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Ant cuticles: A trap for atmospheric phthalate contaminants

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HIGHLIGHTS

- Ants cuticle contaminated by phthalates.
- Ants were able to limit phthalate contamination, probably they metabolize them.
- Phthalates come from the ambient air.
- Phthalates contamination everywhere including all of the insects we investigated.

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ABSTRACT

Phthalates are universal contaminants. We show that they are trapped by the ant cuticles and maintained permanently at a low level, generally less than 1% of cuticular components. They are found throughout the interior of the insect, predominately in the fat body, which suggests that they are adsorbed by the cuticle. In open plastic boxes free of phthalates the ants became more contaminated with phthalates over a period of time, whereas in closed glass jars they did not. This finding suggests that the main source of pollutants is the atmosphere. Different ant species collected from multiple places showed similar levels of contamination. It appeared that in some pristine places the contamination was lower, but this needs to be confirmed. Ants can be considered as bio-indicators of phthalate pollution.

1. Introduction

Phthalate esters are used industrially in many products, including cosmetics, shampoos, soaps, lubricants, pesticides and paints and occupy 80–85% of the plasticizer market. They are also used to make plastics more flexible in a variety of products like polyvinyl chloride (PVC). Several phthalates have been identified and classified as endocrine disruptors possibly due to their estrogenic and anti-androgenic activities in animal models (Colborn, 1998; European Environment Agency, 2012). Because phthalate esters are not chemically bound to the plastics, they can be easily released from products and migrate into food or water that comes into direct contact with them (Jen and Liu, 2006). They are found everywhere in sediments, storm waters (Björklund et al., 2009), and in aerosols (Teil et al., 2006; Alves et al., 2007; Salapasidou et al., 2011 and the review of Babich and Osterhout (2010)). Contamination in water, sediment, soils and biota may be toxic to mammals and aquatic organisms (Zhou et al., 2005). They are hydrophobic and are therefore generally extracted by organic solvents.

The cuticle of terrestrial insect constitutes an important barrier against the environment. It is composed of two major layers: the underlying procuticule with a chitin–protein complex providing the rigidity and the epicuticule with a lipid layer (hydrocarbons, ketones, wax esters, alcohols, free fatty acids, aldehydes) (Hadley, 1981). These lipids protect against desiccation but are also acting as a trap that fixes lipophilic substances from the environment (Gibbs, 1998). In social insects like ants, bees or wasps hydrocarbons serve as signal for nestmate recognition (d'Ettorre and Lenoir, 2010). In numerous studies on nestmate recognition in social insects phthalates are found with cuticular hydrocarbons but considered as universal contaminants and almost systematically neglected (Tissot et al., 2001; Nash et al., 2008; Kather et al., 2011).

In our research on nestmate recognition in the common black ant *Lasius niger* (Lenoir et al., 2009), we found always various phthalates on the ant cuticle and decided to study the ant pollution by these substances. We measured phthalate quantities on the cuticle of ants just after collection in the field. We measured the evolution of phthalate

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amounts of ants in phthalates-free plastic nests in the laboratory. The variation in these tests suggested contamination by the air, so we repeated the tests using closed glass containers. To locate the source of contamination we measured the phthalate quantities present on the substrate (foraging arena and inside the ants nest) and in the air in the laboratory. Finally, to search if the pollution was a general phenomenon, a large survey of ants and other insects from various places in the world was conducted.

2. Material and methods

2.1. Insects

Colonies of *L. niger*, black garden ant, common in temperate European regions, were collected in April 2009 in an orchard near Tours (Azay sur Cher, FR). All colonies were queenless and composed of 300 to 500 workers with brood. Colonies were kept in the laboratory (temperature 25 °C, natural daylight) in plastic boxes (27.5×28.5×9 cm; polystyrene without DEHP-Mino Gaillard ®, FR — theses boxes are not longer manufactured). The absence of phthalates in the boxes was verified with fragments of plastic deposited in hexane, methanol or dichloromethane for several days. Ant nests were made in each box with glass tubes half filled with water. The ants were fed twice a week with mealworm larvae and commercial bumblebee solution (Beehappy ®, Koppert Biological Systems).

Specimens of 17 ant species were collected at different localities in Western Europe and Maghreb (see Table S2, with a myrmecophile beetle). For these ants, the phthalate absolute quantities were not measured, but the relative proportions on the cuticle were calculated. We also assessed phthalate levels on the wood cricket *Nemobius sylvestris* and on honeybees. For honeybees we used only legs, due to the large size of the insects.

