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Ants and their nests as indicators for industrial heavy metal contamination☆

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Ants accumulate heavy metals and respond to pollution with modification in species composition, community structure, altered behaviour and immunity. However, the levels of heavy metals in ants' nests and explicit individual-level responses towards heavy metals have not been revealed. We found that red wood ants *Formica lugubris* accumulate high and correlated values of such heavy metals as *Al*, *Cd*, *Co*, *Cu*, *Fe*, *Ni*, *Pb* and *Zn* both in ants and nest material near cobalt smelter in Finland. Relative differences in metal concentrations were higher in nests than in ants. The highest values were obtained for elements such as *Co* (36.6), *Zn* (14.9), *Cd* (9.7), *Pb* (8.5), *Cu* (7.4), *Ni* (6.4), *As* (4.7), *Cr* (2.9) and *Fe* (2.4) in nest material, and *Co* (32.7), *Cd* (6.3), *Pb* (6), *Fe* (2.8), *Ni* (2.9) and Zn (2.1) in ants. In industrial and reference areas, ants have no differences in size, but differed in dry and residual body mass. In polluted areas, *F. lugubris* had less melanised heads, but not thoraxes. The sensitivity of cuticular colouration in red wood ants subjected to heavy metal pollution might be related to metal-binding properties of melanins. The overall results are useful for the improvement of biomonitoring techniques using ants as indicators of industrial contamination and for further discovery of novel ecotoxicological biomarkers.

1. Introduction

Anthropogenic heavy metal contamination of aquatic and terrestrial ecosystems remains one of the key environmental concerns globally despite of the recent technological advances in pollution management. Moreover, in less developed countries the pollution levels are currently on the rise (Gong and Barrie, 2005; Järup, 2003; Tchounwou et al., 2012). The main sources of heavy metal dispersal into environment are industry, transport, refuse burning, power generation (Agarwal, 2009) and e-waste (Robinson, 2009). Once being released, pollutants penetrate into water, soil or air in particulate or gaseous form. Further they are accumulated in the organisms and transferred through food chains, producing variable adverse effects. Therefore, an improved and timely implemented ecotoxicological risk assessment programmes may prevent further ecosystem poisoning.

With proper methods of preselection and testing, terrestrial invertebrates are reliable bioindicators and biomonitors of environmental, ecological and biodiversity changes, including metal contamination (McGeoch, 1998; Gerlach et al., 2013). Terrestrial invertebrates demonstrate considerable variations in an ability to accumulate heavy metals: high in Isopoda and low in Coleoptera (Heikens et al., 2001; Nummelin et al., 2007). The major driver factors for this ability are behaviour, physiology and diet (Gall et al., 2015). The organisms that are effective at detecting risks for the ecosystem – sentinel species (Berhet, 2013) – are especially useful as bioindicators. So, who are sentinels? To meet the requirements, a species must live permanently in a studied site, possess sufficient population range, be easy to collect and identify, be able to tolerate stress for several years and it must have well known biology for proper differentiation between true signal and background noise (Berhet, 2013). Ants (Formicidae) are widespread, abundant, sufficiently sensitive and at the same time tolerant, highly involved into food chains and do not normally migrate, except army ants (Hölldobler and Wilson, 1990). They accumulate both essential and toxic heavy metals and respond to pollution with alterations at different organisational levels (Stary and Kubizňáková, 1987; Rabitsch, 1995; Eeva et al., 2004; Del Toro et al., 2010). Not surprisingly, when satisfying all the criteria for sentinels and accumulating metals, ants should be proper indicators for industrial heavy metal contamination.

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Several studies confirmed ants as a good bioindicator group for various pollution types (Andersen, 1997; Andersen et al., 2002; Kaspari and Majer, 2000), including soil-heavy metal contamination (Ribas et al., 2012; Gramigni et al., 2013; Khan et al., 2017), aerial phthalate pollution (Lenoir et al., 2014) and land rehabilitation (Majer, 1983; Khan et al., 2017). However, most research was focused on metal levels in ants, not in nests (but see Migula and Głowacka, 1996). Recently it has been confirmed that ant nests do not buffer heavy metal pollution (Jílková et al., 2017). Nevertheless, the available information about quality and quantity of metals in ant nests is still limited. As stated by Andersen et al. (2002), ants will not be widely used as bioindicators in practical land management until the development of simple and efficient protocols for their use. Therefore, revealing the levels of heavy metals in nest material in ant hills will simplify biomonitoring frameworks. Selection of appropriate entities to measure is an essential demand for effective monitoring programmes (Lindenmayer and Likens, 2010). Further search for sensitive responses in ants at different levels of their biological organisation allow the creation of practical environmental indicators and biomarkers of pollution.

Ants tolerate heavy metal pollution better than the many other invertebrate species (Folgarait, 1998; Grześ, 2010). However, pollution may affect ants' biodiversity (Grześ, 2009a) and abundance (Khan et al., 2017), community structure (Grześ, 2009a; Belskaya et al., 2017) and colony size (Eeva et al., 2004). In addition, environmental pollution in some ant species results in an increased frequency of small workers (Grześ et al., 2015b), disturbed immunity (Sorvari et al., 2007) and altered behaviour (Sorvari and Eeva, 2010). The effects may not be only direct. For example, arsenic pollution affects a number of tree species in southern Brazil, which in turn is crucial for the arboreal ants (Ribas et al., 2012).

