morphological alterations of the mandible in *Mus spretus*. These results show that in a relatively short time, heavy metal pollution is interfering with or changing the evolutionary history of the populations, with unknown consequences.

The understanding of the response at the genetic level of the population was only possible because previous work on biomarkers of exposure, susceptibility, and effect at an individual level were performed. The use of several biomarkers allows a holistic and more integrated view of the problem, alerting the various levels of direct and indirect effects that contaminants can have on individuals and populations. This type of study strengthens and enables higher-level studies, such as at the community or ecosystem level, to be possible.

As mentioned earlier, the measurement of the effect at the population level implies biomarkers adjusted to this organizational level. Few studies on the genetic effects of pollution on wildlife populations were performed, especially in terrestrial territories. The present study was the first to investigate the impact of abandoned mines on the population level in *Crocidura russula* and *Mus spretus*. The study of genetic diversity allows us to analyze the implications that heavy metals can have on populations. Several studies have shown that different mechanisms can disturb populations affecting genetic variability (Bickham, 2011). As seen in the present work different characteristics of mines can lead to different population responses. Populations exposed to heavy metals may result in population bottleneck events; increased mutation rates due to contaminants (having a deleterious effect on individuals' fitness); an increase in genetic diversity due to the gene flow through introducing new alleles into the gene pool of the population; or adapt by becoming resistant populations to contaminants. Whatever the population response to the contaminants, this can create costs at the ecological level, which may affect other trophic levels.

Evolutionary toxicology studies are complex studies since different mechanisms may lead to similar responses and similar mechanisms leading to different responses (van Straalen and Timmermans, 2002). The comparison of the various studies is difficult, since there are a number of variables that determine the response of the population to: types of metals, degree of disturbance (concentration, duration of exposure, route of entry, length of exposure, magnitude), exposure conditions (single or metal mixtures), the marker used in the study, the different ecosystems and species susceptibility (Mussali-Galante et al., 2014). The fact that there are few works in terrestrial mammals and several factors that can interact and influence the response of the population makes it difficult to predict the genetic response of the particular species to a contaminated environment.

The chronic exposure of populations can change the dynamics of populations and may have irreversible costs. Population genetic studies allow an evolutionary view of the impact that anthropogenic stress can have on wild populations. However, only through a growing number of studies that it is increasingly possible to understand population responses under different conditions better. This type of work is fundamental for the realization of environmental protection programs and reclamation of recovery plans for areas that could be potential sources of contamination. This study reinforces the importance of the rehabilitation of abandoned mining areas, mainly areas that do not seem to be serious environmental problems or have been deactivated for a long time.

Several studies on the health risks of abandoned mines have been carried out but they mainly focused on environmental compartments or the individual effect. Nonetheless, few have presented a holistic approach, allowing a comprehensive evaluation of the effects of heavy metal exposure to medium-long term, and this is one of the strengths of the present study. With this work, we reinforce the importance of using sentinels to monitor the possible health risk of resident human populations near abandoned mines, since these sites continue to have harmful effects even after several years of inactivation. These sites may cause adverse effects on people's health, and may in the long run translate, for instance, into an increase in the number of cancer cases (Coelho et al., 2007). Few studies have established a relationship between abandoned mines and the problems in human health, namely in the number of cancers in resident populations near of these areas due to the high number of variables influencing the analysis. However, following our findings, an effort should be made to do so to gain a broader understanding of the real problem of long-term abandoned mines in human health.

Once again, the importance of toxicological studies should be reinforced, not only in the preservation and conservation of wild populations but also in protection of human populations' health living near these sites. Since rapid changes occur in a short time with possible ecological costs, only

through continuous biomonitoring is it possible to alert and act efficiently to avoid adverse effects both individually and at other organizational levels.

4.2. Concluding remarks

1. Major knowledge of the negative impact that long-term chronic exposure has on wildlife populations.
2. Abandoned mines deactivated over an extended period remain sources of contamination.
3. Study of two biomarkers at the population level: morphological and genetic variation.
4. The first population study on genetic diversity in *Crocidura russula* and *Mus spretus*, living in two abandoned mines, unpredictable sources of environmental contamination.
5. The genetic structure and genetic diversity may be used as a population level biomarker to elucidate changes in genetic patterns of exposed populations.
6. Reference values for the analysed biomarkers allowing future comparisons.
7. The first study of geometric morphometry of the mandible in *Mus spretus*.
8. Methodological proposal to study fluctuating asymmetry in populations with antisymmetry.
9. The fact that FA has not been observed, but rather morphological variations and genetic variations in populations reinforce the use of more than one biomarker as a monitoring tool.
10. Apparently, the existence of antisymmetry in the mandible of *Mus spretus*, which may be a characteristic of the species.
11. Strengthening the importance of monitoring sites with potential risks to wildlife and human health.
12. Potential use of *Crocidura russula* and *Mus spretus* as a species for monitoring environmental quality since in both species genetic population responses to polluted environmental using two mtDNA genetic biomarkers (*Cytb* and CR) were found, as well as morphological variation in *Mus spretus*.