2.2. Chemical analysis

Lasius samples were made of individual workers killed in the freezer and deposited in 100 µl of pentane. After 1 h the samples were removed and 5 µl of pentane containing 50 ng of eicosane (C20) was added as an internal standard. We verified that pentane was uncontaminated with phthalates. The solvent was then evaporated until 3 µl remained. Samples were injected to a FID gas-chromatograph (VGM250Q system, Perkin-Elmer) equipped with a split/splitless injector and flame ionization detector using a BP-1 fused silica capillary column (Perkin-Elmer, 25 m, 0.32 mm, 0.5 µm). The temperature was kept at 150 °C during the initial splitless 2 min, raised from 150 °C to 300 °C at 5 °C/min and held at 300 °C for the last 10 min. The non-volatile cuticular lipids of *L. niger* had been identified previously (Lenoir et al., 2009). Contaminants were identified using the same GC device coupled to a Perkin-Elmer MS operating 70 EV; and verified with Chimatzu QP2010+ with a SLB-5MS (Supelco, 30 m, 0.25 mm, 0.25 µm) column. The GC and the two GC–MS used the same temperature program.

We calculated the quantities of substances (ng/ant) using their relative proportions compared to the eicosane internal standard. Tetracosane was used as standard for analysis of carbonaceous aerosols (Alves et al., 2007) or an external mixture of phthalates was used by (Teil et al., 2006). We used eicosane as the standard for hydrocarbon analysis and maintained it for our analyses.

We assessed phthalate amounts in the following conditions:

1) On ants collected in the field without any direct contact to plastics (neither container nor tools) and deposited directly in pentane vials.
2) On ants reared later in the laboratory in open plastic boxes free of phthalates.
3) On ants reared in glass vials kept closed to prevent air contamination (the vials were just open for a few seconds to add fresh food). Analyses in 2 and 3 were conducted 8, 15 days and 2 months after collection.
4) On various parts of dissected ants: fat body, ovaries, digestive track.
5) On the substrate in *Lasius* nests. For these tests, we used Solid Phase Micro Extraction (SPME) because liquid extracts were contaminated by many substances. A polydimethylsiloxane (PDMS) 7 µ fiber was rubbed on the substrate (or on the ant's cuticle) for 5 min. The fiber was desorbed in the GC under the same conditions as for liquid extracts (Lenoir et al., 2009). The SPME fiber was coated with 20 ng of eicosane as an internal standard using a micro-syringe.
6) As the air was a potential source of contamination we searched the presence of phthalates in the air of the laboratory. For this test, we simply left the same clean SPME fiber in contact with surrounding air and after 3 or 8 days it was desorbed in the GC. PDMS fibers are known to trap air phthalates (Stenerson, 2012).
7) We also searched if phthalates were present on multiple ant species, wood cricket and honeybees in various places to check if the pollution was a general phenomenon.
8) Finally we also checked the chromatograms for other contaminants to verify that the cuticle was really acting as a trap for lipophilic substances.

All data are presented as means and standard errors (SE).

3. Results

3.1. Phthalates in L. niger ants just collected in the field

Three phthalate esters were identified on the cuticle of *Lasius* ants: DEHP (Di(2-ethylhexyl) phthalate, CAS 117-81-7; =DOP), DBP (Dibutyl phthalate, CAS 84-74-2) and its isomer DiBP (Diisobutyl phthalate; CAS 84-69-5). We found a total for the 3 phthalates of 2.11 ng/ant (±0.48 SE, n=8; i.e. 1/1000 of the ant, or 1 mg/kg fresh weight). The most abundant phthalate was DiBP. The three phthalates represent 0.59% of the cuticular substances (±0.23, mini 0.15, maxi 2.02) (see details in Table S1).

3.2. Evolution of phthalates quantities in L. niger ants in open nests in the laboratory

After ants were transferred to laboratory open PVC nests, phthalate quantities increased. After 15 days the increase was significant (9.15 ng/ant) but after 2 months, this difference disappeared (Fig. 1 and Table S1 for details) (ANOVA $F_{4,12}=3.55$, $P<0.001$). The

