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Metal accumulation and apoptosis in the alimentary canal of *Lumbricus terrestris* as a metal biomarker

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Abstract

The chloragogenous tissue and the intestinal epithelium of adult earthworms, *Lumbricus terrestris*, sampled from sites with and without volcanic activity in the Azores were submitted to hematoxylin/eosin staining, autometallography and TUNEL-test in order to quantify the radial thickness of both tissues, their relative abundance of metals and apoptosis levels. Metals were visualized, through light microscopy, as black silver deposits (BSD) mostly in the chloragogenous tissue. The lowest radial thickness values of both tissues were found in the active volcanic sites, as well as the highest BSD and apoptosis levels. The BSD extent in the chloragogenous tissue, semi-quantified by stereology, exhibited a positive correlation with the apoptosis levels and a negative one with the radial thickness of both tissues. Thus, the variation of the radial thickness of both tissues, but especially of the chloragogenous tissue, which could reflect different cellular turnover rates caused by exposure to metals, is suggested as a biomarker of effect for metal exposure in terrestrial worms inhabiting volcanic environments.

Introduction

Volcanic activity that can manifest through lava emissions, degassing soils and hydrothermal sources, is one of the main inputs of metals, such as Al, As, Hg, Pb, and Zn, in the soils (Ferreira & Oskarsson 1999; Kelepertsis *et al.* 2001). This enrichment in metals renders a high fertility to volcanic soils making volcanic regions densely inhabited and therefore important scenarios for the study of effects of metal pollution.

Previous studies have shown how earthworms are able to tolerate high concentrations of metals in the soil (Ireland 1977; Stürzenbaum *et al.* 1998; Langdon *et al.* 1999, 2001) and that the posterior alimentary duct is their main site of metal accumulation, where the chloragogenous tissue separating the absorptive epithelium from the coelom is a major metal sink (Fischer & Molnar 1992; Morgan *et al.* 2002). The chloragogenous tissue is composed of pedunculated cells and its main functions are (Jamieson 1992): (a) synthesis of hemoglobin; (b) homeostasis of cation composition in the blood and coelomic fluid; (c) maintenance of a balanced pH level; (d) storage of nutrients and waste; and (e) uptake and detoxification of toxic cations. Additionally to chloragogenous tissue, the intestinal epithelium also reveals a great ability for metal accumulation as Morgan *et al.* (2002) found in the oligochaete *Dendrodrilus rubidus*.

Metals cannot be degraded by biological organisms and therefore persist in these as well as in the environment. The determination of the toxicity of metals is difficult because of the complex nature of their interactions with biological systems. Furthermore, for living organisms many metals are vital elements since they play important

roles in the control of gene transcription, redox reactions and oxygen transport. However, essential and non-essential metals may also be prejudicial causing mutagenic (Filipic & Hei 2004; Hei & Filipic 2004), carcinogenic (Waalkes 2003; Waisberg *et al.* 2003) and teratogenic (Calevro *et al.* 1998) effects, which may all be related to apoptosis (Krug 2002). For example, Cd induces disruption of DNA repair leading to mutations that together with increased cell proliferation and blocked apoptosis could result in tumor formation (Waalkes 2003; Waisberg *et al.* 2003; Hei & Filipic 2004). Another example is Zn that has an optimal intracellular range above or below which internucleosomal DNA cleavage, chromatin condensation, and nuclear fragmentation are induced (Krug 2002).

Apoptosis, or programmed cell death, controls the number of cells for different types of organs or tissues and directs the morphological reorganization, avoiding mispatterning during development (Hwang *et al.* 2004). It subserves a general homeostatic function in regulating the size of cell populations under both normal and pathological conditions (Kerr 2002; Zhang & Xu 2002), it is involved in cellular turnover in normal adult animals, it accounts for both normal involution and pathological atrophy of tissues, and it occurs spontaneously in malignant tumors (Kerr 2002). Apoptosis is also present in numerous biological processes like the embryogenesis, the lymphocytic selection within the thymus or the involution of the mammary gland after a lactation period (Seve *et al.* 2002), and it is a well-conserved mechanism throughout metazoan evolution (Hwang *et al.* 2004). Agents that cause necrosis may also trigger apoptosis (Kerr 2002), but unlike necrosis, apoptosis requires energy (Seve *et al.* 2002). Apoptosis by metals can be caused by (a) Ca^{2+} overload, (b) DNA-damaging species and an increase in p53, (c) direct mitochondrial alterations that are upstream of caspase activation, (d) the direct activation of caspases, and/or (e) early activation of death receptors (Krug 2002).