Despite multiple studies on ecosystem- and community-level effects, the explicit individual-level morphological responses to pollution have only rarely been studied. Several attempts failed to reveal the impact of pollution on body size (Eeva et al., 2004; Grześ et al., 2016) or on developmental instabilities (Rabitsch, 1997a; Grześ et al., 2015a). We assume individual-level responses in the traits, which are controlled more by environmental than genetic factors like body mass and colouration, may be more sensitive to environmental stress (Hill, 1995, 2011) by comparison to functionally important traits. Especially since it had been confirmed that pollution affects colouration in a taxonomically diverse range of species (Lifshitz and St Clair, 2016).

We assume that in the case of organic mound building ants, sampling ants with nest material will simplify biomonitoring. Then we suggest, that focusing an attention on individual-level responses in traits with flexible evolutionary function in metal-tolerant organisms will further help to reveal novel biomarkers of environmental pollution (Skaldina and Sorvari, 2017a). The wood ant *Formica lugubris* (Zetterstedt, 1838), which is quite common in benign and anthropogenically altered habitats of Central and Northern Europe, rarely was chosen as a model organism for revealing colony- and individual-level responses to industrial heavy metal contamination.

2. Materials and methods

2.1. Study area

We carried out our field sampling in Western Finland in one industrial area and one reference area without industrial activities in 10th June 2014 (Fig. 1). Kokkola Ykspihlaja industrial area (WGS84, 63°51: 23°3) harbours a metal smelter, cobalt factory, and sulphur dioxide factory. It has a known history of pollution with multiple heavy metals such as *As*, *Cd*, *Co*, *Cu*, *Fe*, *Hg*, *Ni* and *Zn* (Laita et al., 2008; Huuskonen et al., 2013). The reference area was located in the nearby Lohtaja region (WGS84, 63°59: 23°26) 21–25 km northeast of Ykspihlaja in a similar coastal sandy region. The forests in both areas are dominated by Scots pine (*Pinus sylvestris*) mixed with birches (*Betula pubescens*, *B. pendula*) and Norway spruce (*Picea abies*). The ground layer was dominated primarily by the scrubs *Vaccinium vitis-idaea*, *Calluna vulgaris* and various grass species (*Poaceae*).

2.2. Study species

The northern red wood ant *F. lugubris* belongs to the *Formica rufa* species group. It is one of the most common red wood ants in Central and Northern Europe, where it is particularly common in young successional stage forests and islands (Punttila, 1996; Sorvari, 2018). It appeared to be abundant in the study area in the coastal region of western Finland. In Europe, *F. lugubris* has highly variable social structure, from monogynous and monodomous to polygynous and polydomous (Bernasconi et al., 2005; Pamilo et al., 2016). In Finland, each colony of this species occupies typically one nest and has one or only few reproducing queens (Pamilo and Rosengren, 1983). Colonies of *F. rufa* goup species forage within 30 m of the colony, but sometimes their routes can reach more than 100 m (Savolainen and Vepsäläinen, 1988; Sorvari, 2009). A previous study related to heavy metal pollution and red wood ants, revealed a very low abundance of *F. lugubris* in the Harjavalta industrial area located in the southwest of Finland (Eeva et al., 2004). Rarely found in Harjavalta, this species was the most common representative of the *F. rufa* group in Kokkola and Lohtaja. We conducted sampling in summer time, as previously it has been shown that there is a seasonal variation in metal levels in red wood ants: lower in spring and higher during summer (Rabitsch, 1995). In addition, we collected samples within one day to avoid seasonal changes in body mass of similarly sized workers (Rabitsch, 1997b).

2.3. Sampling and preparation of ants and nest material

We studied 20 colonies of *F. lugubris* located from 0.4 to 25 km from the metal smelter and the cobalt refinery. Ten of the studied colonies were located close to the factories in the Ykspihlaja peninsula within 0.4–0.85 km from the factories. Ten colonies were 21–25 km away from the factories located in the Lohtaja region (Fig. 1). From each colony we took nest material samples with live ants from the top of the nest mounds, placed them into 0.75 L plastic containers and brought to the university campus in Kuopio. Different categories of red wood ant worker accumulate different levels of heavy metals (Maavara et al., 1994). For biomonitoring purposes, surface workers and foragers fit the best, as they take in the highest quantities of chemicals among all functional groups of ants (Maavara et al., 1994; Migula and Głowacka, 1996). Anyhow, they are the easiest group of worker ants to be collected by a non-specialist person. The ants were allowed to come out of the containers to larger, 5 L transparent closed containers. When the majority of ants had moved to the larger container, they were freeze killed. After all, two colonies had a limited number of workers, so we used ants from 18 colonies for morphological data analyses. Eight individuals from each of the nests were randomly selected for further morphometric and colour analyses ($N = 142$). The other ants (30–50 workers) were put in open plastic tubes and the nest materials in open paper bags and dried in an oven set to 55 °C for 48 h. The dried ants were pulverised with a plastic pestle in a plastic tube. An unused pestle and tube was used for each nest sample to avoid contamination. Dried nest material was sieved through clean 1.5 mm plastic sieves to get homogenous fine material samples and stored in 15 ml plastic with screw cap.