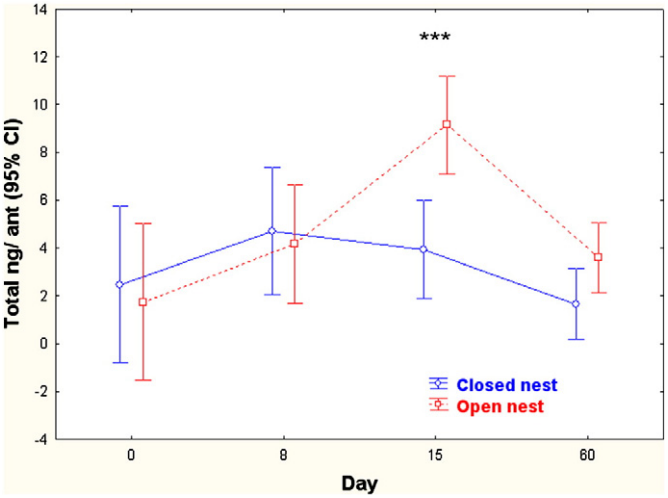

Fig. 1. Total phthalate quantities (ng/ant±95%IC) in ants reared in plastic boxes (plastic) or closed glass jars (glass). Asterisks indicate significant statistical difference.

phthalate quantities on the ant cuticle showed high variability in the laboratory. For example, in measurements taken in January 2011, for the same nests, workers had 28.8 ng/ant (±2.65, n = 10).

3.3. Evolution of phthalates quantities in L. niger ants in closed nests in the laboratory

For ants in closed nests, we did not observe any significant changes in the quantities over 2 months (Fig. 1) (ANOVA, F4,12 = 1.37, P = 0.19), and the quantities were comparable to the control levels of ants reared in contact with the air. It confirms that some contamination may originate from the air of the laboratory.

3.4. Phthalates in different parts of the ants

In January 2011, we measured the phthalate quantities in various parts of the body. Phthalates were more concentrated in the fat body (18.5 ng ± 8.2, n = 10) than in other parts — poison gland, digestive track and ovaries (4.2 ± 4.1, n = 7, $p<0.05$, Mann–Whitney *U* test).

3.5. Phthalates on the substrate

Using the SPME fiber, we collected the phthalates on the ant's cuticle and on the nest substrate. On the cuticle we found 0.19% (±0.05, n = 15) which is comparable to the data obtained by pentane washing. Inside the nest, phthalates represent 1.35% (±0.35, n = 15) of total substances on the walls and 5.05% (±1.05, n = 23) on the foraging arena. The differences were statistically significant (P = 0.003, *t* test). It indicates that phthalates were also present on the substrate in relative quantities that increase outside the nest.

3.6. Phthalates in the air

We collected the 3 phthalates from the ambient air in small but quantifiable quantities after 3 days (1.59 ng ± 0.77, n = 9), and in increasing quantities after 8 days (16.74 ng ± 4.01, n = 7), showing that they are present in the atmosphere. DBP and DiBP were captured in higher quantities (61.5% and 31.8% respectively) while DEHP was less abundant (6.61%). SPME only measure the quantity that was adsorbed by the fiber and not the particulate concentration in air.

3.7. Phthalates on various ant species and other insects

We found phthalates in all ant species at each collection site, including those collected in the field without any direct contact with plastic (in France, Hungary, Spain, Morocco Atlas, Andalusia, Greek island Egine, Burkina Faso — see Table S2). Occasionally we found the more volatile DEP (diethyl phthalate, CAS: 84-66-2). DEP was present in southern countries only: Egypt (0.09% ± 0.04, n = 12, i.e. 9.3% of the phthalates), in various places in Andalusia like Seville (*Aphaenogaster gibbosa* 0.65% ± 0.37, n = 4, 56.9% of phthalates) and Sierra Nevada mountains (*Cataglyphis rosenhaueri* 0.05% ± 0.05, n = 6, and 33.3% of phthalates), and as traces in mountains in Morocco (0.009% ± 0.004, n = 4, 3.5% of phthalates).

The phthalates generally totaled less than 1%, but could vary from 0.11 to 2.66%, even within a species collected at different times from the same place. For example, colonies of *Lasius alienus* (a species close to *L. niger*) showed variation from 0.24% in 1.9%. The myrmecophile beetle was contaminated at the same level as their host ants *Aphaenogaster senilis*. We did not find differences between the nesting habits of the ants (arboricolous, strictly terricolous). The level of human activity at a site may be a factor affecting the level of contamination. In mountains like the Alps, *Myrmica scabrinodis* was highly contaminated (1.98%) in a place frequented by tourists. The lowest quantities were found in Morocco (Dades Valley and National Park in Atlas) which may indicate that these places were less contaminated due to low human activity. However, the possible influence of human visitation on phthalate contamination is not yet confirmed.

