Postprint of: Integrative Zoology (2021) doi:10.1111/1749-4877.12562

Relationships between soil pollution by heavy metals and melanin-dependent coloration of a fossorial amphisbaenian reptile

José MARTÍN,[1] Pablo RECIO,[1] Gonzalo RODRÍGUEZ-RUIZ,[1] Isabel BARJA,[2,3] Eduardo GUTIÉRREZ,[4] and Luis V. GARCÍA [4]

[1]Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain, [2]Departamento de Zoología, Facultad de Biología, Universidad Autónoma de Madrid, Madrid, Spain. [3]Centro de Investigación en Biodiversidad y Cambio Global (CIBC-UAM), Universidad Autónoma de Madrid, Madrid, Spain and [4]Departamento de Biogeoquímica, Ecología Vegetal y Microbiana, Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, Sevilla, Spain

Correspondence: José Martín, Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain.
Email: jose.martin@mncn.csic.es

Running title: Melanin coloration and soil pollution

Abstract

Melanin is the basis of coloration in many animals, and although it is often used in communication, thermoregualtion or camouflage, melanin has many other physiological functions. For example, in polluted habitats, melanin can have a detoxifying function. Melanic coloration would help to sequester in the skin the heavy metals contaminants from inside the body, which will be expulsed to the exterior when the skin is sloughed. Moreover, animals should have evolved more melanic colorations in more polluted habitats ("industrial melanism" hypothesis). We examined whether the fossorial amphisbaenian reptile, *Trogonophis wiegmanni*, is able to eliminate heavy metals, derived from soil pollution by seagulls depositions, through sloughing its skin. Our results suggest a covariation between levels of soil pollution by heavy metals and the concentration of heavy metals in the sloughed skins of amphisbaenians. This suggests that amphisbaenians may expel heavy metals from their bodies when they slough the skins. We also tested whether amphisbaenians inhabiting soils with higher levels of heavy metal pollution had darker (melanin-dependent) body colorations. However, contrary to predictions from the "industrial melanization" hypothesis, we found a negative relationship between soil pollution and proportions of melanic coloration. This contradictory result could, however, be explained because heavy metals have endocrine disruption effects that increase physiological stress, and higher stress levels could result in decreased melanogenesis. We suggest that although amphisbaenians might have some detoxifying mechanism linked to melanin in the skin, this process might be negatively affected by stress and result ineffective under conditions of high soil pollution.

Key words: amphisbaenians, coloration, fossorial reptiles, heavy metals, melanin, soil pollution

INTRODUCTION

Soil pollution is one of the major environmental threats with negative consequences for biodiversity (Osman 2013; Larramendy 2016; Tibbett *et al.* 2020). Contamination may be directly produced by humans through the dump of pollutants to the soil or water (Larramendy 2016; Duarte *et al.* 2017), but dissemination of pollutants may also be mediated by animals. For example, some seabirds can act as vectors that move heavy metals from anthropogenic contaminated food sources to the soil of their nesting colonies in natural areas (Headley 1996; Otero 1998; García *et al.* 2002a, b; Blais *et al.* 2005). Irrespective of the pollution mechanisms, heavy metals can be transferred from the soil to plants and invertebrates, and go up the food chain (Walker *et al.* 2012), reaching high toxicological concentrations in tissues of vertebrates such as birds (Manjula *et al.* 2015; Orłowski *et al.* 2015) or lizards and snakes (Loumbourdis 1997; Campbell *et al.* 2001; Márquez-Ferrando *et al.* 2009; reviewed in Grillitsch & Schiesari 2010).

Nevertheless, some animals seem able to "fight" against the toxicological effects of heavy metals. For example, in polluted habitats, some reptiles and birds are able to accumulate in the skin or in the feathers trace elements from the body that are later expelled when the skin is sloughed or the feathers are molted (Hopkins *et al.* 2001; Jones & Holladay 2006; Chatelain *et al.* 2014; Goiran *et al.* 2017). This process would help to detoxify the body of heavy metals. The mechanism is enhanced by increasing melanin concentration in the skin, because melanin can bind and sequester heavy metals (Liu *et al.* 2004; Bridelli *et al.* 2008). Melanic areas of the skin accumulate larger concentrations of heavy metals (Niecke *et al.* 1999, 2003; Goiran *et al.* 2017), which would be expelled with the sloughed skins or shed feathers. As a consequence, it has been suggested, and found in some animals, that proportions of melanin-dependent coloration of the body should increase in populations inhabiting areas highly polluted by heavy metals ("industrial melanism" hypothesis) (Chatelain *et al.* 2014; Goiran *et al.* 2017).

Soil pollution may particularly affect fossorial animals that spend all or most of their life underground (Tibbett *et al.* 2020), such as some rodents or reptiles, but the difficulty of studying these animals (Measey 2006; Henderson *et al.* 2016), may result in that the potential threats for their health state were going unnoticed (How & Shine 1999; Measey *et al.* 2009; Böhm *et al.* 2013). Amphisbaenians are one notorious, but little conspicuous and understudied, group of fossorial reptiles (Gans, 1978, 2005). They have evolved very specialized morphological and functional adaptations to a fossorial life, such as elongated body, narrow head, loss of limbs and reduced vision (Gans 1974, 1978; Navas *et al.* 2004; Baeckens *et al.* 2017). These adaptations, however, constraint many aspects of their little known ecology (e.g., Papenfuss 1982; Martín *et al.* 1991; Colli & Zamboni 1999; Webb *et al.* 2000; Andrade *et al.* 2006).

The checkerboard worm lizard (*Trogonophis wiegmanni* Kaup, 1830) is an amphisbaenian found in the Mediterranean region of NW Africa (Bons & Geniez 1996). This species bases its microhabitat selection patterns on soil characteristics (Civantos *et al.* 2003; Martin *et al.* 2013a). Because it is a strictly fossorial species, its health state seems to be mainly affected by natural and anthropogenic soil alterations (Martin *et al.* 2015, 2017). However, the processes involved in these negative effects and the potential existence of compensatory mechanisms are unknown. In contrast to most amphisbaenian species that are uniformly colored and frequently show loss of pigmentation (Gans 2005), *T. wiegmanni* shows a characteristic 'checkerboard' pattern of coloration, alternating some scattered melanin-dependent black scales with yellow or whitish scales (Fig. 1). Being an underground, almost blind species, it is not plausible that this pattern of coloration could have any function in social interactions, thermoregulation or protection from UV radiation, as occurs in other reptiles (Clusella-Trullas *et al.* 2009; Vroonen *et al.* 2009; Bury *et al.* 2020), although it might have a camouflage function if a predator dug or lifted-off a rock and the amphisbaenian became unintentionally exposed onto the surface. However, melanin can have many other physiological functions linked to its redox, metal chelating, or free radical scavenging properties (Solano 2014). We hypothesized that, as it has been described for other animals (Chatelain *et al.* 2014; Goiran *et al.* 2017), one of the potential roles of melanin coloration of this amphisbaenian might also be using it as a heavy metal detoxifying mechanism. Moreover, because melanic coloration of this amphisbaenian must not have a social signaling or thermoregulatory function underground, we are able to avoid these possible confounding effects when exploring the potential use of melanin to eliminate heavy metals from the body.