4.3. Future perspectives

Portugal has a heavy legacy of abandoned mines. These areas are unpredictable polluted areas of toxicological hazards which make them one of the greatest contemporary environmental problems. Many studies on cellular, molecular and individual effect were performed to understand the adverse effects it has on the individual's health. There is, however, little information about the chronical exposure effect to heavy metals on the level of other biological organizations.

In this way, to understand the impact that heavy metals may have at the population level in these polluted sites, two levels of biomarkers were used in the present study: one genetic and other morphological. This combination of multiple levels of biomarkers reinforces the importance of using a battery of biomarkers to obtain a more holistic view of the problem and more suitable assessment of the response of the populations.

Taking into count the high number of abandoned mines in Portugal and the risks that they represent to the health and sustainability of populations, it is recommendable a continuous monitoring of these areas. These studies must be carried out namely in mines that “apparently” does not have a hazard or are deactivated at a long time. As seen throughout the present study, old mines without rehabilitation continue to be an environmental problem with unpredictable ecological cost. Few works in evolutionary toxicology on terrestrial animals were performed and much less that cover a period that accompanies the evolutionary response of a population. Additionally, since the data from present work have 15 years, it would be interesting to carry out a new biomonitoring adding more reference areas. Incorporate environmental variables and ecological parameters into the data (such as population density, reproduction, and diet), allows a better ecotoxicological characterization and interpretation of the results.

Finally, it is important to emphasize the antisymmetry found in *Mus spretus*. This particularity not yet reported for this species should be confirmed through a wider sampling, including other points of the Iberian Peninsula. The hypothesis of this asymmetry may be linked to behavior effect, reinforces the importance that the behavioral studies have in the interpretation of the evolutionary history of the species.

As seen throughout this work, evolutionary ecotoxicology is a well-developed science but with some complexity due to the limited number of works carried out on wildlife populations and the many factors that may lead to different population responses. Biomonitoring once again proved to be a useful and crucial tool for a rapid and efficient action in species conservation as well as environmental toxicity alert, namely to the man. This work warns for the importance of recovery plans after the mines shutdown and remediation actions for abandoned mines to mitigate adverse impacts.

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ANEXOS

ANEXO I – Protocol of the simmering method

Simmering method for small mammals

1. Cut the head of the specimen with the help of a scissor near the spine so as not to damage any bones of the cranium.
2. Remove the skin from the specimen, starting the cut by the occipital part;
3. Place the specimen in a compress with the respective label. Close the compress as a “candy package” with the help of a thread;
4. Place water in a metal container on a heat plate. Heat the water to 80°. Place the specimens in the water for 15 to 20 minutes, maintaining the water temperature;
5. After the simmering time, remove the sample, place on a petri dish and open the “candy package”;
6. With the aid of a lancet and abundant cool water, manually remove the meat from the bone. The process should be performed slowly and carefully, without forcing movements so that there is no rupture of the bone;
7. After the bone is clean, let it dry for 24 hours.

ANEXO II - Summary of cranium cleaning techniques tested on *Mus spretus*

Methods	Skeleton with soft tissue (small mammals)
Blowflies larvae (Fig.1)	No - In a short time, the bone becomes soft, and the larvae through the movement end up breaking the bone.
Simmering (Fig. 2)	Yes -It is necessary to control the time and temperature of the water so as not to damage the specimen; - Rapid method; - Cleaning multiple specimens simultaneously.
Cold water	No - Becomes a very difficult and time-consuming task, which can lead to the destruction of the specimens
Boil	More or less - The bones become very soft and eventually breaking.
Enzyme (enzyme solution – Neutrase)	No - In very low concentrations the bones break.



Fig. 1 Cleaning of cranium by Blowflies larvae. A) Trapping, placing the trap in a vertical position with pig liver; B) example of some specimens inside the trap; C) production of larvae in the laboratory; D) final cleaning of the skull.