Fragmentation of DNA is a hallmark of apoptosis and besides several biochemical markers that typify programmed cell death, apoptosis is also characterized by morphological markers such as cell and nuclear shrinkage, chromatin condensation, apoptotic body formation followed by phagocytosis of the dying cells (Zhang & Xu 2002), and possibly by morphological cell changes that could alter the feature of the affected tissues.

The capability of earthworms to efficiently retain and compartmentalize metals within tissues may be useful in understanding the basic mechanisms that allow the accumulation of high body burdens (Morgan & Morgan 1989). One of the target tissues for metal accumulation is the chloragogenous tissue (Ireland & Richards 1977; Morgan & Morgan 1989; Morgan *et al.* 2002), which shows a strong plasticity in its morphology provoked by the buildup of high levels of bioavailable metals. Thus, the aim of this study is to investigate if the morphological alterations shown in the chloragogenous tissue and in the intestine epithelium of *Lumbricus terrestris* are related to metal-induced apoptosis.

Materials and methods

Sampling stations

The Azores archipelago is made up by nine islands and is located in the North Atlantic Ocean at the triple junction of Eurasian, African and North American plates, characterized by an intricate tectonic settlement, where the seismic and volcanic occurrences are common (Nunes *et al.* 1993). São Miguel and Santa Maria are the two most eastern islands of the Azores, and the later is the oldest of all nine. Located in São Miguel, which is the largest island (757 km^2), Furnas is a rural parish inside a caldera complex that is considered one of the most active and dangerous volcanoes in the Azores archipelago (Guest *et al.* 1999). Santa Maria, which is one of the smallest islands (92 km^2), is also rural however has no volcanic activity since ca. 3 M.y. ago (Feraud *et al.* 1984).

Earthworms: collection

Forty clitellate *Lumbricus terrestris*, divided by four groups, were collected in autumn/2002. Half of the groups were from two sites within Santa Maria, Feteiras de Baixo (GM) and Aeroporto (FM), and the other half was from two sites in Furnas, Poça da Beija (BM) and near Água Azeda (AM). Earthworms were transferred to the laboratory, where they were depurated of gut contents by maintenance on moistened paper, for 36 h.

Histological processing

The ten specimens of *L. t*. from each site were used for light microscopy morphometry, autometallography and apoptosis analysis. From each worm, a fresh piece, posterior to the clitellum, was fixed in neutral-buffered formaldehyde (Hopwood 1996), dehydrated in alcohol, cleared in methylbenzoate (overnight), rinsed in benzene, embedded in paraffin and sectioned at 7 μm thickness for morphometry and autometallography and at 4 μm thickness for TUNEL-test.

Morphometry

Sections were stained with hematoxylin and eosin (Martoja & Martoja-Pierson 1970). To quantify the radial thickness of the chloragogenous tissue and intestinal epithelium, the first and the fifth sections of all individuals were used and both were theoretically divided into four regions each, meaning a total of eight measurements *per* individual. Measurements were made by a single observer using micrometric eyepieces.

Autometallography

First developed by Danscher (1984), the procedure employed to demonstrate metals in the tissue sections was autometallography, which is a histochemical technique based on principles of photography (Soto *et al*. 1998a). Paraffin sections were dewaxed in xylene, hydrated in ethanol–water mixtures and left in an oven at 37 °C until completely dried. Tissues sections were covered with a photographic emulsion (Ilford Nuclear Emulsion L4) under safety light conditions. After drying for 30 min in total darkness, sections were rinsed in a developer bath (1:5, b/w Ilford PQ Universal) for 15 min, rinsed in a stop bath (1% acetic acid) for 1 min, and finally rinsed in a fixative bath (1:10, b/w Ilford Hypam) for 10 min (Soto *et al*. 1998a). Metal ions were developed as black silver deposits (BSD).