Here, to test the "industrial melanism" hypothesis (Chatelain et al. 2014; Goiran et al. 2017), we designed a field study in island populations of T. wiegmanni amphisbaenians. In some areas of the study populations, nesting seagull colonies are known to increase heavy metal concentration in the soil (García *et al.* 2002a, b; Martín *et al.* 2015), and it is plausible that metals could reach amphisbaenians through their soil invertebrate prey (Martín *et al.* 2013b). We specifically examined 1) whether this amphisbaenian is able to eliminate the heavy metals contaminants of its body through sloughing its skin, and 2) whether increased melanin-dependent dark coloration may facilitate this detoxifying mechanism. For this, we analyzed concentration of heavy metals in the soil and in sloughed skins of amphisbaenians. We also measured the proportions of melanin-dependent dark coloration of live individuals and related them to the levels of soil pollution at the capture sites. We predicted that populations of amphisbaenians inhabiting soils with high levels of heavy metal pollution should have higher concentrations of these trace elements in the sloughed skin and also darker body colorations.

MATERIALS AND METHODS

Study area

We carried out the field study from 4th to 18th October 2018 at the small archipelago of Chafarinas Islands (SW Mediterranean Sea, Spain; 35°11' N, 02° 25' W). These are three small islands (Congreso, Isabel II and Rey Francisco) located 2.5 nautical miles (/ 4.6 km) off the northern Moroccan coast (Ras el Ma). Climate is of a dry and warm Mediterranean type and vegetation consists of sclerophyll and halophilic bushes (*Salsola*, *Suaeda*, *Lycium* and *Atriplex*) (García *et al.* 2002a, b; Martín *et al.* 2013a). In general, the soils are immature and poorly developed with only a thin layer, rich in organic matter, over the volcanic rocks (García 2005; García *et al.* 2007).

In two of the islands (Congreso and Rey), there are large nesting colonies and resting areas of two seagull species; the endangered protected Audouin's gull (*Larus audouinii* Payraudeau, 1826) (221 nests in Rey in the year 2018), and the widespread yellow-legged gull (*Larus cachinnans* Pallas, 1811) (6074 nests in Congreso and 2380 nests in Rey in 2018) (unpubl. data from census made by the technicians of the Spanish National Parks authority), The yellow-legged gull population is increasing considerably since some years ago. Nests are widespread ober all the islands surface, without forming specific nesting patches, and the gulls also use the islands for resting all the year outside of the reproductive season. The other island (Isabel) has lower seabird influence because it is the only one inhabited by man, although some resting groups of gulls can be always found along the island. Depositions of gulls have a negative influence on the chemical properties of the soil, increasing concentration of organic matter and heavy metals, such as Cd, Ni, Cr, Fe and Zn (García *et al.* 2002a, b; Martín *et al.* 2015). These soil alterations are known to increase physiological stress of amphisbaenians (i.e. increased levels of corticosterone metabolites in faeces) (Martín *et al.* unpubl. data), although do not decrease their body conditions, which are mainly negatively affected by increased salinization and soil compaction (Martín *et al.* 2015, 2017).

Captures and observations of amphisbaenians were performed under license by the Organismo Autónomo de Parques Nacionales (Spain). All the procedures were approved by the Ethical Committee ("Comisión Ética de Experimentación Animal", CEEA) of the Museo Nacional de Ciencias Naturales, CSIC.

Soil pollution

To characterize the level of pollution of the soil by heavy metals, we made random transects in the three islands covering all the areas used by amphisbaenians (excluding bare rocky areas without soil) (Martín *et al.* 2011a) and selected a total of 79 random points, geolocalized with a GPS. At each sampling point, we took a bulked soil sample (around 300 g) under a rock like those used by amphisbaenians (Civantos *et al.* 2003), digging between the surface and until 10 cm depth (or less if the soil was less deep), which coincided with the soil layers used by amphisbaenians (Martín *et al.* unpubl. data). In the laboratory, soil samples were air-dried, crushed and sieved (< 2 mm) and then ground to < 60 μm. Approximately 0.5 g of dry soil was disaggregated in a START D microwave digestion system (Milestone Srl, Sorisole, Bergamo, Italy) with a mixture 12 ml of HNO_3:HCl (3:1 ratio) at 150 °C for 20 min. Subsequently, the resulting solution was filtered with a 0.45 μm filter and diluted to 25 ml with deionized water. We used acid digestion because with this method only metals present in the organic fraction, where bioavailable metals were concentrated, were recovered (Cox & Preda 2003). We then determined the soil quasitotal concentration of eight 'heavy-metals' (Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) using inductively coupled plasma optical emission spectrometry (ICP-OES; Varian ICP 720-ES; Varian, Palo Alto, California, USA) (for details of analyses see Sparks 1996). Quality assurance was obtained by analyzing the soil reference material CRM 141R (calcareous loam soil, European Community Bureau of Reference). Recoveries rates from CRM 141R values ranged from 82% to 95%, according to the element.

Heavy metals in sloughed skins of amphisbaenians

Measuring the concentrations of heavy metals found in shed skins of reptiles is considered as a reliable non-destructive method of evaluation of contaminant exposure in the field (Hopkins *et al.* 2001; Jones & Holladay 2006; Goiran *et al.* 2017). Thus, we looked in the field for sloughed skin of amphisbaenians. We haphazardly followed different routes on the three islands between 07:00 and 18:00 (GMT) covering all the locations inhabited by amphisbaenians (Martín *et al.* 2011a; García-Roa *et al.* 2014). We lifted almost all rocks and stones found, where amphisbaenians were abundant and easy to find (Martín *et al.* 2011a, 2013a). We collected fresh sloughed skins of amphisbaenians that were found under these rocks. Sloughed skins were usually found complete in a single piece (i.e., the entire body of the animal), such as it occurs in snakes. This allowed an unambiguous assignation of the skins to amphisbaenians, avoiding confusion with species of geckos, snakes, skinks and lizards that could also been found there (García-Roa *et al.* 2014). The location point of each skin was measured with a GPS (GPSmap 60CSx, Garmin). Skins were kept in eppendorf tubes and later conserved in a freezer until further analyses.

