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RESEARCH ARTICLE

Ant cuticular response to phthalate pollution

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Abstract Phthalates are common atmospheric contaminants used in the plastic industry. Ants have been shown to constitute good bioindicators of phthalate pollution. Hence, phthalates remain trapped on ant cuticles which are mostly coated with long-chain hydrocarbons. In this study, we artificially contaminated *Lasius niger* ants with four phthalates: dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), di(2-ethylhexyl) phthalate (DEHP), and benzyl butyl phthalate (BBP). The first three have previously been found on ants in nature in Touraine (France), while the fourth has not. The four phthalates disappeared rapidly (less than 5 days) from the cuticles of live ants. In contrast, on the cuticles of dead ants, DEHP quantities remained unchanged over time. These results indicate that phthalates are actively absorbed by the cuticles of live ants. Cuticular absorption of phthalates is nonspecific because eicosane, a nonnatural hydrocarbon on *L. niger* cuticle, was similarly absorbed. Ants are important ecological engineers and may serve as bioindicators of ecosystem health. We also suggest that ants and more generally terrestrial arthropods may contribute to the removal of phthalates from the local environment.

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Introduction

Phthalates are important environmental pollutants used in the industrial manufacturing of a wide array of products including cosmetics, shampoos, soaps, lubricants, pesticides, and paints. They are also commonly employed to increase the flexibility of plastic products such as polyvinyl chloride (PVC). Because phthalate esters are not chemically bound to the plastics into which they are mixed, they can easily be released into the environment and contaminate sediments, water, and food (Jen and Liu 2006; Olujimi et al. 2010; Bono-Blay et al. 2012). Moreover, as they are semivolatile, they evaporate into the atmosphere, adsorb to atmospheric particles forming aerosols (El Haddad et al. 2009; Bono-Blay et al. 2012; Cecinato et al. 2012; Wang et al. 2012). Although they are rapidly degraded, within a few days or weeks, either by photoxidation or by anaerobic or aerobic biodegradation, they are released in such quantities that they can locally reach relatively high concentrations in the environment (Staples et al. 1997; Liang et al. 2008). For example, concentrations of di(2-ethylhexyl) phthalate (DEHP) of 4 to 60 ng per cubic meter of air have been recorded in Paris (Teil et al. 2006). In other cities around the world, total phthalate concentration can reach more than 5,000 ng per cubic meter of air (Cecinato et al. 2012; Blanchard et al. 2013). As a consequence, animals, including humans, are widely and permanently exposed to these substances, which enter the body via ingestion, inhalation, and dermal absorption (Koch and Calafat 2009; Blanchard et al. 2013). Several phthalates have been classified as endocrine disruptors due to their estrogenic and antiandrogenic activity in animal models, for example, disturbing sexual development and individual fertility of vertebrates (Colborn 1998;

European Environment Agency 2012). Very few studies have analyzed the impact of phthalates on invertebrate species, and especially on terrestrial insects (see Jensen et al. 2001; Oehlmann et al. 2009). In a recent study, we showed that DEHP is reducing fecundity of *Lasius niger* ant queens and provokes a stress response of workers with ecological chronic doses (Cuvillier-Hot et al. 2014).

Ants are dominant animals in most terrestrial ecosystems worldwide, providing important services such as soil regeneration and oxygenation and plant dispersal. They are also used as bioindicators of environmental contamination in mines (Majer 2009). Moreover, they were recently proposed as potential tools in the monitoring of phthalate pollution (Lenoir et al. 2012). Hence, their cuticle, which is mostly composed of long-chain saturated and unsaturated hydrocarbons, traps phthalates from the atmosphere. Although they generally represent less than 1 % of all cuticular compounds, they are present in all studied species so far, including some from remote areas such as Moroccan deserts (Lenoir et al. 2012). Phthalates are also found inside the ant body and seem to be more concentrated in the fat body than in other tissues. This result suggests that phthalates might be absorbed by the ant cuticle or internalized through self-grooming and sequestrated in adipocytes in accordance with their highly lipophilic nature (Lenoir et al. 2012). The aim of the present study was to better understand the process of phthalate internalization in ants. To that end, we first deposited large amounts of four common phthalates on the cuticle of live ants and followed the dynamics of their disappearance during the following days. Then, we compared the dynamic of disappearance of DEHP deposited on the cuticles of live and dead ants. If DEHP degradation depends on ant metabolism, it should be much faster on live than on dead ants. In contrast, if phthalate disappearance just results from its natural degradation in the atmosphere, no difference should be observed between live and dead ants. DEHP was chosen as it is one of the phthalates most frequently found in the environment (Chen et al. 2012; Sioen et al. 2012; Blanchard et al. 2013) and it is commonly used to manufacture PVC (Gaudin et al. 2011).