The wood cricket *Nemobius sylvestris* showed a distinctive cuticular profile with a few heavy hydrocarbons on the C35 group (A. Lenoir, unpublished). We also found phthalates (188.10 ± 10.66 ng/cricket, n = 4; i.e. 2.76 ± 0.27% of cuticular substances), representing 9.41 ± 0.83 mg/kg fresh weight. This high concentration may be due to the particular composition of the cuticle of this insect which changes the properties of the epicuticle. DEHP was the most concentrated (1.94 ± 0.46%). Phthalates were also found on honeybee legs: 28.05 ± 10.39 ng/bee (0.73 ± 0.11%, n = 4).

3.8. Other contaminants

Squalene (CAS: 111-02-4) was regularly found on the ant cuticles. In our *L. niger* colonies it was equivalent to phthalates contamination (0.25 ± 0.11%, n = 29), but its presence was unpredictable as only 29.5% of the ants were contaminated at a quantifiable level. Squalene was also found in the nest material. Oleamide (9-octadecenamide, oleyl amide, CAS: 301-02-0) was also found in some samples.

4. Discussion

Our results confirm that the ant's cuticle traps lipophilic substances that are not produced by their metabolism. All ants and other insects in all examined localities studied were spontaneously contaminated by phthalates and ants can be considered as a good witness of phthalate pollution. It is possible, but not confirmed, that contamination is lower in places isolated from human impact like deserts and mountains in Morocco. Further research will test whether contamination also occurs in tropical forest very far from any human activity.

Some other contaminants were also found like squalene. This alkene is not produced by insects but is found regularly on their cuticle (Juarez and Blomquist, 1993; Chapman et al., 2000; Santomauro et al., 2004; Cvacka et al., 2006; Nash et al., 2008; Yusuf et al., 2010), or in the silk of spiders (Xiao et al., 2009). This product is frequently found in the human skin lipids (Wisthaler and Weschler, 2010). We suggest that it is a simple contamination with human manipulated insects or apparatus. Oleamide was found also in the spider silk (Xiao et al., 2009), but it comes from laboratory plastic ware (McDonald et al., 2008), and we verified that it was absent when we used oleamide-free plastic ware.

We showed here that the level of phthalate contamination on ants changes in the laboratory if the ants have contact with the laboratory air; this contamination may come from other plastics in the laboratory which diffuse in the atmosphere. For example we observed that the platform of a binocular microscope was coated with plastic rich in DEHP; the simple contact of ants on this substrate elevated the phthalate content of the ants (data not shown). It is known that many phthalates are present as aerosols in the atmosphere (see references in Introduction) and they are found in particle emissions from vehicles (El Haddad et al., 2009). They are very concentrated in indoor air in all places where they were measured, for example 100 to 1000 ng/m^3 for DBP (Rudel and Perovich, 2009). They are captured by storm waters (Björklund et al., 2009). They also change during the year, increasing in the winter (Teil et al., 2006; Alves et al., 2007). We therefore suggest that phthalate contamination comes from the air and that the lipophilic compounds are deposited spontaneously on the substrate and on the insect cuticle, which traps them.

We also showed that the contamination level does not change greatly in the laboratory. It is well-known that the cuticle rapidly adsorbs the surface components, so we hypothesize that phthalates were adsorbed and subsequently concentrated in the fat body. Are phthlates metabolized by ants? We cannot answer to this question as we would expect a decrease in contamination of ants kept in closed boxes. Some phthalates are already known to be quickly metabolized;

in humans 70% of DEHP is excreted within 24 h (Frederiksen et al., 2007). Phthalates are also degraded by microorganisms and no bio-concentration is known (Staples et al., 1997). Aerobic bacteria in mangrove sediments in Taiwan were found to degrade phthalic acid in a few days (Yuan et al., 2010). Ants are known to regulate their levels of other environmental toxins. For instance, *L. niger* ants in cadmium-polluted areas can limit the amount of cadmium in their bodies. As soil pollution increases, ant body Cd concentrations show an initial increase, but then plateau, remaining stable even at higher concentrations of soil Cd (Grzes, 2009). It suggests that the cuticle tolerates only a small level of phtalates (less than 1%) and only above this level they are adsorbed.

The long-term effects of phthalate contamination in ants are unknown. The accumulation of heavy metals is known to disturb the immune response of *Formica* ants (Sorvari et al., 2007) and also affects intra-specific aggressiveness (Sorvari and Eeva, 2010). The effects of phthalate contamination on model vertebrates and on freshwater animals are well-known (Oehlmann et al., 2009) but few data are available for terrestrial insects (Kristensen et al., 2004; Oehlmann et al., 2009). We would like to investigate the effects of phthalates on ant reproduction and immune responses. Further studies will be needed to detect phthalate diffusion and study the associated physiological impact on ants.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.10.003.