In the laboratory, skins were carefully cleaned with distilled water to remove dirt and adsorbed trace elements, cut into pieces and dried. Then, skins were ground using a manual agate mortar. Because a minimum weight of sample (200 mg) was required for these analyses and individual skin samples were much more lighter (< 15 mg), we had to pool skins of different individuals from nearby locations in single samples. Therefore, we only obtained average measures for the population of amphisbaenians of each site. Samples were digested by wet oxidation with concentrated HNO_3 under pressure in a microwave digester. Analyses of heavy metal concentrations in the skin were made with a Varian ICP 720-ES device, as for the soil samples (see above). The accuracy and precision of the analytical methods were assessed by routine analyses of the ERM® certified reference material (mussel tissue ERM-CE278; from Sigma-Aldrich). Recovery rates for reference samples ranged from 90 to 100% depending of the element.

Melanin-dependent coloration of amphisbaenians

During the field surveys (see above), we also captured by hand live amphisbaenians that were found under rocks. Location of capture points were determined with a GPS (GPSmap 60CSx, Garmin). We used a metallic rule to measure snout-to-vent length (SVL) and tail length of amphisbaenians and determined sexes by examining the presence of hemipenis in the cloacae (Martín *et al.* 2011b).

In the field, we took photographs of the captured adult amphisbaenians (N = 104; 36 males and 68 females). We used a white plastic board as a background and included an individual numeric code and a small metallic rule in the picture for reference. Amphisbaenians were gently held against the board in a straight position and we took pictures using a digital camera (Nikon D3000; 3872 x 2592 pixels of resolution and 4096 colors per channel), with a Nikkor AF-S DX 18-55 mm f/3,5-5,6 lens, placed at about 10 cm over the amphisbaenian. We used natural sunlight in the shade (with a filling flash light) for taking pictures of three zones ('anterior', just after the head, 'medium', at the middle of the body, and 'posterior', before the cloaca) in the dorsal area and the equivalent three zones in the ventral area of the body of amphisbaenians.

After being measured and photographed, amphisbaenians were marked individually with PIT-tags as a part of a population study (Recio *et al.* 2019), which allowed to avoid sampling twice the same individual in different surveys, and released them within a few minutes under the same rock where they were found.

Pictures of amphisbaenians were processed using Adobe Photoshop CS4 software. We first cut and enlarged one part of the picture representing approximately a body area of 8 x 3 mm^2, which included about 70 scales on average. In the cropped area, we estimated the proportion of dark coloration by counting the number of dark-black scales in relation to the total number of scales (approx. 40 % overall), which also included yellow (40%) and white scales (20%) (Fig. 1). The color of each scale was easily determined. Because all dorsal and ventral scales were rectangular and of similar size (approx. 0.25 x 0.75 mm^2) and were regularly distributed in columns and rows, this counting method provided a highly reliable estimation of the surface area with black coloration. We followed this method for each of the six different photographs of the different body areas and zones of each individual. We calculated the 'proportion of melanin-dependent dark coloration' in the entire body as the average of the relative proportions of black scales in the three zones of the dorsal area and the three zones of the ventral area.

Data analyses

We first used a General Lineal Model (GLM) to estimate variation in individual proportions (square root transformed) of melanin-dependent dark coloration of adult amphisbaenians as the response variable, depending of the body area (dorsal vs. ventral) and zone (anterior vs. medium vs. posterior), both as repeated measures factors, and including sex (male vs. female) as a fixed factor. As there were no differences between sexes (see results), we pooled data of males and females for subsequent analyses.

Then, we referred the GPS location of each amphisbaenian to the nearest soil sampling point. As amphisbaenians show high site fidelity and moved over very small areas (Martín et al. 2021), we could confidently relate their locations to a given soil point. We designated each of these soil sampling points as a block, and since these points were selected randomly, we considered that we had a randomized complete block design. Thus, each of the samples of amphisbaenians could be considered as replicates, capturing all the variance of the response variable (melanin proportion). Because amphisbaenians were unequally distributed in the islands (Martín *et al.* 2011a), we collected valid information related to 25 of the soil sampling points (number of amphisbaenian samples per point, mean ± SE = 4 ± 1 indiv., range = 1-18).

We then explored the relationship between heavy metals and melanin coloration. Because there was a high correlation between the concentrations of the eight heavy metals measured in the soil, we used a principal component analysis (PCA) that, after a varimax rotation, produced a PC-1 (eigenvalue = 5.03, proportion of variation explained = 63.8%), with strong positive loadings with most of all heavy metals (Cd = 0.91, Cr = 0.83, Cu = 0.72, Fe = 0.94, Mn = 0.90, Ni = 0.71, Zn = 0.90) except Pb (= 0.02), The PCA also produced a PC-2 (eigenvalue = 1.50, variation explained = 18.7%) with only a positive significant loading with Pb (= 0.89) and much lower values for the rest of metals (= 0.04 to 0.38 in absolute values).

Thereafter, we made a Generalized Linear Models (GLZ), with a normal distributional and a log link function, with proportions (square root transformed) of melanin-dependent coloration of amphisbaenians as the response variable, and the two PC scores describing soil heavy metal concentrations as continuous fixed variables. We conducted Chi-square Wald’s tests to estimate the significance of the soil variables. Statistical analyses were made with the Statistica 8.0 software (StatSoft Inc. Tulsa, OK).

RESULTS

Heavy metals in the soil and in sloughed skins of amphisbaenians

We found appreciable amounts of heavy metals in both the samples of soil (the average of 25 soil samples/site) and, although in a much lower magnitude, in the sloughed skins of amphisbaenians (single pools of skins of around 15 individuals/site). The average concentrations varied clearly among four distinct study sites of the islands (Fig. 2). This variation seemed to be mainly linked to the densities of nesting, and resting, seagulls at each site, being the concentrations of heavy metals higher in sites with more seagulls.(i.e. sites 3 and 4). The comparison of the proportions of most of the different trace elements in soil and in sloughed skins at each site suggested that, in general, concentrations in the skin of amphisbaenians increased when the concentrations of the same heavy metals in the soil also increased (Fig. 2).

Relationships between soil pollution and melanin-dependent coloration of amphisbaenians

The proportion of melanin-dependent coloration was significantly higher in the dorsal than in the ventral body area (GLM, $F_{1,102} = 118.93$, $P < 0.0001$) (Fig. 3), but it did not vary significantly among the anterior, medium and posterior zones ($F_{2,204} = 1.23$, $P = 0.29$), However, the interaction between body area and zone was significant ($F_{2,204} = 10.22$, $P <$

0.0001), showing that, in the dorsal body area, melanin proportion was significantly higher in the anterior than in the medium zone (Tukey's test, $P = 0.022$), but the rest of comparisons were not significant ($P > 0.09$ for both). However, in the ventral body area, the proportion of melanin was significantly lower in the anterior than in the posterior zone ($P = 0.006$), but there were no significant differences between other zones ($P > 0.28$ for both comparisons). In addition, there were no significant differences in melanin coloration between sexes ($F_{1,102} = 0.011$, $P = 0.92$), for any of the body areas or zones (sex x area: $F_{1,102} = 2.02$, $P = 0.16$; sex x zone: $F_{2,204} = 0.47$, $P = 0.62$; sex x area x zone: $F_{2,204} = 2.38$, $P = 0.095$) (Fig. 3).