Materials and methods

Insects

Colonies of *L. niger*, the European black garden ant, were collected in the spring of 2010, 2011, and 2012 in a personal orchard near Tours (A. Lenoir, Azay sur Cher, FR). It is not an endangered or protected species, and its collection does not require permission. All colonies were queenless and composed of 500 to 1,000 workers with brood. Colonies were kept in the laboratory (25 °C, natural daylight) in phthalate-free polystyrene boxes (27.5×28.5×9 cm; Mino Gaillard ®). The absence of phthalates in the rearing boxes was verified by gas chromatography–mass spectrometry (GC-MS); polystyrene fragments that had been soaked in hexane, methanol, or dichloromethane for several days were analyzed. Within the boxes, the ants could shelter in glass tubes, which were half filled with water retained by a cotton plug. The ants were fed mealworm larvae, oranges, and a commercial bumblebee solution (Beehappy ®, Koppert Biological Systems) twice a week.

Experiments

Phthalates We used three phthalate esters that had previously been found on the cuticle of *L. niger* in Touraine: DEHP (CAS 117-81-7, 99 % purity), dibutyl phthalate (DBP, CAS 84-74-2, 99 % purity), and DBP's isomer diisobutyl phthalate (DiBP, CAS 84-69-5, 99 % purity) (Lenoir et al. 2012). We also used benzyl butyl phthalate (BBP, CAS 85-68-7, 98 % purity), which has never been found on the cuticle of this ant species in Touraine. All the phthalates were purchased from Sigma-Aldrich. In a first experiment, we used pure phthalates dissolved in pure methanol (2,000 ng/μl). Pure methanol was used on the control ants. The ants were immobilized using soft forceps, and 1 μl of a given phthalate solution or methanol was deposited on the gaster. We used this large quantity of phthalates (2,000 ng) to mimic pollution as we showed that the ants were rapidly contaminated with phtalates after simple contact with plastic toys (A. Lenoir, unpublished). This treatment was not lethal on the short term since the mortality rate was less than 1 % after several days in all the groups, including the control. The phthalate-treated and control ants were placed individually in Petri dishes containing wet cotton (to maintain humidity) and were regularly fed. They were kept in the dish for up to 5 days, depending on the compound with which they were treated. To prevent pseudoreplication, we used in each experiment three different colonies both for controls and treatments.

We also looked at the disappearance of DEHP, the most frequent phthalate, from the cuticle of dead ants. After the ants were first freezer-killed (−18 °C for two hours), their bodies were left at room temperature for half an hour, and 2 μl of DEHP (200 ng/μl) was deposited on the abdomens. We used a smaller concentration of DEHP (20-mg/kg fresh weight) to simulate levels in nature (1 mg/kg; see Lenoir et al. 2012). As in the case of the live ants, the dead ants were placed in a Petri dish for 1 to 5 days before chemical analyses took place.

Eicosane In order to determine if the insect cuticle has a generalized ability to absorb lipids, we examined the disappearance of eicosane (n-C20) from the cuticle of live ants. This hydrocarbon is not naturally present on the cuticle of *L. niger* (Lenoir et al. 2009); we verified this finding before proceeding. We placed 200 ng of eicosane dissolved in 1 μl of

pentane on the abdomen of live and dead ants. Such small amount of pentane is well tolerated, and survival was not affected by the solvent. Control ants received pentane only.

