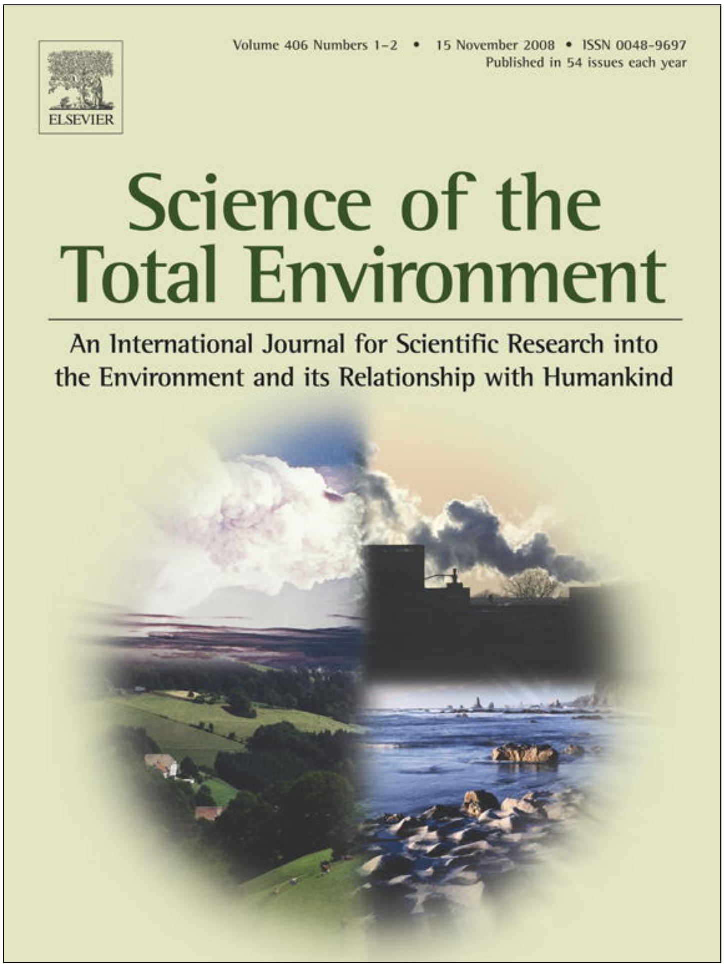
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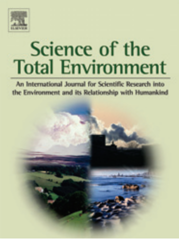

Bioavailability of heavy metals and their effects on the midgut cells of a phytopaghous insect inhabiting volcanic environments

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ABSTRACT

Organisms living in volcanic environments are chronically exposed to metals, either as particles or associated with gases, from volcanic emissions, being therefore potential sentinels of the effects derived from such exposure. Concentrations of Ca, Cd, Cu, Mg, Mn, Pb, Rb, and Zn were measured in soil, grass (*Lolium perenne*), and larvae of *Pseudaletia uninpuncta* captured in sites exposed and non-exposed to volcanic activity. The midgut epithelial cell morphometry and apoptosis of *P. unipuncta* larvae were also analyzed. Larvae from the site with volcanic activity showed higher levels of Cu, Mn, Rb and Zn. Metals such as Pb, Cd and Mg levels of *P. unipuncta* larvae were similar between sites. Apoptosis was higher in cells from digestive epithelium of larvae exposed to volcanic activity. Soils and grass not exposed to volcanic activity showed higher levels for most of the analyzed elements with the exception of Rb. Such result when compared with metal levels of larvae may reveal that bioavailability of elements differs between sites. The higher levels of Cd, Zn and Mg in soils and grass from the site with no volcanic activity are probably related to the severe artificial fertilization in the studied pastures. Such result, when compared with metal levels of larvae, suggest that the bioavailability of metals differs between sites.

1. Introduction

In São Miguel Island (Azores, Portugal) volcanic activity is manifested, among other ways, by the occurrence of hydrothermal vents, and soil degassing (Cruz, 2003; Ferreira et al., 2005). Consequently, organisms living in such environment may be continually exposed to metals, either as particles or associated with gases (Hansell et al., 2006). The association between changes in the digestive epithelium and the occurrence of apoptosis under chronic metal and metalloid exposure, originated by volcanic activity, has been previously found in terrestrial (e.g., earthworms, and snails) and marine invertebrates (e.g., limpets) (Amaral and Rodrigues, 2005; Zaldibar et al., 2006; Cunha et al., 2008). Thus, it is important to look for organisms that can be used as biological indicators or sentinels for the presence of those pollutants under such conditions, and in which one can rapidly measure several biomarkers of exposure and/or effects.