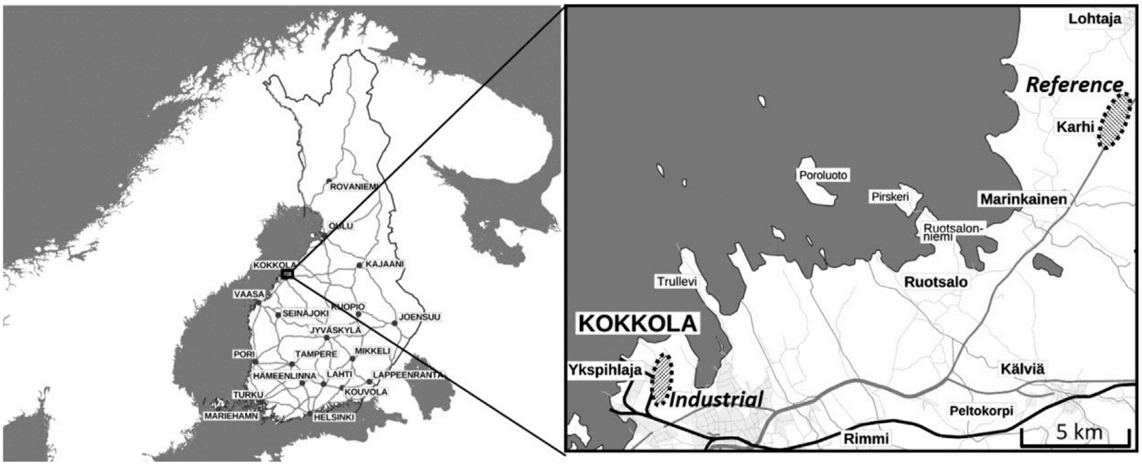

Fig. 1. Locations of the study areas in western Finland. Industrial area has zinc, cobalt and sulphuric acid factories. The reference area represents similar habitat without factories.

2.4. Heavy metal analyses

The heavy metal analyses were conducted for *Al*, *As*, *Cd*, *Co*, *Cr*, *Cu*, *Fe*, *Ni*, *Pb*, *Sr*, *V* and *Zn*. Ant samples (42.5–95.3 mg) and nest material samples (225.0–283.7 mg) were accurately weighed, transferred into Teflon digestion vessel and 8.0 mL of conc. HNO_3 (TraceMetal Grade, Fisher Chemical) was added. Microwave digestion was performed using MARS 6 iWave instrument. After the digestion samples were diluted to 100 ml with ultrapure Milli-Q-water. The determination of concentrations of the analytical elements was done with ICP-MS spectrometer (PerkinElmer NexION 350 D). The calibration of the instrument was done with certified multielement solution (TraceCERT Periodic table mix 1 for ICP) from Sigma-Aldrich.

2.5. Morphometric, digital photographing and melanisation analyses

Of the ant samples, eight random individuals per nest were dried individually in an oven set to 55 °C for 48 h and weighed with a Metler Toledo scale to the nearest 0.001 mg. After that, each individual ant was dissected into head and thorax. Each body part was carefully mounted to a wooden base with wood glue to reduce the further possible error in position offset. They were separately photographed using a Nikon DS-Fi1 microscope camera (5 megapixel CCD) and a DS Camera Control Unit attached to an Olympus SZX9 trinocular microscope, and the photos were captured using NIS-Elements BR imaging software version 3.2. The photos of the anterior side of the head (face) and dorsal side of the thorax were taken at 17 ms exposure, and magnification × 25 for both. We performed the photography under stable lighting conditions in a windowless room. The photographs were saved as uncompressed JPG files and later used for digital image analyses.

For the analyses of the degree of darkness, we converted the initial RGB photos to grey scale photos in Adobe Photoshop CS5. The head width (HW) above compound eyes was measured as an estimator of an individual body size. We measured it from digital pictures using the software ImageJ. Degree of melanisation (MD) was scaled as a rectangular area from head and oval area from thorax, namely pronotum (Pn) and mesonotum (Mn), a score of zero was counted as white and 255 was counted as black. We performed the MD measurement two times and took mean values for the final analyses. The measurements were conducted in two temporally separated occasions, to reduce the possible manual measurement error. The method for MD measurements is described in detail in Skaldina and Sorvari (2017b). The variation of *F. lugubris* head colour morph was very low as the majority (c.a. 95%) of the individuals in both the industrial and the reference area belonged to the same variants: head – V and pronotum – V, as described in Skaldina and Sorvari (2017b). Therefore, we did not check the effect of environmental pollution on head and pronotum colour polymorphism in *F. lugubris* wood ants.

2.6. Statistics

Statistical analyses were carried out using SAS statistical software, version 9.4 (SAS Institute Inc.). The correlation of heavy metals between nest materials and worker ants was analysed using Pearson correlations. Analyses on the difference in concentrations of heavy metals in industrial and reference habitats was analysed using t tests using a Satterthwaite corrected test when the equality of variances were not met. We carried out Principal component analysis for the nine intercorrelated heavy metal concentrations in ant samples using procedure FACTOR and Varimax rotation.