Proportions of melanin dark coloration in the skin of amphisbaenians were significantly and negatively related with soil concentration of most of heavy metals, all but Pb (GLZ, PC-1 scores: estimate ± SE = -0.028 ± 0.006, Wald's $\chi^2 = 21.12$, $P < 0.0001$; Intercept: estimate ± SE = -0.468 ± 0.008, Wald's $\chi^2 = 3613.14$, $P < 0.0001$), but melanin dark coloration was not significantly related to Pb concentration (PC-2 scores: estimate ± SE = -0.009 ± 0.006, Wald's $\chi^2 = 1.76$, $P = 0.19$). Therefore, the amphisbaenians inhabiting soils with higher levels of soil pollution by heavy metals, all except Pb, had lower proportions of melanin-dependent coloration on their bodies (Fig. 4a), while the pollution by Pb did not seem to affect melanin coloration in a significant way (Fig. 4b).

DISCUSSION

The results of this study suggest that there was a relationship between the soil pollution by heavy metals and the concentration of heavy metals in the sloughed skins of the populations of amphisbaenians that live in those soils. This covariation suggests that amphisbaenians may be able to expulse heavy metal contaminants from their bodies when they slough the skins. In addition, there was a relationship between soil pollution and melanin-dependent coloration, but it was contrary to predictions from the "industrial melanization" hypothesis (Chatelain *et al.* 2014; Goiran *et al.* 2017). Thus, we found a negative relationship, such that amphisbaenians had more melanin-dependent dark coloration when the soils had lower concentrations of heavy metals.

We firstly found that the concentrations of heavy metals in the soil at a given site seemed to be related to concentrations of heavy metals in the sloughed skin of amphisbaenians (although with a very different, much lower magnitude). In several epigeal species of reptiles, it has been found a relationship between heavy metals in blood, muscle or other body tissues and metals in their food or the environment (e.g., Campbell & Campbell 2001; Márquez-Ferrando *et al.* 2009; Nasri *et al.* 2017; Doya *et al.* 2020), and their toxicological effects are also known (reviewed in Grillitsch & Schiesari 2010). Also, some studies found a similar relationship between environmental pollution and metals in sloughed skins of reptiles (Hopkins *et al.* 2001; Goiran *et al.* 2017). For example, concentrations of 13 heavy metals are higher in skins of turtle-headed seasnakes (*Emydocephalus annulatus* Krefft, 1869) from polluted urban-industrial areas (Goiran *et al.* 2017). We do not have data on concentrations of heavy metals in internal body tissues of amphisbaenians because, in this study, we did not intend to measure accurately the ecotoxicological levels of heavy metal pollution in amphisbaenians or their effects. However, the apparent covariation observed between concentrations of heavy metals in soil and sloughed skins suggests that amphisbaenians may detoxify their bodies, at least partly, when the skin is shed. The importance and efficacy of such detoxifying mechanism and the potential role of melnnin, are, however, not known and deserve further analyses that estimate the actual threat of soil pollution for health state of amphisbaenians. Alternatively, higher concentrations of heavy metals in the skin could potentially be simply the result of a higher integumentary direct absorption from more polluted soils, rather than of expulsion from the body. However, studies of other animals and humans (Grillitsch & Schiesari 2010; Walker *et al.* 2012) show that most metals are poorly permeable across the skin (except a few such as Pb), and this integumentary route is generally not regarded to be of significance, with most metals entering the body mainly through the gastrointestinal route (i.e., food or direct soil ingestion), Nevertheless, the integumentary route has been little explored in reptiles (Grillitsch & Schiesari 2010) and future studies should address the importance of the potential transfer of metals through the reptilian skin and lungs, specially for fossorial species that live in intimate contact to soil.

We also expected to find a positive relationship between soil pollution and melanin coloration. This would have indicated that increasing melanin in the skin might help to sequester more metals in the skin, making easier the detoxifying mechanisms that likely occurs when the skin is sloughed. This result would have been similar to the "industrial melanization" effect observed in other species (Chatelain *et al.* 2014; Goiran *et al.* 2017). Thus, for example, in some birds, there is a positive correlation between melanin-dependent plumage coloration and concentrations of some heavy metals (e.g., Zn, Mn) (Niecke *et al.* 1999, 2003; Dauwe & Eens 2008; Chatelain *et al.* 2014, 2017; Zduniak *et al.* 2014), but not of others (e.g., Pb, Cd) (Gochfeld *et al.* 1991; Chatelain *et al.* 2014, 2017). Also in turtle-headed seasnakes, melanic morphs are more common in urban-industrial areas, while dark and light banded or blotched morphs are more common in less heavily polluted sites (Goiran *et al.* 2017). Moreover, concentration of metals is higher in dark than in light skin bands of the same individual seasnakes (Goiran *et al.* 2017), supporting the metal-binding role of melanin.

Surprisingly, we found the opposite relationship, with darker amphisbaenians inhabiting soils with less pollution. In fact, in previous surveys, we even found in one of the most polluted areas two individuals with xanthic coloration (i.e., predominantly yellow color without black scales; García-Roa & Martín 2016). There might be several explanations to this unexpected finding. First, the mechanism of increased melanization in response to pollution might be physiologically impossible or too costly to produce in this amphisbaenian if there were trade offs with other functions of melanin. Also, the melanic coloration of this species might not show enough phenotypic plasticity to respond adequately to quick temporal changes in environmental conditions. Because the increase of seagull populations in these islands, and the derived soil pollution of anthopic origin, are recent phenomena in the evolutionary time,, this mechanism might require a longer time of selection to evolve and be observed in the population. However, in all of those cases above, we would expect a complete lack of relationship between contamination and melanization, while we observed a clear negative relationship.