The relationship between the accumulation of metals and their biomagnification in edible plants, insects, such as Lepidoptera larvae, and higher consumers, such as predators, has been shown in recent studies (Vickerman and Trumble, 2003; Dauwe et al., 2004; Mulder and Breure, 2006). Such studies indicate the important role of insects as links in metal transport

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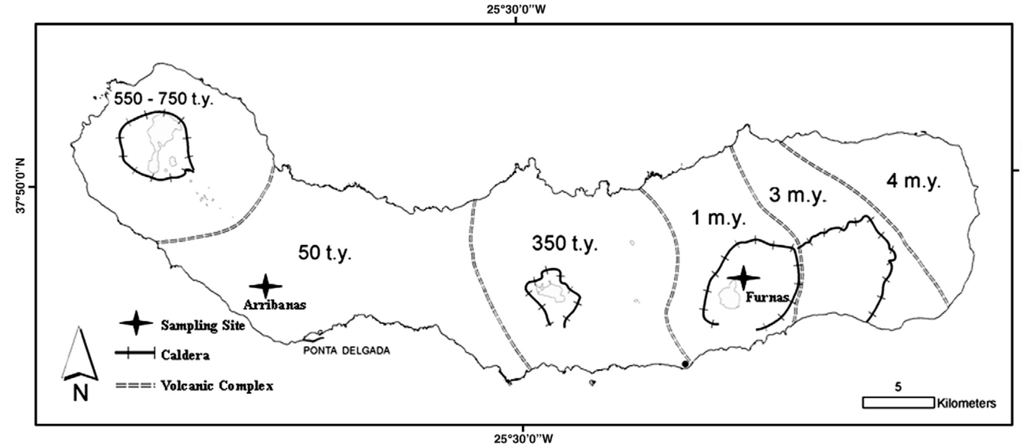

Fig. 1 – Sampling sites in São Miguel Island (Azores). Geological age expressed in million of years (m.y.) and thousands of years (t.y.).

chains between trophic levels in many food webs. Moreover, insects possess several mineral bioaccumulation structures in various organs, such as the midgut, being therefore of particular interest among invertebrates to what metal storage and effects is concerned (Ballan-Dufrançais, 2002).

The armyworm, *Pseudaletia* (=*Mythimna*) *unipuncta* (Haworth) (Lepidoptera: Noctuidae), is an important cosmopolitan pest of cereal which is known to forage crops in North America (McNeil et al., 2000) and Europe (Bues et al., 1986). In the Azores, this pest is a polivoltine species (Raposo et al., 2003), and its larvae are usually found in pastures throughout all seasons (Vieira et al., 2004). Since the larval stages develop and feed usually on grass within a restricted area inside the pastures (Reis et al., 2003; Silva et al., 2003), these organisms, their food and inhabiting soils could be used to evaluate bioavailability and biotransference of trace metals in extreme environments of volcanic origin. Bearing this in mind, and since it is known that metals can promote and originate changes in the composition and diameter of digestive epithelia of several invertebrates that can be measured through variations in the mean radial epithelial thickness (Amaral et al., 2006a; Marigomez et al., 2006; Cunha et al., 2008), but also with apoptosis, another cellular response that may be triggered by metal stress. Apoptosis is involved in cell turnover, and has been suggested as a biomarker of the effect of bioavailable metals when its rates vary from normal (Amaral and Rodrigues, 2005; Cunha et al., 2008). The present study aimed to determine whether larvae exposed and not exposed to volcanic activity show distinct metal body burdens, but also to analyze the effects of such metals on the midgut cell morphometry and apoptosis.