In the analyses of individual ant morphology, we used linear mixed models (LMM, procedure MIXED in SAS). Since there were eight workers per nest, the nest of origin was used as a random factor in all analyses combined with Kenward-Roger calculation for the degrees of freedom. All means are presented with 95% confidence limits, unless specified otherwise.

3. Results

The ICP-MS detected *Al*, *As*, *Cd*, *Co*, *Cr*, *Cu*, *Fe*, *Ni*, *Pb*, *Sr*, *V* and *Zn* from nest materials and *Al*, *Cd*, *Co*, *Cu*, *Fe*, *Ni*, *Pb*, *Sr* and *Zn* from ant body homogenates. Several heavy metal levels such as *Al*, *Cd*, Co, Fe, Ni, *Pb* and *Zn* correlated strongly between ants and ant nest material (Table 1). Correlation for *Cu* was much weaker (Pearson $r = 0.47$, $p = 0.034$), and levels of *Sr* did not correlate between ants and nest material (Pearson $r = 0.12$, $p = 0.624$).

In our sample, there was significant difference in levels of all these heavy metals between industrial and reference areas (Tables 2 and 3), except *Al, Cr* and *Sr* in nest material and *Al* and *Sr* in ants (Tables 2 and 3). Concentration of cadmium was roughly ten times higher in ant workers than in ant nests in both habitat types, being clearly higher in industrial site.

We calculated relative differences between industrial and reference sites by dividing the mean metal concentration of

Table 1
Correlation in levels of heavy metals between ants and ant nest material sampled from industrial site (Kokkola) and reference site (Lohtaja) in western Finland.

Heavy metal	*r*	*p*
Al	0.76	<0.0001
Cd	0.69	0.0007
Co	0.86	<0.0001
Cu	0.47	0.034
Fe	0.88	<0.0001
Ni	0.66	0.0015
Pb	0.69	0.0006
Sr	0.12	0.624
Zn	0.66	0.0015

Table 2
Mean concentrations, presented with confidence limits (95% CL) of heavy metals (mg/g) in ant nest material from industrial site (Kokkola) and reference sites (Lohtaja) in western Finland and the results of *t* tests.

Element	Habitat type	Concentration	95% CL	df	*t*	*p*
Al	industrial	2.7458	1.0681	18	1.66	0.114
	reference	1.8954	0.5957			
As	industrial	0.0047	0.0018	8.48	4.62	0.0015
	reference	0.0010	0.0003			
Cd	industrial	0.0029	0.0010	8.16	5.88	0.0003
	reference	0.0003	0.0001			
Co	industrial	0.0696	0.0274	8.01	5.71	0.0004
	reference	0.0019	0.0005			
Cr	industrial	0.0104	0.0072	8.46	2.18	0.059
	reference	0.0035	0.0012			
Cu	industrial	0.0438	0.0156	8.10	5.59	0.0005
	reference	0.0059	0.0012			
Fe	industrial	4.9461	0.6795	18	6.94	<0.0001
	reference	2.0690	0.6391			
Ni	industrial	0.0198	0.0067	8.34	5.73	0.0004
	reference	0.0031	0.0009			
Pb	industrial	0.0439	0.0018	8.20	5.02	0.0009
	reference	0.0052	0.0019			
Sr	industrial	0.0110	0.0038	10.69	1.94	0.079
	reference	0.0077	0.0015			
V	industrial	0.0062	0.0020	18	2.46	0.024
	reference	0.0038	0.0012			
Zn	industrial	0.6862	0.2652	8.02	5.57	0.0005
	reference	0.0459	0.0075			

industrial site samples by mean metal concentrations of reference site samples. The largest relative differences in metal levels (mg/g) between industrial and reference study areas were revealed for elements such as *Co* (36.6), *Zn* (14.9), *Cd* (9.7), *Pb* (8.5), *Cu* (7.4), *Ni* (6.4), *As* (4.7), *Cr* (2.9) and *Fe* (2.4) in nest material, and *Co* (32.7), *Cd* (6.3), *Pb* (6), *Fe* (2.8), *Ni* (2.9) and Zn (2.1) in ants.

Principal component analysis made for heavy metals, detected from ant body homogenates, identified two principal components that had an eigen value over one (PC1 and PC2). Component loadings are 64.6% of variance for PC1 and 12.9% of variance for PC2. All other metals except *Al* correlated positively (>0.65) with the PC1, whereas *Al* correlated strongly positively and *Fe* weakly positively with PC2. The other metals did not do so (Table 4). Therefore, PC1 effectively describes general heavy metal pollution, and PC2 that of *Al*. The overall level of heavy metals in ants (PC1) differed significantly between industrial area and reference sites ($t = 8.33$, $df = 19$, $p < 0.0001$; but PC2 did not do so ($t = 0.78$, $df = 19$, $p = 0.4456$; Fig. 2). Therefore, PC1 was used as an explanatory variable for further analyses related to the morphology of ants.