TUNEL-test

The detection of apoptotic nuclei in the intestinal epithelium and in the chloragogenous tissue was performed using a FragEL^{TM} kit (Oncogene, USA). Briefly, tissue sections were dewaxed and rehydrated. Sections were then washed in Tris-buffered saline and treated with 20 μg/ml proteinase K for 20 min at room temperature. Tissues were treated with 3% H_2O_2 for 5 min to inactivate endogenous peroxidase, and their DNA was labeled at 3′ ends with biotin-dUTP by incubation with the reaction buffer containing terminal deoxynucleotidyl transferase enzyme for 90 min at 37 °C. The sections were further incubated with peroxidase streptavidin conjugate to detect biotinylated nucleotides for 30 min at room temperature. Diaminobenzidine reacted with the labeled samples to generate an insoluble colored substrate at the site of DNA fragmentation. Finally, sections were counterstained with methyl green to aid in the morphological evaluation and characterization of normal and apoptotic cells.

Statistical analyses

Radial thickness differences determined by morphometry in the earthworm chloragogenous tissue and intestinal epithelium from the four sites were examined by a one-way ANOVA, while the extent of BSD and apoptotic nuclei differences were examined by the Mann–Whitney test. Pearson correlations between variables, and both tests were determined using the statistical package SPSS 11.5 (SPSS Inc., Microsoft).

Results

Individuals of Santa Maria and Furnas differed significantly not only on the radial thickness of the intestinal epithelium but also on the radial thickness of the chloragogenous tissue, with the highest values in the former (Figures 1 and 2). One should note that the radial thickness of the chloragogenous tissue of the individuals of Santa Maria presented values four or five-fold higher than the ones of Furnas (Figure 2).

The individuals of Santa Maria and Furnas also differed significantly on the volumetric density of BSD found in the chloragogenous tissue. But now the highest levels of BSD extent were found in Furnas, which were approximately four-fold higher than the ones of Santa Maria (Figures 1 and 3).

After the application of the TUNEL-test, one observed that in the individuals of Santa Maria no nuclei were found apoptotic (Figure 4 and 5).