2. Materials and methods

2.1. Experimental design and sampling sites

P. unipuncta larvae were collected early in the summer (late June–early July 2007) by randomly sampling two distinct areas of pasture grass within each site (Silva et al., 2003), until 20 larvae of the third, fourth and fifth instar were obtained. Larvae were collected from two sites separated 50 km in São Miguel Island (Azores) (Fig. 1), and differing in the presence or not of hydrothermal volcanic activity: (i) Água Azeda which is a natural pasture, chronically exposed to soil degassing and hydrothermal vents; and (ii) Arribanas, which do not show volcanic activity and is a heavily and artificially fertilized pasture. From each site, a set of 10 larvae were captured for chemical analyses, and another set of 10 larvae were collected for histological processing and apoptosis tests. For each site, two samples (20 m apart) of *Lolium perenne* L. leaves (150 g wet weight each) and two of soil (500 g wet weight each, collected from an area of 200 m^2 and 20 cm depth) were collected for the chemical analysis of metal contents and soil properties. Soils from the active volcanic site had lower pH and clay-silt percentage than soils from the non-active volcanic site (Table 1).

Table 1 – Physico-chemical properties of the analyzed soils from the site with volcanic activity (Água Azeda) and the other with no volcanic activity (Arribanas)

Soil		%		
	pH (H_2O)	Clay-silt	Moisture	
Água Azeda	5.7	30.88	31.78	Amaral et al. 2006b
Arribanas	6.2	64.67	34.9	Unpublished data

2.2. Metal analysis

The soft tissues of the thorax and the abdomen of *P. unipuncta* larvae were dried (130 °C) for 48 h, digested in aqua regia at 95 °C for 2 h, and then microwave digested inside closed vessels for 2 min. Resultant sample solutions were diluted and analyzed on a Finnegan Mat Element 2 High Resolution ICP/MS (Actlabs, Canada) for the quantification of Ca, Cd, Cu, Mg, Mn, Pb, Rb and Zn. The same procedure was used for soil and grass samples from each site. The concentrations are expressed in μg/g (dry weight). For quality control, internal standards and reference materials were run together with the samples, and no less than six different reference materials covering all the elements in study were used. Duplicate samples were also used in order to determine precision of the analysis. For each element, a minimum of three standards were used to cover the analytical working range of the instrument. Ultrapure water was used to prepare blanks and calibration standards, and three replicate assessments were performed for each sample.

A pool of several fertilizers frequently used in pastures of the site with no volcanic activity (Arribanas) was also analyzed.

2.3. Midgut epithelial cell morphometry and apoptosis

The digestive tract of each 10 larvae per site was used for light microscopy, morphometry and apoptosis studies (TUNEL test). Larvae were fixed in neutral-buffered formaldehyde, dehydrated in an increasing series of ethanol, cleared in methylbenzoate, rinsed in benzene, and embedded in paraffin. Histological sections of the midgut were cut with 5 μm thickness and stained with hematoxylin and eosin (Martoja and Martoja-Pierson, 1970).

To quantify the radial thickness of the midgut digestive epithelium, a planimetric procedure was applied on sections of the digestive tube (Amaral et al., 2006a). From each individual, two tissue sections, 25 μm apart from each other, were used by taking a mean value of four measures in each section, for a total of eight measurements per individual, while their digestive tract profiles were captured using a CoolSNAP-cf camera (Photometrics GmbH, Germany) coupled to a light microscope, and analyzed with Image Pro-Plus 5.0 software (Media Cybernetics, Silver Springs). For statistical analysis, it was considered the average of the eight measurements per individual, being each individual a replicate.

The detection of apoptotic nuclei in the digestive tract was performed using a DeadEnd™ kit (Promega, USA). Briefly, two tissue sections (25 μm apart from each other) from the midgut digestive epithelium were dewaxed and rehydrated. Sections were then washed in phosphate buffered saline and treated with 20 μg/ml proteinase K for 20 min at room temperature. DNA of the tissue sections was labeled at 3′ ends with biotin-dUTP by incubation with the reaction buffer containing terminal deoxynucleotidyl transferase, for 60 min at 37 °C. Tissues were then treated with 3% hydrogen peroxide for 5 min, and incubated 30 min at room temperature with streptavidin horseradish peroxidase to detect biotinylated nucleotides. Diaminobenzidine reacted within the labeled nuclei to generate an insoluble brown substrate at the site of DNA fragmentation. As a negative control, terminal deoxynucleotidyl transferase was replaced by bi-distilled water (results not shown). Finally, sections were counterstained with 0.3% methyl green to allow the morphological evaluation and characterization of normal and apoptotic cells. The percentage of apoptotic nuclei was graded as follows: 0 (no staining); 1 (>0 a 50%); 2 ($>50\%$).