The analyses of morphological traits in ants from industrial and reference areas revealed that the ant head widths did not differ between zones (industrial area: 1.80 ± 0.06 mm, reference sites: 1.84 ± 0.05 mm; $F_{1,\ 16.1} = 0.85$, $p = 0.37$). However, significant

Table 3
Mean concentrations presented with confidence limits (95% CL) of heavy metals (mg/g) in ants from the industrial site (Kokkola) and reference sites (Lohtaja) in western Finland and the results of *t* tests.

Element	Habitat type	Concentration	95% CL	df	*t*	*p*
Al	industrial	0.1464	0.0820	19	1.41	0.18
	reference	0.1075	0.0913			
Cd	industrial	0.0253	0.0035	11.18	12.80	<0.0001
	reference	0.0040	0.0012			
Co	industrial	0.0098	0.0019	9.18	11.3	<0.0001
	reference	0.0003	0.0002			
Cu	industrial	0.0186	0.0021	19	2.64	0.0160
	reference	0.0142	0.0030			
Fe	industrial	0.3287	0.0919	19	4.63	0.0002
	reference	0.1175	0.0514			
Ni	industrial	0.0023	0.0006	19	3.62	0.0018
	reference	0.0008	0.0006			
Pb	industrial	0.0060	0.0014	19	6	<0.0001
	reference	0.0010	0.0012			
Sr	industrial	0.0103	0.0025	19	2.09	0.0505
	reference	0.0065	0.0012			
Zn	industrial	0.8075	0.0950	19	7.28	<0.0001
	reference	0.3921	0.0865			

Table 4
Correlation coefficients of heavy metals and the varimax rotated principal components (PC1 and PC2) obtained from principal component analysis of *Formica lugubris* workers that were collected from an industrially polluted site (in Kokkola) and reference sites (in Lohtaja) in western Finland.

Metal	PC1	PC2
Al	0.45	0.85
Cd	0.85	−0.04
Co	0.88	−0.26
Cu	0.76	−0.28
Fe	0.84	0.41
Ni	0.85	0.06
Pb	0.90	0.07
Sr	0.65	−0.30
Zn	0.93	−0.17

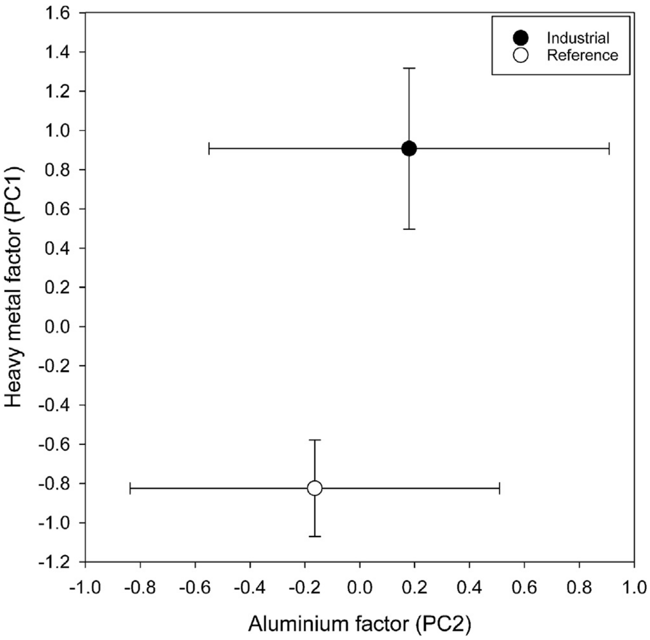

Fig. 2. Estimated marginal means and 95% CL for heavy metal factor (PC1) and aluminium factor (PC2) of ants from industrial site (Kokkola) and reference sites (Lohtaja) in western Finland. The values are obtained from linear mixed models after principal component analysis. Component loadings are 64.6% of variance for PC1 and 12.9% of variance for PC2.

difference in dry body mass was found. The workers from close to the metal industry area had less body mass compared to the workers from further away (industrial area: 3.76 ± 0.42 mg, reference area: 4.65 ± 0.38 mg; $F_{1,\ 16} = 11.12$, $p = 0.0042$). Residual body mass (residuals from a regression between body mass and head width; $r^2 = 0.36$, df = 1, $t = 8.90$, $p < 0.0001$) was lower in industrial area compared to that of the reference area ($F_{1,\ 15.9} = 8.96$, $p = 0.0087$; Fig. 3).

Ants from industrial area had lighter heads than those from the reference site ($F_{1,\ 16} = 8.29$, p = 0.0109; Fig. 4). In addition, head melanisation degree was significantly affected by the levels of heavy metals (PC1) ($F_{1,\,16} = 11.38$, $p = 0.0039$) (Fig. 5). However, no association between habitat type, levels of heavy metals (PC1) and darkness of thorax was revealed (Pn: $F_{1,\,15.9} = 0.78$, $p = 0.39$; Mn: $F_{1,\ 15.3} < 0.01$, $p = 0.99$).