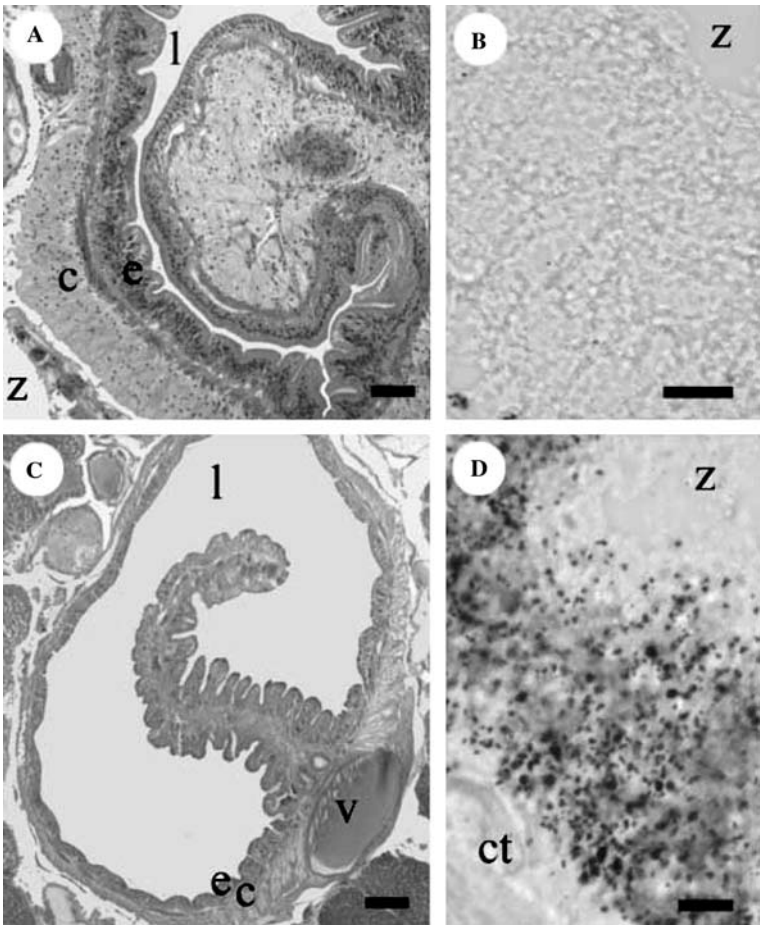

Figure 1. (a) Section of the intestine of a specimen from Santa Maria, stained with hematoxylin and eosin, showing high chloragogenous tissue and intestinal epithelium. Scale bar = 100 μm. (b) Chloragogenous tissue of a specimen from Santa Maria, stained with autometallography, showing few BSD. Scale bar = 10 μm. (c) Section of the intestine of a specimen from Furnas, stained with hematoxylin and eosin, showing reduced chloragogenous tissue and intestinal epithelium. Scale bar = 100 μm. (d)- Chloragogenous tissue of a specimen from Furnas, stained with autometallography, showing many BSD. Scale bar = 5 μm. Chloragogenous tissue = c; connective tissue = ct; intestinal epithelium = e; lumen = l; dorsal blood vessel = v; coelomic space = z.

However, in the individuals of Furnas both tissues studied, intestinal epithelium and chloragogenous tissue, presented apoptotic nuclei (Figures 4 and 5). Also, some coelomocytes presented apoptotic nuclei.

The radial thickness of the chloragogenous tissue was positively and significantly correlated with the radial thickness of the intestinal epithelium. Both were also negatively and significantly correlated with the BSD extent in the chloragogenous tissue and with the apoptotic nuclei of the same tissue. One found that the BSD extent was also correlated with the apoptotic nuclei of the chloragogenous tissue but not with the ones of the intestinal epithelium (Table 1).

Discussion

Volcanic soils, through natural volcanic activity, may be enriched in metals such as Al, As, Hg, Pb, and Zn (Ferreira & Oskarsson 1999; Kelepertsis *et al.* 2001), rendering a source of these metals to living organisms that may accumulate them in different parts of their bodies. Autometallography has been formerly reported to be a valuable technique for the localization and quantification of Cd, Cu, Zn, and Hg deposits in cellular compartments of marine and terrestrial animals (Hemelraad & Herwig 1988; Holwerda 1991; Marigómez *et al.* 1996; Soto *et al.* 1996, 1998b). In the present study, metals weakly bound to proteins were readily

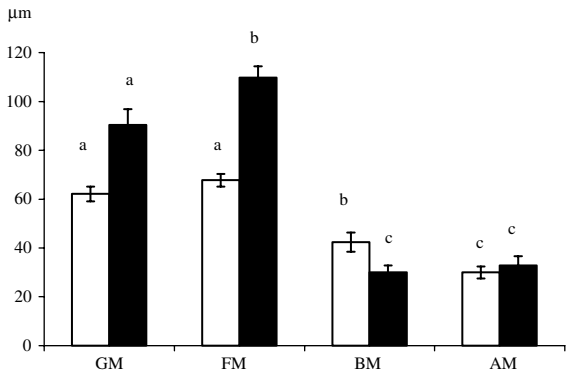

Figure 2. Radial thickness [$\bar{\times}$ ± se] (μm) of the intestinal epithelium (white bars) and chloragogenous tissue (black bars) of *L. t*. from Santa Maria (GM and FM) and Furnas (BM and AM). Different letters over the bars indicate significant differences at $P \leq 0.05$.

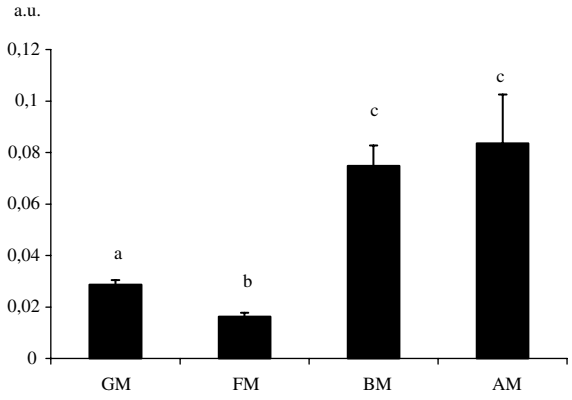

Figure 3. (Semi)quantification, by means of volume density, of metals (V_{VBSD}) [$\bar{\times}$ ± se] in the chloragogenous tissue of *L. t*. from Santa Maria (GM and FM) and Furnas (BM and AM). a. u. = arbitrary units. Different letters over the bars indicate significant differences at $P \leq 0.05$.