There is a more likely alternative explanation related to the toxicological effect of heavy metals as powerful endocrine disruptors, which leads to abnormal circulating hormone levels and increased physiological stress (Giesy *et al.* 2003; Ottinger *et al.* 2005; Meillère *et al.* 2016). The expression of melanism, although is usually under genetic control (Lin & Fisher 2007), may also be condition dependent (McGraw 2008), and increased stress may negatively affect melanogenesis (Zhang *et al.* 2020). Thus, experimental manipulations showed that increased supplemented corticosterone, which increased physiological stress, negatively affected melanin-based coloration of nestling barn-owls, *Tyto alba* (Scopoli, 1769) (Roulin *et al.* 2008) or common lizards, *Zootoca vivipara* (Lichtenstein, 1823) (San José & Fitze 2013). Also, in mice (*Mus musculus* L., 1758), chronic stress suppresses the expression of some elements of the cutaneous hypothalamic–pituitary–adrenocortical (HPA) axis and melanogenesis, leading to decreased skin pigmentation (hair graying) through fast depletion of melanocyte stem cells (Bin *et al.* 2020; Pang *et al.* 2014 Zhang *et al.* 2020). Therefore, it seems that stressful factors may induce a rise in circulating corticosterone, which would inhibit the secretion of melanocortins and tyrosinase and decrease melanin production (Roulin *et al.* 2008; Pang *et al.* 2014; Zhang *et al.* 2020). Interestingly, in a previous study we found that heavy metal pollution in the soil is related to increased physiological stress (estimated from fecal corticosterone metabolite levels) of *T. wiegmanni* amphisbaenians that live buried in those soils (Martín *et al.* unpubl. data). Therefore, we suggest here that the lower melanin-dependent coloration of amphisbaenians in the most polluted sites may be explained by the presumably higher levels of physiological stress induced by the endocrine disruption effect of heavy metals, which would result in decreased melanogenesis.

In contrast to most heavy metals measured in this study, there was an apparent lack of effect of lead (Pb) on melanin coloration. Also, in some birds, Pb did not seem to affect melanin coloration (Gochfeld *et al.* 1991; Chatelain *et al.* 2014). In addition to a potential unknown lack of effect of Pb on physiology of amphisbaenians related to melanogenesis, this result might be also explained in our study area by some confounding effects due to the existence of multiples sources of contamination. Thus, pollution by Pb does not only come from depositions of seagulls, but, in men inhabited areas, Pb pollution is also associated to direct anthropogenic activities (Markus & McBratney 2000; Martín *et al.* 2017).However, in these areas used by humans, other heavy metals in the soil are scarce due to a low influence of seagulls. Also, levels of pollution by Pb in these islands do not seem to be so high (i.e., most samples were below the limits allowed for agricultural soils; L.V. García, unpubl. data) as to have an appreciable effect on stress and melanogenesis of amphisbaenians.

Our results reveal a potential limitation of the detoxifying mechanism associated to sloughing the skin. Higher levels of heavy metal pollution would require allocating more melanin to the skin to increase the detoxifying effect. However, high levels of heavy metals increase physiological stress, which would negatively affect melanogenesis. Consequently, the efficiency of the detoxifying effect would decrease, being probably lower than required to avoid the negative ecotoxicological effects of heavy metals. This result has implications for conservation of populations of these animals, and also probably of other vertebrates, in polluted habitats. In spite of the possible existence of compensatory physiological detoxifying mechanisms, the health state of fossorial vertebrates may be greatly affected by the soil characteristics and its conservation state.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for helpful commnets and the field station of the ZEC “Islas Chafarinas” for use of their facilities. We thank J.I. Montoya, J. Díaz, G. Martínez, A. Sanz, F. López, and A. Ruiz for support and friendship in the Islands. Financial support was provided by the Spanish Ministerio de Ciencia, Innovación y Universidades project PGC2018-093592-B-I00 (MCIU/AEI/FEDER, UE). Research procedures were approved by the "Comisión Ética de Experimentación Animal (CEEA)" of the Museo Nacional de Ciencias Naturales, CSIC.

REFERENCES

Andrade DV, Nascimento LB, Abe AS (2006). Habits hidden underground: a review on the reproduction of the Amphisbaenia with notes on four neotropical species. *Amphibia-Reptilia* **27**, 207-17.

Baeckens S, García-Roa R, Martín J, Ortega J, Huyghe K, Van Damme R (2017). Fossorial and durophagous: implications of molluscivory for head size and bite capacity in a burrowing worm lizard. *Journal of Zoology* **301**, 193-205.

Bin BH, Bhin J, Yang SH, Choi DH,, Park K, Shin DW, Lee AY, Hwang D, Cho EG, Lee TR (2014). Hyperosmotic stress reduces melanin production by altering melanosome formation. *PLoS ONE* **9**, e105965.

Blais JM, Kimpe LE, McMahon D, Bronwyn EK, Mallory ML, Douglas MSV, Smol JP (2005). Marine arctic seabirds transport marine-derived contaminants. *Science* **309**, 445.

Böhm M, Collen B, Baillie JEM *et al.* (2013). The conservation status of the world's reptiles. *Biological Conservation* **157**, 372–85.

Bons J, Geniez P (1996). *Amphibians and Reptiles of Morocco*. Asociación Herpetológica Española, Barcelona.

Bridelli M, Crippa P (2008). Theoretical analysis of the adsorption of metal ions to the surface of melanin particles. *Adsorption* **14**, 101–109.

Bury S, Tomasz D. Mazgajski TD, Najbar B, Zając B, Kurek K (2020). Melanism, body size, and sex ratio in snakes—new data on the grass snake (*Natrix natrix*) and synthesis. *Naturwissenschaften* **107**, 22.

Campbell KR, Campbell TS (2001). The accumulation and effects of environmental contaminants on snakes: a review. *Environmental Monitoring and Assessment* **70**, 253–301.

Campbell KR, Campbell TS, Burger J (2005). Heavy metal concentrations in northern water snakes (*Nerodia sipedon*) from East Fork Poplar Creek and the Little River, East Tennessee, USA. *Archives of Environmental Contamination and Toxicology* **49**, 239–48.

Chatelain M, Gasparini J, Jacquin L, Frantz A (2014). The adaptive function of melanin-based plumage coloration to trace metals. *Biology Letters* 10, 20140164.

Chatelain M, Pessato A, Frantz A, Gasparini J, Leclaire S (2017). Do trace metals influence visual signals? Effects of trace metals on iridescent and melanic feather colouration in the feral pigeon. *Oikos* **136**, 1542-1553.

Civantos E, Martín J, López P (2003) Fossorial life constrains microhabitat selection of the amphisbaenian *Trogonophis wiegmanni*. *Canadian Journal of Zoology* **81**, 1839–44.

Clusella-Trullas S, van Wyk JH, Spotila JR (2009). Thermal benefits of melanism in Cordylid lizards: a theoretical and field test. *Ecology* **90**, 2297-312.

Colli GR, Zamboni DS (1999). Ecology of the worm lizard *Amphisbaena alba* in the cerrado of central Brazil. *Copeia* **1999**, 733–42.

Cox M, Preda M (2003) Trace metal distribution and relation to marine sediment mineralogy, Gulf of Carpentaria, Northern Australia. *Marine Pollution Bulletin* **46**, 1615–29.