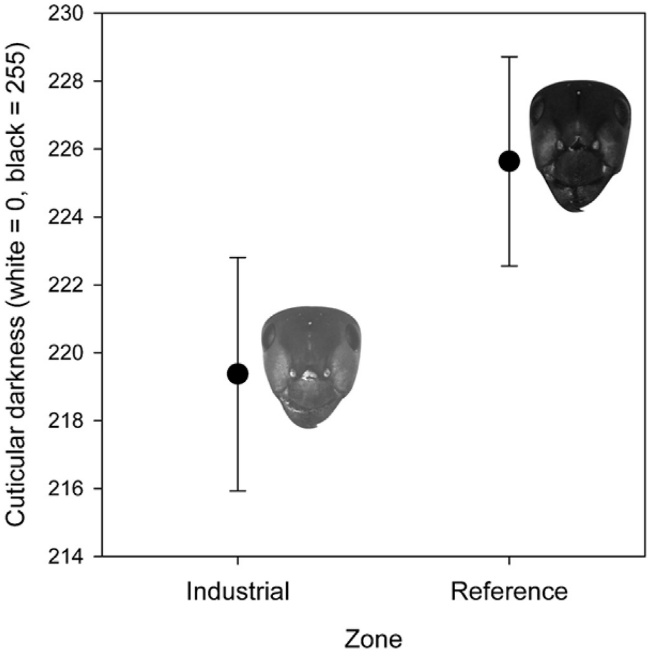

Fig. 4. Estimated marginal means and 95% CL of cuticular darkness of *Formica lugubris* ant worker foreheads in an industrial site (in Kokkola) and reference sites (in Lohtaja) in western Finland. Cuticular darkness was measured from grayscale photos using grayscale values of ImageJ software.

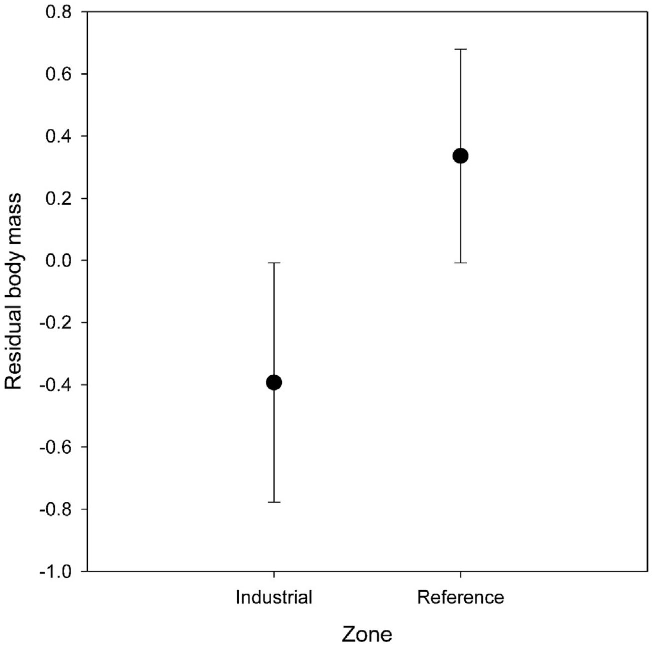

Fig. 3. Estimated marginal means and 95% CL of residual body mass, a body size-free estimate of body mass, of *Formica lugubris* ant workers in an industrial site (in Kokkola) and reference sites (in Lohtaja) in western Finland. The values are obtained from linear mixed models after taking residuals from a regression between head width and body mass of individual ants.

4. Discussion

Our results confirmed that both nest material and worker ants of *F. lugubris* can indicate industrial pollution, as they accumulate detectable and correlating levels of heavy metals. Therefore, for revealing the presence of these metals in the terrestrial ecosystems, it is possible to sample nest material and ants. However, further studies revealing the interrelation between metals in anthills and soils across the other smelters of the similar type would result in more generalized conclusions. Relative difference in heavy metal levels between industrial and reference areas were generally higher in ant nest material than in ant homogenates. We found the highest relative differences for *Co*, *Zn* and *Cd* in nest material, and for *Co*, *Cd* and *Pb* in ants. Recently Jílková et al. (2017) have revealed that the content of heavy metals in ant nests is not lower from those in the surrounding soils, therefore the ant nests do not buffer environmental pollution. It was shown that metal concentrations in soil samples are more heterogenous than those in soil invertebrates (Notten et al., 2005). Moreover, it was pointed out that in the common Mediterranean ant *Crematogaster scutellaris,* the quality and quantity of heavy metal loads in ants increases with an increase of those in the soil (Frizzi et al., 2017). It would be beneficial to study interrelation between metal concentrations in soils (depending on the soil type) and further transfer of metals from soil to fungi and plants, associated with red wood ants. When constructing nests, red wood ants use both mineral soil and organic