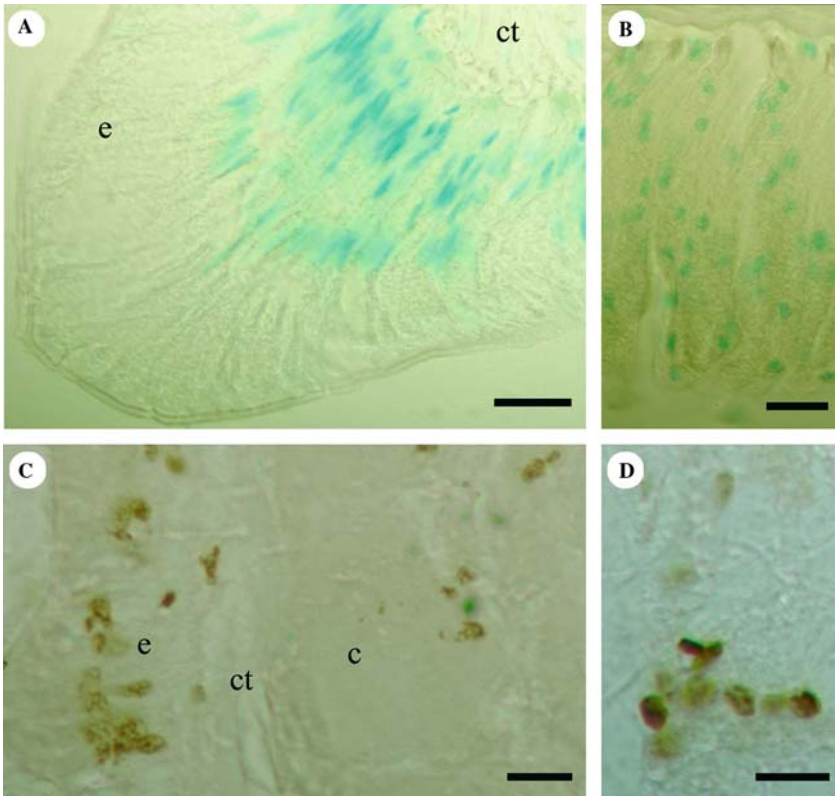

Figure 4. (a) Intestinal epithelium of a specimen from Santa Maria, stained with TUNEL-test, showing no apoptotic nuclei. Scale bar = 20 μm. (b) Chloragogenous tissue of a specimen from Santa Maria, stained with TUNEL-test, showing no apoptotic nuclei. Scale bar = 20 μm. (c) Intestinal epithelium and chloragogenous tissue in the tiflosole of a specimen from Furnas, stained with TUNEL-test, showing several apoptotic nuclei. Scale bar = 10 μm. (d) Chloragogenous tissue of a specimen from Furnas, stained with TUNEL-test, showing apoptotic nuclei. Scale bar = 10 μm. Chloragogenous tissue = c; connective tissue = ct; intestinal epithelium = e.

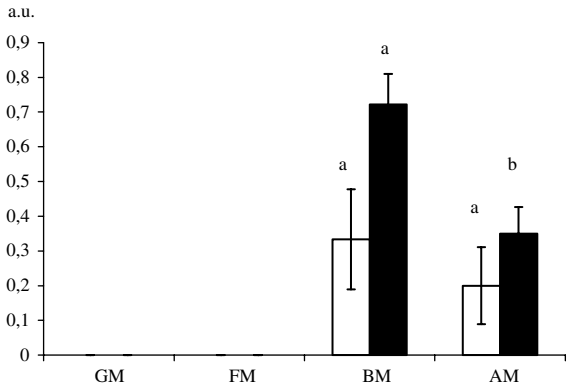

Figure 5. (Semi)quantification of apoptotic nuclei [$\bar{\times}$ ± se] of the intestinal epithelium (white bars) and chloragogenous tissue (black bars) of *L. t.* from Santa Maria (GM and FM) and Furnas (BM and AM). a. u. = arbitrary units. Different letters over the bars indicate significant differences at $P \leq 0.05$.

Table 1. Pearson's correlation between radial thickness of the chloragogenous tissue (RTC), radial thickness of the intestinal epithelium (RTIE), apoptotic nuclei of the chloragogenous tissue (ANC), apoptotic nuclei of the intestinal epithelium (ANIE), and black silver deposits (BSD) found in the chloragogenous tissue.