Dauwe T, Eens M (2008). Melanin- and carotenoid-dependent signals of great tits (*Parus major*) relate differently to metal pollution. *Naturwissenschaften* **95**, 969–73.

Doya R, Nakayama SMM, Nakata H, Toyomaki H, Yabe J, Muzandu K, Yohannes YB, Kataba A, Zyambo G, Ogawa T, Uchida Y, Ikenaka Y, Ishizuka M (2020). Land use in habitats affects metal concentrations in wild lizards around a former lead mining site. *Environmental Science & Technology* **54**, 22

Duarte A, Cachada A, Rocha-Santos T (2017). *Soil Pollution. From Monitoring to Remediation*. Academic Press, California.

Gans,C (1974). *Biomechanics: an Approach to Vertebrate Biology*. Lippincot, Philadelphia.

Gans C (1978). The characteristics and affinities of the amphisbaenia. *Transactions of the Zoological Society of London* **34**, 347–416.

Gans C (2005). Checklist and bibliography of the amphisbaenia of the world. *Bulletin of the American Museum of Natural History* **280**, 1–130

García LV (2005). Suelos de las Islas Chafarinas y sus relaciones ecológicas. *Ecosistemas* **14**, 135–139.

García LV, Marañón T, Clemente L (2002a). Animal influences on soil properties and plant cover in the Chafarinas Islands (NW Africa). In: Rubio JL, Morgan RPC, Asins S, eds. *Man and Soil at the Third Millennium Vol. 1*. Geoforma, Logroño, Spain, pp 705–12.

García LV, Marañón T, Ojeda F, Clemente L, Redondo R (2002b). Seagull influence on soil properties, chenopod shrub distribution, and leaf nutrient status in semi-arid Mediterranean islands. *Oikos* **98**, 75–86.

García LV, Clemente L, Gutiérrez E, Jordán A (2007). Factores condicionantes de la diversidad edáfica en las islas Chafarinas. In: Bellinfante N, Jordán A. eds. *Tendencias Actuales de la Ciencia del Suelo*. Universidad de Sevilla, Sevilla, Spain, pp 828–33.

García-Roa R, Martín J (2016). Xantismo en la culebrilla mora (*Trogonophis wiegmanni*) en las Islas Chafarinas. *Boletín de la Asociación Herpetológica Española* **27**, 12-4.

García-Roa R, Ortega J, López P, Civantos E, Martín J (2014). Revisión de la distribución y abundancia de la herpetofauna en las Islas Chafarinas: datos históricos vs. tendencias poblacionales. *Boletín de la Asociación Herpetológica Española* **25**, 55-62.

Giesy JP, Feyk LA, Jones PD, Kannan K, Sanderson T (2003). Review of the effects of endocrine-disrupting chemicals in birds. *Pure and Applied Chemistry* **75**, 2287–303.

Gochfeld M, Saliva J, Lesser F, Shukla T, Bertrand D, Burger J (1991). Effects of color on cadmium and lead levels in avian contour feathers. *Archives of Environmental Contamination and Toxicology* **20**, 523–6 .

Goiran C, Bustamante P, Shine R (2017). Industrial melanism in the seasnake *Emydocephalus annulatus*. *Current Biology* **27**, 2510–3.

Grillitsch B, Schiesari L (2010). The ecotoxicology of metals in reptiles. In: Sparling DW, Linder G, Bishop CA, Krest SK, eds. *Ecotoxicology of Amphibians and Reptiles*. CRC Press, Florida, pp. 337–448.

Headley AD (1996). Heavy metal concentrations in peat profiles from the high Arctic. *Science of the Total Environment* **177**, 105-111

Henderson RW, Powell R, Martín J, López P (2016). Sampling techniques for arboreal and fossorial reptiles. In: Dodd Jr CK, ed. *Reptile Ecology and Conservation. A Handbook of Techniques*. Oxford University Press, Oxford, pp 139-53.

Hopkins WA, Roe JH, Snodgrass JW, Jackson BP, Kling DE, Rowe CL, Congdon JD (2001). Nondestructive indices of trace element exposure in squamate reptiles. *Environmental Pollution* **115**, 1–7.

How RA, Shine R (1999). Ecological traits and conservation biology of five fossorial 'sand-swimming' snake species (*Simoselaps*: Elapidae) in south-western Australia. *Journal of Zoology* **249**, 269–82 .

Jones DE, Holladay SD (2006). Excretion of three heavy metals in the shed skin of exposed corn snakes (*Elaphe guttata*). *Ecotoxicology and Environmental Safety* **64**, 221–5.

Larramendy M (2016). *Soil Contamination. Current Consequences and Further Solutions*. IntechOpen, London.

Lin JY, Fisher DE (2007). Melanocyte biology and skin pigmentation. *Nature* **445**, 843–50.

Liu Y, Hong L, Kempf V, Wakamatsu K, Ito S, Simon J (2004). Ion-exchange and adsorption of Fe(III) by *Sepia* melanin. *Pigment Cell & Melanoma Research* **17**, 262–9.

Loumbourdis NS (1997). Heavy metal contamination in a lizard, *Agama stellio stellio*, compared in urban, high altitude and agricultural, low altitude areas of north Greece. *Bulletin of Environmental Contamination and Toxicology* **58**, 945–52.

Manjula M, Mohanraj R, Devi MP (2015). Biomonitoring of heavy metals in feathers of eleven common bird species in urban and rural environments of Tiruchirappalli, India. *Environmental Monitoring and Assessment* **187**, 1–10.

Márquez-Ferrando R, Santos X, Pleguezuelos JM, Ontiveros D (2009). Bioaccumulation of heavy metals in the lizard *Psammodromus algirus* after a tailing-dam collapse in

Aznalcollar (Southwest Spain). *Archives of Environmental Contamination and Toxicology* **56**, 276-85.

Markus J, McBratney AB (2000). A review of the contamination of soil with lead. I. Origin, occurrence and chemical form of soil lead. *Progress in Environmental Science* **24**, 291-318.

Martín J, López P, Salvador A (1991). Microhabitat selection of the amphisbaenian *Blanus cinereus*. *Copeia* **1991**, 1142–6.

Martín J, Polo-Cavia N, Gonzalo A, López P, Civantos E (2011a). Distribución, abundancia y conservación de la culebrilla mora (*Trogonophis wiegmanni*) en las Islas Chafarinas. *Boletín de la Asociación Herpetológica Española* **22**, 107–12.

Martín J, Polo-Cavia N, Gonzalo A, López P, Civantos E (2011b). Structure of a population of the amphisbaenian *Trogonophis wiegmanni* in North Africa. *Herpetologica* **67**, 250–7.

Martín J, López P, García LV (2013a). Soil characteristics determine microhabitat selection of the fossorial amphisbaenian *Trogonophis wiegmanni*. *Journal of Zoology* **290**, 265–72.