2.4. *Statistical analysis*

Differences in cell morphometry as well as the extent of apoptotic nuclei in the midgut epithelium were examined by a *t*-test assuming equal variances and considered as significant when $p \leq 0.05$. Statistical analyses were made using SPSS 15.0 (SPSS Inc., Chicago, U.S.A.).

3. Results

3.1. *Metal analysis*

All measured elements were detectable in soil, grass and larvae samples from both sites (Table 2). Soils from the site with no volcanic activity (Arribanas) showed higher levels of all tested trace elements, except Rb (higher in Água Azeda) and Zn (values were similar in both sites). In Arribanas, higher concentrations of Cd, Mg and Zn were also observed in grass samples, being the Cd concentration seven times higher than in the samples from Água Azeda (Table 2). Although richness of Zn was particularly higher in the Arribanas grass, larvae from the pasture with volcanic activity (Água Azeda) revealed a higher concentration of this metal. Also, a higher concentration of Cu was detected in larvae from Água Azeda, while in grass concentrations from both sites were similar (Table 2). Rb was the only element always present at higher concentrations in soil, grass and larvae samples collected in the pasture with volcanic activity (Table 2).

Although Mn concentration in soil from Arribanas was almost two fold higher, grass and larvae samples from the site with volcanic activity showed concentrations two- to three-fold higher than those from the former site.

Metal content of the fertilizers can be seen in Table 3.

Table 2 – Concentrations of metals (μg element/g of dry weight) measured by HR-ICP/MS in the soils, grass and the larvae of *P. unipuncta* from a site with volcanic activity (Água Azeda) and other with no volcanic activity (Arribanas)

Elements	Soil		Grass		Larvae	
	Volcanic activity		Volcanic activity		Volcanic activity	
	No	Yes	No	Yes	No	Yes
Ca	12,328	7611	6315	3295	1565	1390
Cd	0.8	<0.5	0.7	<0.1	0.1	0.1
Cu	29	15	9	8	15.2	28.1
Mg	6500	3400	3600	1415	4770	4690
Mn	2400	1400	96	314	110.2	287
Pb	54	42.5	0.1	0.1	0.1	0.1
Rb	83	187	66.7	128	30.9	34.8
Zn	160	153	94	33	165.3	189

Table 3 – Average concentrations of metals (μg element/g of dry weight) measured by ICP/MS of several fertilizers that are frequently used in pastures of the site with no volcanic activity (Arribanas)

Elements	Fertilizers
Ca	41,880
Cd	9.7
Cu	16.7
Mg	2433.3
Mn	17.3
Pb	3.5
Rb	<15
Zn	176.3

3.2. *Midgut cell morphometry and apoptosis*

Mean epithelium thickness showed highest values in larvae from Água Azeda [56.04 ± 1.95 (±S.E.) μm]. The mean epithelium thickness of larvae from Arribanas was 51.84 ± 2.42 (±S.E.) μm. Nevertheless, the mean digestive epithelium thickness did not differ significantly between both sites (*t*-value = −1.35; $p = 0.185$).