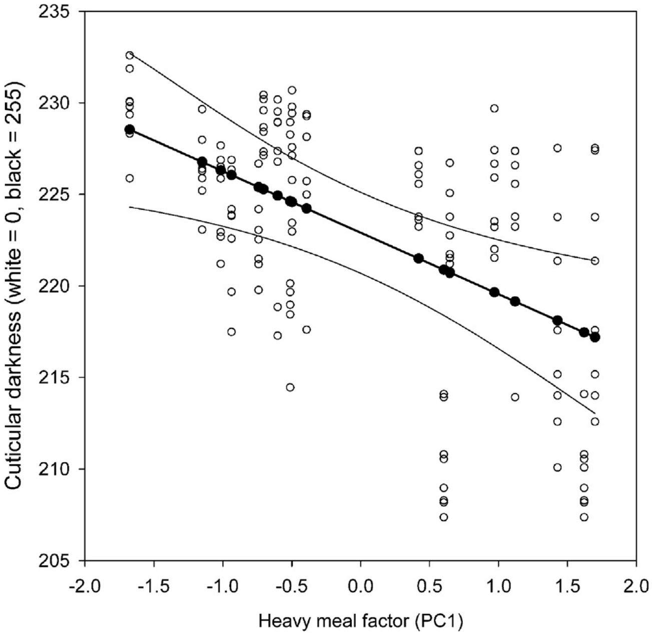

Fig. 5. Predicted association of cuticular darkness of forehead and the heavy metal factor (PC1) with 95% CL (filled symbols and lines) and the individual raw data (open symbols). The predicted values are obtained from a linear mixed model after a principal component analysis.

matter such as conifer needles, leaves, grasses, twigs, small branchlets, etc. In addition, ant nests store aphid honeydew, remains of invertebrates and plant seeds (Risch et al., 2016). Accumulating such diverse types of organic materials, ant nests may provide sufficient information about general quality of the surrounding environment at the radius of effective foraging area, which is typically about 30 m in *Formica rufa*-group red wood ants (Savolainen and Vepsäläinen, 1988). One can consider ant nests as biodiversity hotspots for particular ecosystems (Hughes et al., 2008). Due to accumulation of various environmental material and high loads of heavy metals, red wood ant nests can act as bioindicator “hotspots” in monitoring surveys.

Formica lugubris has earlier been used in a national heavy metal survey in Finland (Ukonmaanaho et al., 1998) and was shown to be useful in that kind of surveys. Heavy metal concentrations in ants depend on the biological role of a metal, its distribution in the ecosystem, and the type and source of exposure (Migula and Głowacka, 1996). Therefore, the presence or concentrations of different metals may vary between closely related species and between study locations. In a previous study using *F. aquilonia* in Harjavalta copper smelter site in southwestern Finland, Eeva et al. (2004) found the largest differences between industrial and reference sites for the elements such as *As*, *Ni*, *Cu* and *Pb* while the largest relative differences in our current study with *F. lugubris* in Kokkola zinc smelter and cobalt refinery site in western Finland, were obtained for *Co*, *Cd*, *Pb*, *Ni* and *Fe* concentrations in ant homogenates. Interestingly, the zinc concentrations were almost 14 times higher in Kokkola industrial site nest materials compared to that of reference sites, but only times higher in workers. Zinc is a common metal in the earth's crust, having on average concentration about $75\ \mathrm{mg\ kg^{-1}}$ in soil (Emsley, 2001). Insects, such as *F. lugubris* in our study, may have evolved to have effective regulation mechanisms for zinc, because it is so common, and thus, may not accumulate zinc in relatively mildly polluted sites like Kokkola Ykspihlaja.

While toxic in high concentrations, elements such as *Cu*, *Fe*, *Mn*, *Se* and *Zn* are essential micro nutrients for normal metabolism (Bodgen and Klevay, 2000) and are also needed for proper development of ants. For example, in the leaf-cutting ant *Atta sexdens*, the hardness of the mandibles correlates with *Zn* content, therefore sufficient levels of *Zn* are crucial for the soldiers (Schofield et al., 2002). So, moderately elevated levels of some heavy metals may even be beneficial for the ants. It has been shown that red wood ants are able to tolerate pollution and maintain reproducing colonies in heavily contaminated sites (Eeva et al., 2004; Grześ, 2010). The other chemicals, such as *Cd*, *Co*, *Pb*, *Hg* are useless, even poisonous, depending on concentration, exposure time or combined effect with the other metals. Migula et al. (1997) found that under elevated levels of *Cd* exposure, red wood ants develop biochemical compensatory mechanisms. However, prolonged exposure of *F. aquilonia* red wood ants to *Cd* results in metabolic disorders both in workers and pupae; and an exposure to both *Cd* and *Hg* increases the propensity of colony mortality (Migula et al., 1997).

Our results revealed high relative differences for *Co* and *Cd*, both in ants and their nests. Cadmium accumulation by ants is consistent with the other studies (Stary and Kubizňáková, 1987; Rabitsch, 1995; Gall et al., 2015). However, explicit individual-level responses in ants have not been revealed so far.

Heavy metals may affect different developmental parameters in taxonomically diverse group of insects. For example, excess of *Cd* and *Cr* decreases the pupation rate in black soldier fly larvae *Hermetia illucens* (Diptera, Stratiomyidae; Gao et al., 2017). Cadmium pollution results in decreased body length, body mass and deformations in digestive and reproductive systems in the ground beetle *Blaps polycresta* (Coleoptera, Tenebrionidae; Osman et al., 2015). Copper and lead decreases body length (Görür, 2006) and produces developmental instabilities (Görür, 2009) in the cabbage aphid *Brevicoryne brassicae* (Hemiptera, Aphididae). Elevated levels of *Cd*, *Pb* and *Zn* led to decreased size of wings in the females of the red mason bee species *Osmia bicornis* (Hymenoptera, Megachilidae; Szentgyörgyi et al., 2017). Therefore, we suppose that further search for individual-level responses in ants towards pollutants may contribute to development of biomarkers – biological responses towards chemical contaminants, measured in an organism or its products and not occurring in non-exposed organisms (Romeo and Giamberini, 2013).