Martín J, Ortega J, López P, Pérez-Cembranos A, Pérez-Mellado V (2013b). Fossorial life does not constrain diet selection in the amphisbaenian *Trogonophis wiegmanni*. *Journal of Zoology* **291**, 226–33.

Martín J, López P, Gutiérrez E, García LV (2015). Natural and anthropogenic alterations of the soil affect body condition of the fossorial amphisbaenian *Trogonophis wiegmanni* in North Africa. *Journal of Arid Environments* **122**, 30-6.

Martín J, Gutiérrez E, García LV (2017). Alteration effects of ornamental whitewashing of rocks on the soil properties and body condition of fossorial amphisbaenians that live under them. *Herpetological Conservation and Biology* **12**, 367–72.

Martín J, Ortega J, García-Roa R, Jiménez-Robles O, Rodríguez-Ruiz G, Recio P, Cuervo JJ (2021). Going underground: short- and long-term movements may reveal the fossorial spatial ecology of an amphisbaenian. *Movement Ecology* **9**, 14.

McGraw KJ (2008). An update of the honesty of melanin based color signals in birds. *Pigment Cell and Melanoma Research* **21**, 133–8.

Measey GJ (2006). Surveying biodiversity of soil herpetofauna: towards a standard quantitative methodology. *European Journal of Soil Biology* **42**, S103-10.

Measey GJ, Armstrong AJ, Hanekom C (2009). Subterranean herpetofauna show a decline after 34 years in Ndumu Game Reserve, South Africa. *Oryx* **43**, 284–7.

Meillère A, Brischoux F, Bustamante P, Michaud B, Parenteau C, Marciau C, Angelier F (2016). Corticosterone levels in relation to trace element contamination along an urbanization gradient in the common blackbird (*Turdus merula*). *Science of the Total Environment* **566–567**, 93–101.

Nasri I, Hammouda A, Hamza1 F, Zrig1 A, Selmi1 S (2017). Heavy metal accumulation in lizards living near a phosphate treatment plant: possible transfer of contaminants from aquatic to terrestrial food webs. *Environmental Science and Pollution Research* **24**, 12009–14.

Navas CA, Antoniazzi MM, Carvalho JE, Chaui-Berlink JG, James RS, Jared C, Kohlsdorf, T, Pai-Silva MD, Wilson RS (2004). Morphological and physiological specialization for digging in amphisbaenians, an ancient lineage of fossorial vertebrates. *Journal of Experimental Biology* **207**, 2433–41.

Niecke M, Heid M, Krüger A (1999). Correlations between melanin pigmentation and element concentration in feathers of white-tailed eagles (*Haliaeetus albicilla*). *Journal of Ornithology***140**, 355–62.

Niecke M, Rothlaender S, Roulin A (2003). Why do melanin ornaments signal individual quality? Insights from metal element analysis of barn owl feathers. *Oecologia* **137**, 153–8.

Orłowski G, Kamiński P, Karg J, Baszyński J, Szady-Grad M, Koim-Puchowska B, Klawe JJ (2015). Variable contribution of functional prey groups in diets reveals inter-and intraspecific differences in faecal concentrations of essential and non-essential elements in three sympatric avian aerial insectivores: a re-assessment of usefulness of bird faeces in metal biomonitoring. *Science of the Total Environment* **518**, 407–16.

Osman KT (2013). *Soil Degradation, Conservation and Remediation*. Springer, Netherlands .

Otero XL (1998). Effects of nesting yellow-legged gulls (*Larus cachinnans* Pallas) on the heavy-metal content of soils in the Cies Islands (Galicia, north-west Spain). *Marine Pollution Bulletin* **36**, 267-72.

Ottinger MA, Quinn MJ, Lavoie E, Abdelnabi MA, Thompson N, Hazelton JL, Wu JM, Beavers J, Jaber M (2005). Consequences of endocrine disrupting chemicals on reproductive endocrine function in birds: establishing reliable end points of exposure. *Domestic Animals Endocrinology* **29**, 411–9.

Pang S, Wu H, Wang Q, Cai M, Shi W, Shang J (2014). Chronic stress suppresses the expression of cutaneous hypothalamic–pituitary–adrenocortical axis elements and melanogenesis. *PLoS ONE* **9**, e98283.

Papenfuss TJ (1982). The ecology and systematics of the amphisbaenian genus *Bipes*. *Occasional Papers of the Califonian Academy of Sciences* **136**, 1–42.

Recio P, Rodríguez-Ruiz G, Ortega J, Martín J (2019). PIT-Tags as a technique for marking fossorial reptiles: insights from a long-term field study of the amphisbaenian *Trogonophis wiegmanni*. *Acta Herpetologica* **14**, 101-7 .

Roulin, A., Almasi, B., Rossi-Pedruzzi, A., Ducrest, A.-L., Wakamatsu, K., Miksik, I., Blount, J.D., Jenni-Eiermann, S., & Jenni, L. (2008). Corticosterone mediates the condition-dependent component of melanin-based coloration. *Animal Behaviour***75**,1351–8.

San-Jose LM, Fitze PS (2013). Corticosterone regulates multiple colour traits in *Lacerta* [*Zootoca*] *vivipara* males. *Journal of Evolutionary Biology* **26**, 2681–90.

Solano F (2014). Melanins: skin pigments and much more — types, structural models, biological functions, and formation routes. *New Journal of Science* **2014**, 498276.

Sparks DL (1996). *Methods of Soil Analysis. Part 3. Chemical Methods*. Soil Science Society of America and American Society of Agronomy. Madison, MK.

Tibbett M, Fraser TD, Duddigan S (2020). Identifying potential threats to soil biodiversity. *PeerJ* **8**, e9271.

Vroonen J, Vervust B, Van Damme R (2013) Melanin based colouration as a potential indicator of male quality in the lizard *Zootoca vivipara* (Squamata: Lacertidae). *Amphibia-Reptilia* **34**, 539–49.

Walker CH, Sibly RM, Hopkin SP, Peakall DB (2012). *Principles of Ecotoxicology. 4th ed.* CRC Press, Florida.

Webb JK, Shine R, Branch WR, Harlow PS (2000). Life underground: food habits and reproductive biology of two amphisbaenian species from South Africa. *Journal of Herpetology* **34**, 510–6.

Zduniak P, Surmacki A, Erciyas-Yavuz K, Chudzińska M, Barałkiewicz D (2014). Are there different requirements for trace elements in eumelanin- and pheomelanin-based color production? A case study of two passerine species. *Comparative Biochemistry and Physiology A* **175**, 96–101.