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Supplementary Table S1.. Evolution of phthalate quantities (ng/ant) in 2 different rearing conditions (open plastic boxes or closed glass jars).

	Control		Open 8 days		Open 15 days		Open 2 months		Closed 8 days		Closed 15 days		Closed 2 months	
	ng/ ant	SE	ng/ ant	SE	ng/ ant	SE	ng/ ant	SE	ng/ ant	SE	ng/ ant	SE	ng/ ant	SE
DBP	0.553	0.226	0.153	0.153	1.071	0.333	2.022	0.331	0.027	0.025	1.198	0.517	0.383	0.178
DIBP	0.901	0.156	3.060	0.667	5.711	1.703	0.912	0.318	4.154	1.700	2.069	0.834	0.793	0.464
DEHP	0.655	0.274	0.954	0.279	2.368	0.836	0.666	0.102	0.531	0.079	0.677	0.220	0.492	0.096
Total	2.108	0.484	4.167	0.932	9.150	1.830	3.601	0.624	4.712	1.717	3.943	0.880	1.667	0.530
% cut subst	0.589	0.226	0.300	0.056	1.052	0.208	0.909	0.396	1.058	0.364	0.632	0.184	0.430	0.092
n=	8		7		10		20		7		10		20	

Supplementary Table S2

Phthalates relative proportions on the cuticle of different ant and other insect species (mean ± SE)

In bold lower quantities found

Name	Locality, date	% mean	se	n=	
Cataglyphis savignyi	Edfu (Egypt) Feb 2010	0.47	0.11	6	with DEP
Cataglyphis savignyi	Assouan (Egypt) Feb 2010	1.44	0.41	6	with DEP
Cataglyphis bombycina	Imassine, Dades valley (Morocco) May 2009	**0.012**	0.011	3	Desert, only DBP - 1310m
Cataglyphis rosenhaueri	Sierra Nevada (Spain) May 2009	0.16	0.09	4	1270m, with DEP
Cataglyphis rosenhaueri	Bonares (near Sevilla - Spain) May 2010	0.11	0.03-0.18	2	
Cataglyphis emmae	Imini (Atlas - Morocco) may 2009	0.27	0.08	4	1430m, with DEP traces
Cataglyphis mauritanica	National Park Ifrane (Morocco) May 2010	**0.035**	0.016	4	Leg Serge Aron, 1780m
Cataglyphis aenescens	Kiskunsag national park (Hungary) Sept 2009	0.67	0.54	5	Leg Lazlo Galle
Cataglyphis sp.	Bobo Dioulasso (Burkina Faso) Dec 2006	0.22	0.06	3	
Cataglyphis viatica	Azenmour (Morocco) May 2005	2.66	1.41-3.90	2	
Lasius alienus	Azay sur Cher (FR) May 2006	1.9	0.41	21	
Lasius alienus	Azay sur Cher (FR) July 2007	0.24	0.04	31	
Lasius alienus	Samoëns (FR) August 2008	1.98	0.76	4	1700m
Camponotus fallax	Montlouis sur Loire (FR) March 2008	0.24		1	arboricolous
Myrmecina graminicola	Chinon forest (FR) July 2006	0.15	0.06	10	strictly terricolous
Aphaenogaster gibbosa	El Pedroso (near Sevilla, Spain) Oct 2007	1.14	0.47	4	with DEP
Aphaenogaster gibbosa	National Park Cazorla (Spain) May 2000	0.19	0.29	3	
Aphaenogaster simonellii	Egine island (Greece) March 2009	0.38	0.1	5	
Aphaenogaster subterranea	Lassalle (FR) Aug 2008	0.72	0.18	6	strictly terricolous
Aphaenogaster senilis	Donana national park (Spain) Nov 2009	0.22	0.16	5	
Sternocoelis hispanus (Histeridae)	Donana national park (Spain) Nov 2009	0.26	0.03	19	Myrmecophile of *A. senilis*
Pheidole pallidula	Bruniquel (FR) April 2010	1.68	0.28	38	Leg Denis Fournier
Nemobius sylvestris	Sauvagnon (near Pau, FR) Sept 2010	2.76	0.27	4	Forest
Apis mellifera (legs)	Morillon (Haute-Savoie, FR) July 2008	0.73	0.11	4	