We found that ants from industrial sites have decreased body mass, but not body size. This finding may be related to *Cd* accumulation, as cadmium pollution results in decreased body mass in ground beetles (Osman et al., 2017), mason bees (Moroń et al., 2014), cotton bollworm (Zhan et al., 2017). However, such responses have not been reported for the ants. In insects, heavy metals distributed among tissues unevenly. High concentrations are frequently found to accumulate in the intestines (Lukàň, 2009). Metals can be accumulated and stored in different body parts of the ants, for example *Mn* and *Zn* in exoskeleton and mandibles (Schofield et al., 2002), *Fe* in the fat body, *Zn* in the gut wall, *Zn* and *Sr* in the Malpighian tubules (Gramgini et al., 2010). As shown by Rabitsch (1997c) such elements as *Cd*, *Cu*, *Fe*, *Mn* and *Pb* in ants reach the highest concentrations first in midgut, then in malpighian tubules and the hindgut. Therefore, we conclude that more studies, related to localisation of heavy metals in red wood ants, will help to reveal direct interrelation between pollution and decreased body mass.

Not all the heavy metal accumulating ants can be used as bio indicators in biomonitoring surveys. For example, *Lasius niger* can rapidly accumulate high levels of *Cd*, however also rapidly downregulate its level when the rate of pollution is increased (Grześ, 2009b). Generally, regulatory mechanisms of metal tolerance include: a) avoidance of polluted resources; b) overexpression of genes related to pollution; c) ability to store metals in non-soluble forms and d) an ability to excrete them (Hopkin, 1989). As it was pointed out, that the largest source of heavy metal uptake for red wood ant is honeydew from aphids (Stary and Kubizňáková, 1987). Therefore, red wood ants more likely do not avoid polluted resources like, for example, some grasshoppers do (Migula and Binkowska, 1993). Tolerance of heavy metals in ants may either be related to regulatory metabolic mechanisms, occurrence of metal-binding proteins (Rabitsch, 1995) or some other amino acid derivatives, like complex polymer melanins (McGraw, 2003). Therefore, changes in melanisation may be useful indicators of heavy metal pollution.

Colouration traits as reflectors of individual quality have been much more intensively studied in vertebrates, especially in birds, as compared to invertebrates (Roulin, 2016). As we currently do not know about any responses in insects with altered colouration traits towards heavy metal pollution, we can derive some hypothesis using the examples from the other taxonomic groups.

Different pigments such as ommochromes, pterins, bile pigments and melanins produce colours in insects (Fuzeau-Braesch, 1972). Melanin pigment produce grey-to-black (eumelanins) and red-to-brown (feumelanins) colours in the majority of insect species (Fuzeau-Braesch, 1972). Besides the enzyme tyrosinase, biosynthesis of melanin requires stimulatory action of metal ions, especially *Cu*, *Fe* and *Zn* (Prota, 1993). As pointed out by McGraw (2003), melanin-based colouration can be an honest and costly signal of an individual quality, entailing accumulation, stimulatory action and detoxification of metal ions. McGraw (2003) predicted that individuals, possessing higher levels of metal concentrations in their bodies, should develop more melanised patterns concerning either area, intensity or both. This was later confirmed for great tit

Parus major (Dauwe and Eens, 2008; Giraudeau et al., 2015). However, detailed analyses of the effects of heavy metals on melanin-based colouration in the same species revealed their ability for both increment and decrement. For great tit, it was found that *Cu* affected melanin colouration positively and *Cr* negatively, however no physiological mechanism for these effects have been discovered (Giraudeau et al., 2015).

McGraw (2003) noticed that across different species, the effect of various metals on colour traits may differ significantly. In *F. lugubris*, we found that ants from polluted areas had lighter heads, but not thoraxes, which was related to the levels of heavy metals. Different results for colour traits on different parts of the body of red wood ants can be explained with the modularity of colour in red wood ants (Skaldina and Sorvari, 2017b). It is worth of noting that melanin-based colour traits are highly species-specific (Stoehr, 2006). The finding that heavy metal pollution affects red wood ant colouration opens the door to a further search for eco-toxicological biomarkers in ants' colouration. However, detailed investigations about physiological and biochemical mechanisms underlying this sensitivity is necessary.

To summarise, *F. lugubris* is a proper bioindicator species to monitor industrial heavy metal pollution. Both ants and nests accumulate detectable and correlated levels of heavy metals. Thus, for the simplification of biomonitoring techniques, it is possible to sample nest material without separating ants to get an overall value of ecosystem contamination or sample nest material and ants separately as indicators of litter contamination and bioavailability of heavy metals. At the individual level *F. lugubris* responds to heavy metal pollution with decreased body mass and decreased melanisation of the head. These explicit individual-level responses may contribute to creating novel biomarkers of industrial heavy metal pollution.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.04.134.

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