Zhang B, Ma S, Rachmin I, He M, Baral P, Choi S, Gonçalves WA., Shwartz Y, Fast EM, Su Y, Zon LI, Regev A, Buenrostro JD, Cunha TM, Chiu IM, Fisher DE, Hsu Y-C (2020).

Hyperactivation of sympathetic nerves drives depletion of melanocyte stem cells. *Nature* **577**, 676–81.

Figure 1 Checkerboard worm lizard (*Trogonophis wiegmanni*) as it was found under a rock (head to the right), and detail (inserted picture) of the pattern of scales in the dorsal area.

Figure 2 Heavy metals quasitotal concentrations in the soil (mg/kg soil; the average of 25 soil samples/site) and sloughed skins of amphisbaenians (mg/kg dry weight; single pools of skins of around 15 individuals/site) from the same study sites. Due to the different magnitudes, concentrations in soil and skin are represented at different scales to facilitate the comparison.

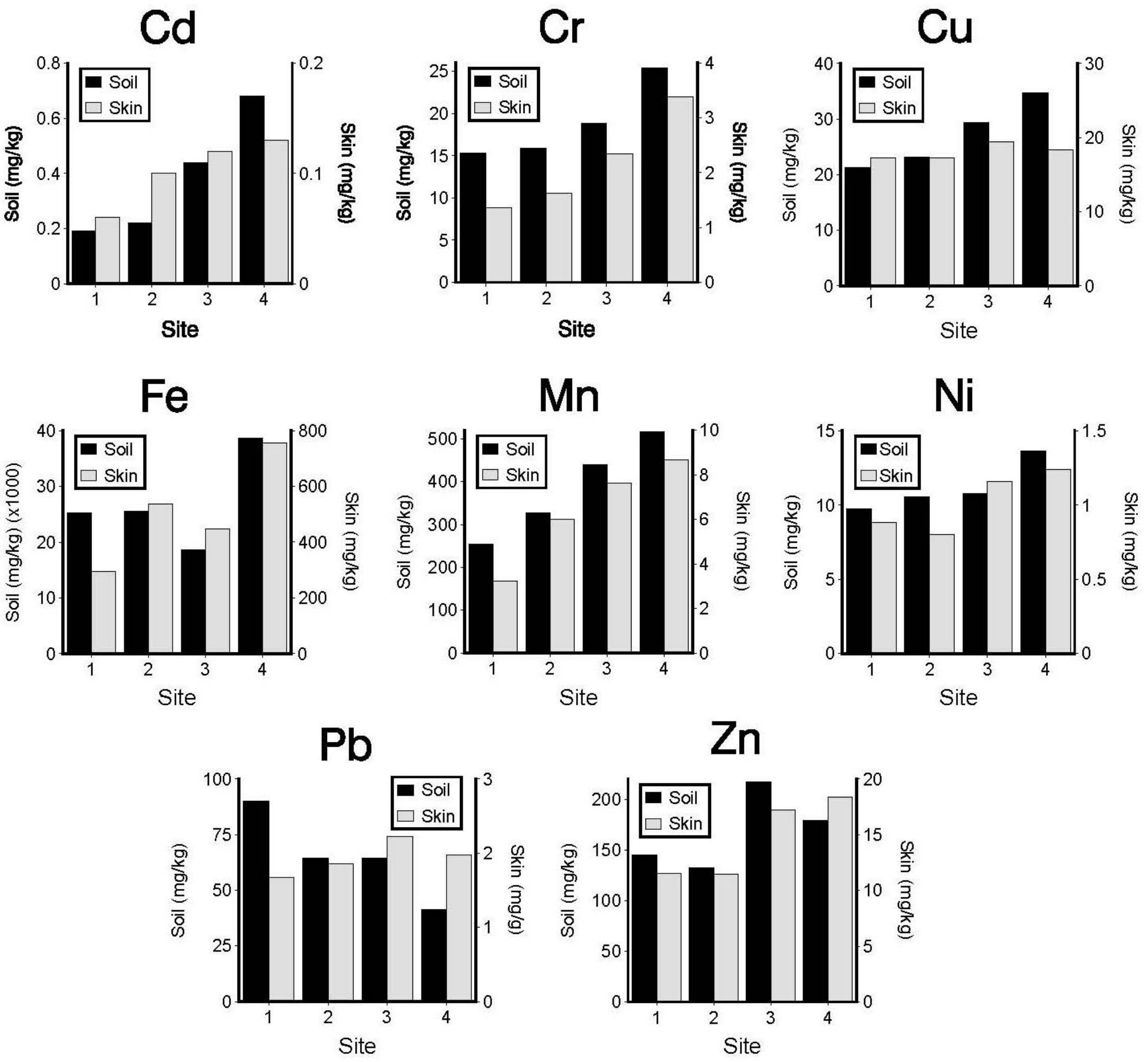

Figure 3 Proportion (mean ± SE) of melanin-dependent black coloration (i.e. relative proportion of black scales) in three different zones of the dorsal body area and three zones of the ventral body area of male and female amphisbaenians.

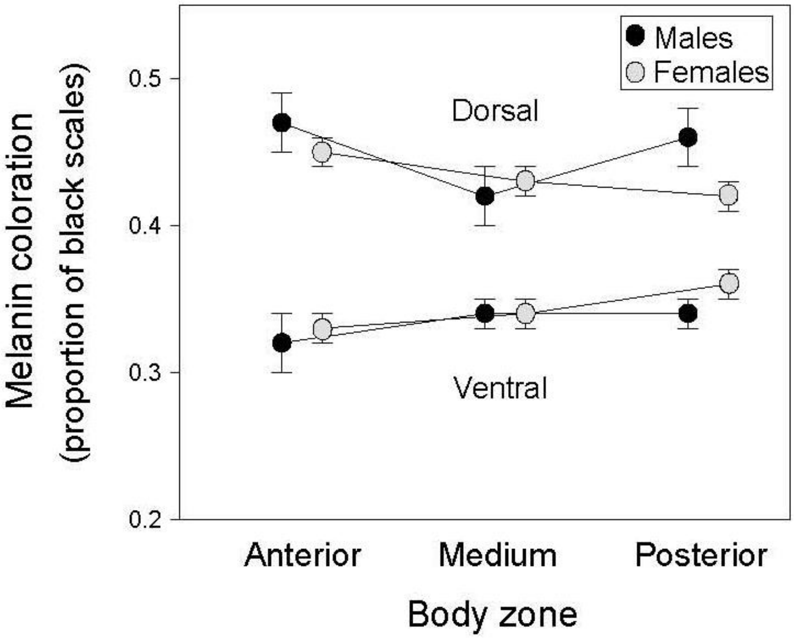

Figure 4 Relationship between principal components (PC) scores describing heavy metal concentrations at a soil sampling point and melanin-dependent black coloration (i.e. relative proportion of black scales) in the body of the amphisbaenians found close to that point.