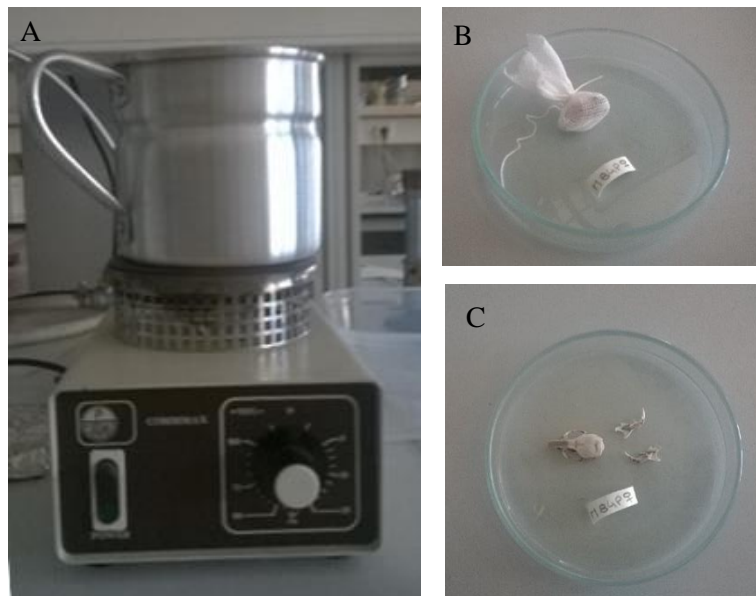
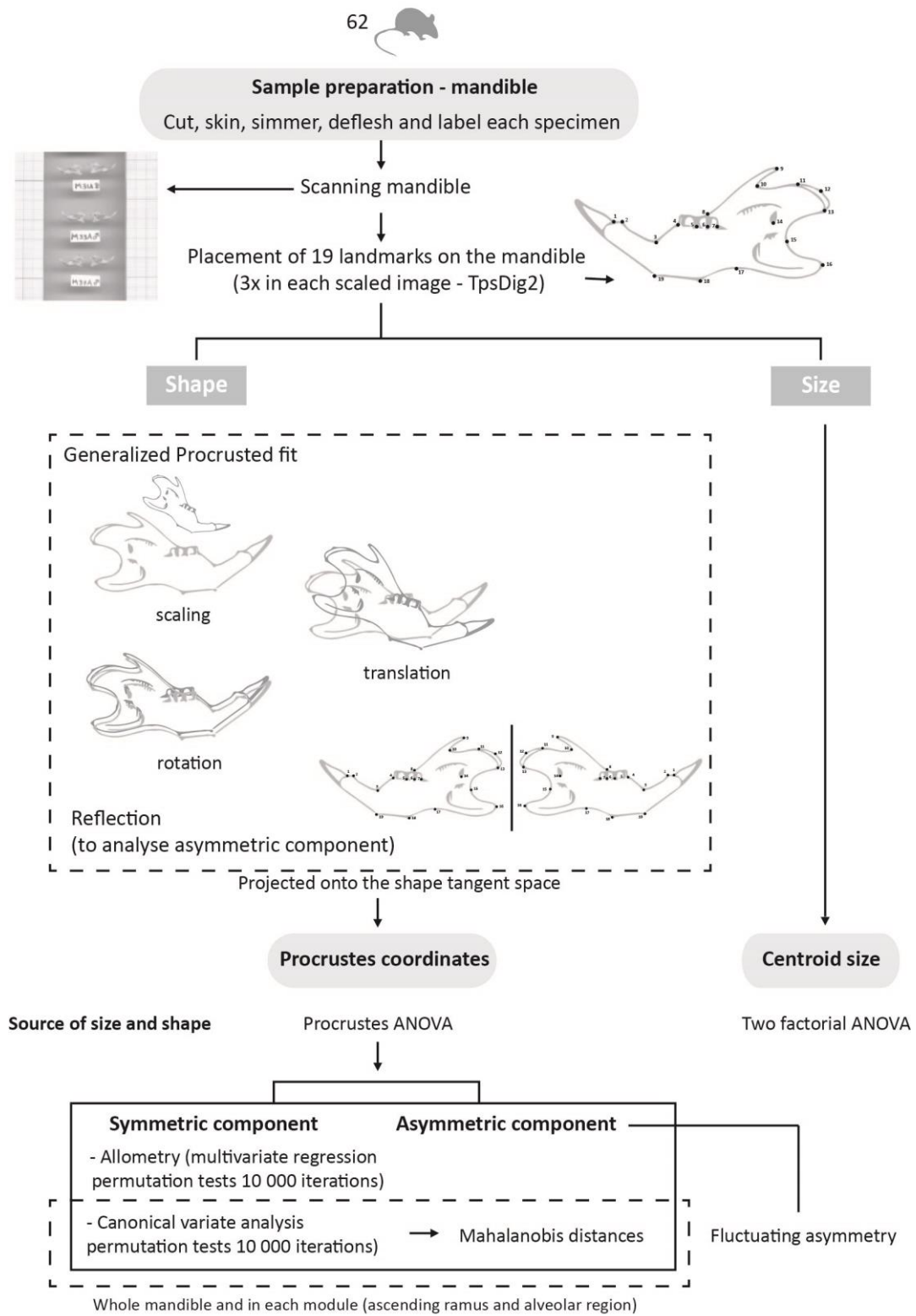


Fig. 2 Simmering method A) Water heated between 70 and 80 ° C; B) Cranium packed in a compress and labeled; C) perfectly cleaned mandible and skull.

ANEXO III - Flowchart of the Geometric Morphometry analysis

Geometric Morphometric analysis



 *Mus spretus*

ANEXO III - Flowchart of the Genetic analysis