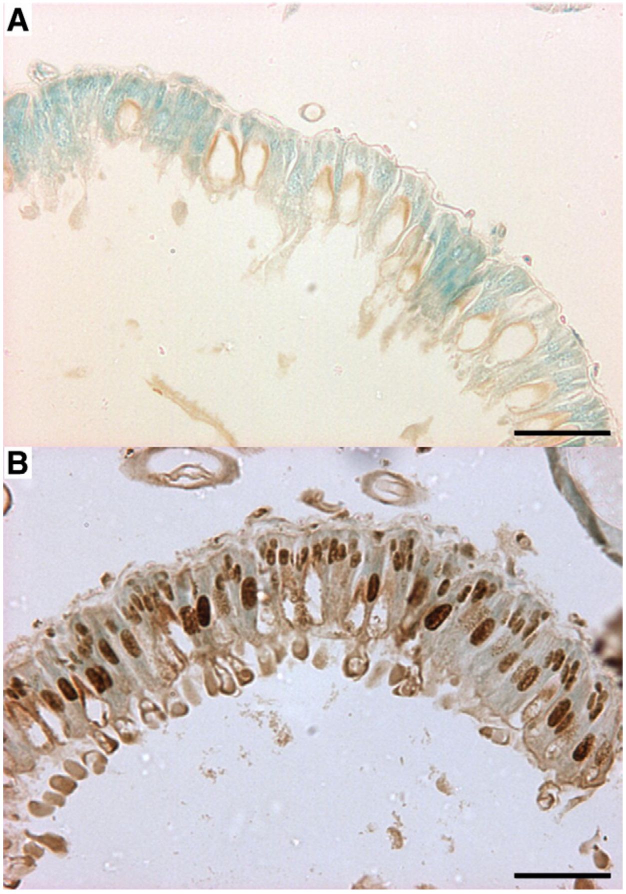

Fig. 2 – A – Digestive epithelium of a specimen from a site free of volcanic activity (Arribanas), stained with TUNEL test, showing no apoptotic nuclei. B – Digestive epithelium of a specimen from a site with volcanic activity (Água Azeda) stained with TUNEL test, showing numerous apoptotic nuclei. Apoptotic nuclei stained brown. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

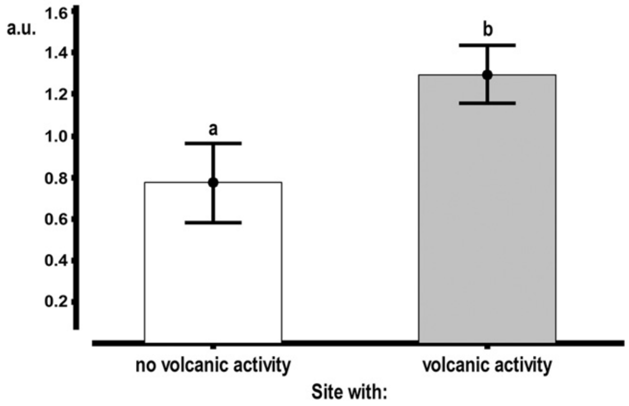

Fig. 3 – (Semi)quantification of apoptotic nuclei [mean ± SE] of digestive epithelium of *Pseudaletia unipuncta* from a site with volcanic activity (Água Azeda) and other with no volcanic activity (Arribanas); a.u. = arbitrary units. Different letters over the bars indicate significant differences at $p < 0.05$ (*t*-test).

Differences in apoptotic levels found in these larvae were not due to larval stages variability, which revealed no significant differences ($F = 0.77$; $p = 0.473$, two-way ANOVA). Also, neither the interaction between larval stages and sites was significant ($F = 1.38$; $p = 0.268$, two-way ANOVA). Pooled data from both sites revealed that apoptosis was more prominent in cells from the digestive epithelium of *P. unipuncta* larvae captured in the site with volcanic activity (Água Azeda) (Fig. 2), revealing significant differences (t-value $= -2.31$; $p = 0.027$) (Fig. 3).

4. Discussion

Soil and grass samples from environment without volcanic activity showed higher concentrations of most of the considered elements probably due to soil enrichment by fertilizers (see Table 3). In fact, in all agricultural areas of São Miguel Island, including Arribanas, artificial fertilizers are applied to pastures in an uncontrolled manner, often inappropriately and in large amounts, which are also the cause of severe eutrophication of ponds and lakes (Depledge et al., 1992). Although concentration of Cd was seven times higher in the grass from the inactive volcanic site, the levels of Cd in larvae were similar between sites, suggesting the occurrence of detoxification processes that lower the Cd levels in the insect body. According to Borowska et al. (2004) a possible via of detoxification of xenobiogenetic metals (as Cd and Pb) in insects is by allocating metals to the surface of the chitinous exoskeleton (cuticule), which is periodically shed during larval molts. On the other hand, in the active volcanic site the higher concentration of Cd in larvae than in the grass could be due to other uptake via (e.g., cutaneous and/or respiratory) related to the increased bioavailability of this element in this extreme environment, as previously suggested by Amaral et al. (2007) for mice (*Mus musculus*).