	RTC	RTIE	ANC	ANIE	BSD
RTC	–				
RTIE	0.843*	–			
ANC	−0.702*	−0.536*	–		
ANIE	−0.381**	−0.379**	0.506*	–	
BSD	−0.680*	−0.691*	0.452*	0.053	–

* Correlation is significant at the 0.01 level.
** Correlation is significant at the 0.05 level.

visualized at the light microscope as BSD in the earthworm chloragocytes. However, the individuals of the volcanically-active area, i.e., Furnas, presented a much higher BSD extent than the ones of the inactive volcanic area, i.e., Santa Maria. In fact, the BSD extent in the lysosomal compartment has been proposed as a metal exposure biomarker that reflects the levels of bioavailable metals in the environment (Soto & Marigómez 1997; Soto *et al.* 1998b, 1999; Da Ros *et al.* 2000; Porte *et al.* 2001). According to a previous study, there is a higher bioavailability of zinc and cadmium in the soils of Furnas when compared with Santa Maria.

The principal biological effects observed in earthworms inhabiting in both areas were alterations in the radial thickness of the chloragogenous tissue and in the intestinal epithelium. The currently observed morphological alterations were strongly and negatively correlated to the BSD extent, and may be interpreted as an adaptation of the earthworms to the accumulation of bioavailable metals, meaning perhaps that high levels of metals may cause depletion of both tissues. Analogous adaptations in the digestive epithelium thickness of mollusks have been previously reported as an effect of changes in environmental quality as increased bioavailability of pollutants (Lowe *et al.* 1981; Vega *et al.* 1989; Marigómez *et al.* 1990, 1991, 1992; Cajaraville *et al.* 1992). Marigómez *et al.* (1996, 1997) also found a clear evidence of severe adaptative changes in the digestive gland of mollusks following long-term/chronic exposure to pollutants. According to Morgan *et al.* (2002), the earthworm chloragocyte suffers morphological alterations to cope with larger quantities of metals.

The TUNEL-test showed that DNA fragmentation, which is a marker of apoptosis, was occurring in both chloragogenous tissue and intestinal epithelium and exclusively in the individuals of Furnas, where the mean radial thickness of both tissues was lower, possibly induced by the higher levels of BSD extent found in those. This way, the high levels of BSD extent in Furnas may correspond to high bioavailability of metals that stressed the cells of the intestinal epithelium but especially of the chloragogenous tissue and provoked apoptosis, that lead to a depletion and consequent regeneration of both tissues. Like a variety of environmental organic and inorganic chemical stressors that can produce qualitative and quantitative changes in the chloragocytes

(Ireland & Richards 1977; Fischer & Molnar 1992; Vogel and Seifert 1992), metals such as zinc and cadmium may have apoptotic and/or necrotic effects over cells of different organs (Seve *et al*. 2002; Hwang *et al*. 2004). *In vivo* data suggests that zinc-induced oxidative stress may result in apoptosis, leading to a reduction in neurons in the substantia nigra, followed by reduced dopaminergic function in the nigrostriatal dopaminergic system (Lin *et al*. 2003). A study of Hwang et al. (2004) on planarian apoptosis shows that this mechanism can occur in normal conditions and in regenerating parts of the body of the planarian. Fischer and Molnar (1992) found an almost total depletion of the chloragogenous tissue in paraquat toxicated earthworm *Eisenia fetida*, and Fischer (1989) also found that when the worms were put in untoxicated medium the chloragogenous tissue renewed itself. Thus, the depletion of the chloragogenous tissue found in this study might be explained by an augmented rate of turnover of chloragocytes related with an increased apoptotic cell death (Vogel & Seifert 1992), and perhaps a higher rate of chloragocyte–eleocyte transformation (Fischer 1989; Fischer & Molnar 1992) since Cancio *et al*. (1995) suggests that an extrusion of whole chloragocyte may occur as a way of eliminating toxic metals.

One may conclude that the volcanic environment poses stress to organisms that inhabit areas volcanically active forcing them to adapt by changing their rates of programmed cell death and consequently the morphometric characters of some of their organs, such as the chloragogenous tissue. Thus, the quantitation of the observed morphological changes and of the apoptotic nuclei, especially in the chloragogenous tissue, can be used as a reliable biomarker of effect to bioavailable metals in natural populations of *L. t*. inhabiting active volcanic environments.

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