In contrast with heavy metal contents in soils and grass, larvae from the active volcanic environment presented higher concentrations of Cu, Mn, Rb and Zn, similarly to that observed by Cunha et al. (2008) in the digestive gland of marine limpets from shallow water hydrothermal vents. Higher levels of Rb in organisms after volcanic exposure are not uncommon (Widom et al., 1997; Claude-Ivanaj et al., 2001; Durand et al., 2004), so the presence of Rb in samples from Água Azeda were expected, and thus the levels of this trace element could be used as a biomarker of exposure to volcanic environments, as previously suggested (Durand et al., 2004; Amaral et al., 2008). In larvae from Água Azeda, values observed for Cu and Zn were particularly high and clearly greater than the observed by Dauwe et al. (2004) for Lepidoptera larvae (22.5 and 157.2 μg/g for Cu and Zn, respectively) collected in a site near (0–0.35 km) a metallurgic plant, thus heavily contaminated by heavy metals. Furthermore, according to Borowska et al. (2004) and contrarily to the observed, insects should be more efficient in the elimination of biogenetic (Cu and Zn) than xenobiogenetic elements (Cd and Pb). Such results suggest that the active volcanic environment, characterized by a lower pH and a lesser clay-silt percentage (see Table 1), and a damp atmosphere rich in sulfur gases, may increase the availability of Cu and Zn to the larvae, as suggested in previous studies by Amaral et al. (2006a, 2007). This result is exemplified by the concentrations of Zn in the Arribanas grass and larvae, which contrast with Zn levels of those from the pasture with volcanic activity (Água Azeda), indicating sources of contamination other than the ingestion of grass. Dermal absorption of this element from the air resulting from the volcanic degassing is one of the possible explanations for this. Also, the higher concentration of Cu in larvae from Água Azeda may be explained by a similar via of contamination.

Contrarily to the concentrations of Mn observed for soil samples, the higher levels of this element in grass (314 μg/g) and larvae (287 μg/g) from the site with volcanic activity suggest a higher availability of this element for plants due to the lower concentration of Mg and Ca in soil. This phenomenon could be potentiated by the lower pH of the soil in this local (Table 1), as it was pointed out by Davis (1996) and CTAHR-Cooperative Extension Service (1998). According to data published by Lei et al. (2007), Mn concentration in grass samples from the active volcanic environment configures a high Mn chronic stress situation, being above Mn phytotoxicity thresholds for sheep fescue species (*Festuca ovina*) (>200 ppm) (Paschke et al., 2005). On the other hand, in Arribanas Ca and Mg application by manual fertilization could result in an important reduction in Mn uptake in grass, as sustained by Davis (1996).

Morphometric changes in digestive tissues have been used as common biomarkers for pollution exposure of anthropogenic and volcanic origin (Marigomez et al., 1996; Vega et al., 1989; Amaral et al., 2006a; Cunha et al., 2008). However, the present study shows that this trait is not reliable as a marker of the chronic metal exposure of *P. unipuncta* larvae, since the mean digestive epithelium thickness did not differ between both sites.

The presence of Cu has been shown to disturb the elemental composition of the epithelial cells of housefly midgut (i.e., cellular concentration decreases of K, Mg, P, and S), particularly after the exposure to 5 μg/g of Cu in the larvae rearing medium (Tylko et al., 2005). Since levels of Cu in *P. unipuncta* larvae were considerably high, especially for larvae from the active volcanic environment (28 μg/g), it is possible that the elemental composition of the midgut cells was disturbed, increasing the apoptotic events as a possible via of detoxification. Loeb et al. (2000) have demonstrated that apoptosis is probably a normal method for biological disposal of older differentiated midgut cells of Lepidoptera larvae (*Heliothis virescens*) and that when midgut cell cultures are exposed to adverse environments, such as poisonous media, down-regulation of cell populations is induced by apoptosis. Nevertheless, programmed cell death and autophagy are natural and intense processes during larval development of lepidoptera (Tettamanti et al., 2007), which were considered during data treatment and analysis. Differences in apoptotic levels found in the larvae were not due to variability between larval stages nor the interaction between larval stages and sites. Therefore, pooled data (Zar, 2007) was used to compare both sites, which revealed that apoptosis was more prominent in cells from the digestive epithelium of *P. unipuncta* larvae captured in the site with volcanic activity (Água Azeda).

On the other hand, Zhang et al. (2001) refer that in contrast to other metals, Zn is not deposited efficiently in cells, being a less potent inducer of metallothioneins in insects (Diptera), which are important proteins involved in metal detoxification. Thus, Zn levels could act synergistically with other metals, such as Cd and Cu, in promoting apoptosis. Since active volcanic environment seems to be a source of chronic metal stress, particularly Zn, the higher levels of apoptosis found in organisms from Água Azeda may be again associated to apoptotic detoxification processes as a cellular response and adaptation to this particular extreme environment of volcanic origin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scitotenv.2008.07.069.

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