Chemical analysis

Live individual ants were freezer-killed (10 min at −18 °C). Freezer-killed and dead ants were placed in 100 μl of pentane for 1 h to avoid extraction of internal lipids; samples were removed; 2 μl of pentane containing 400 ng of eicosane (C20) was added as an internal standard. The solvent was then evaporated until 10 μl remained. Two microliters was subsequently injected into a gas chromatograph (VGM250Q system, Perkin-Elmer) equipped with a flame ionization detector; a BP-1 fused silica capillary column (25 m, 0.32 mm, 0.5 μm) was used. Injection was splitless for 2 min, and the temperature was kept at 150 °C for the first 2 min following injection of the sample. It was then raised from 150 to 300 °C at 5 °C/min and held at 300 °C for the last 10 min. Phthalate identities and retention times were verified using GC-MS (Perkin-Elmer) conducted under the above-mentioned chromatographic conditions. The problem using a universal detector, such as FID, is that peaks frequently contain the analyte (phthalate) and also other substances that are the interferences. DEHP particularly comes in the chromatograms with 5MeC25 in many ant species. But, it is not the case of *L. niger* which does not have 5MeC25; so, FID can be used without interference.

An external mixture of phthalates is generally used to quantify PAEs (Teil et al. 2006). Eicosane is frequently used as the standard in hydrocarbon analyses, and we utilized it in our present analyses to remain consistent with past work (Lenoir et al. 2012). We calculated the quantity of each compound relative to the eicosane internal standard. In the eicosane experiments, it was not possible to use eicosane as internal standard; so, we calculated the quantities remaining relative to direct injections of eicosane used as external standard. All the data were presented in nanogram per milligram of dry weight (DW) which is the most precise measure as the fresh weight can change considerably according to the state of the individual (see for example, Marlier et al. 2004). The limit of quantification (LOQ) in our FID conditions was 0.35 ng for each phthalate.

Preliminary analyses were done to verify that all used solvents (pentane and methanol) were not contaminated by phthalates.

Statistics

All data are presented as means±standard errors (SEs). All comparisons were conducted using analysis of variance (ANOVA) and Newmann-Keuls post hoc tests with Statistica6 ®.

Results

DEHP

The mean quantity of DEHP present on the cuticles of treated ants decreased progressively during the following days while that of the control (solvent only) remained equally low (Fig. 1a; ANOVA $F_{3,\ 41}=12.64$, $P<10^{-4}$). Hence, the difference between control and treated ants was significant at day 0 ($P<10^{-4}$), day 1 ($P<10^{-4}$), day 3 ($P=0.0007$) but not day 5 ($P=0.40$).

DBP

DBP quantity decreased rapidly over time (ANOVA $F_{2,27}=40.0$, $P=0.001$); after 48 h, treated ants did not differ from control ants (Fig. 1b). The differences between control and treated ants were for day 0, $P=0.000001$; day 1, $P=0.00005$; and day 2, $P=0.59$ NS.

DiBP

The change in DiBP was comparable to that for DBP: Quantities decreased rapidly and reached control levels in 2 days (ANOVA $F_{2,25}=274.4$, $P<10^{-5}$) (Fig. 1c). The differences between control and treated ants were significant at day 0 ($P<10^{-5}$) and day 1 ($P<10^{-5}$), but not at day 2 ($P=0.86$).

BBP

We verified that BBP was never present on live ants, and therefore, all control values were 0. BBP disappeared very quickly (ANOVA $F_{2,21}=59.8$, $P=0.00001$). After 24 h, some traces remained, but the mean quantity did not differ from that of control ants (7.8 ng/ant±3.2 SE vs 0, $P=0.35$). After 2 days, it had disappeared completely (Fig. 1d).

DEHP on dead ants

It appears that the quantity of DEHP remaining was very stable during the 5 days; it did not decrease significantly over time (ANOVA $F_{1,4}=1.67$, $P=0.19$), and it was always significantly greater for treated ants than control ants (Fig. 2).

Eicosane

Eicosane quantities decreased over time and differed between live and dead ants (Fig. 3; ANOVA $F_{\text{time}}=12.38$; $P=0.00001$; $F_{\text{treatment}}=11.44$, $P=0.001$ and interaction $F=84.3$; $P=0.00000$). Hence, while eicosane evaporated very slowly (after a few days) from dead

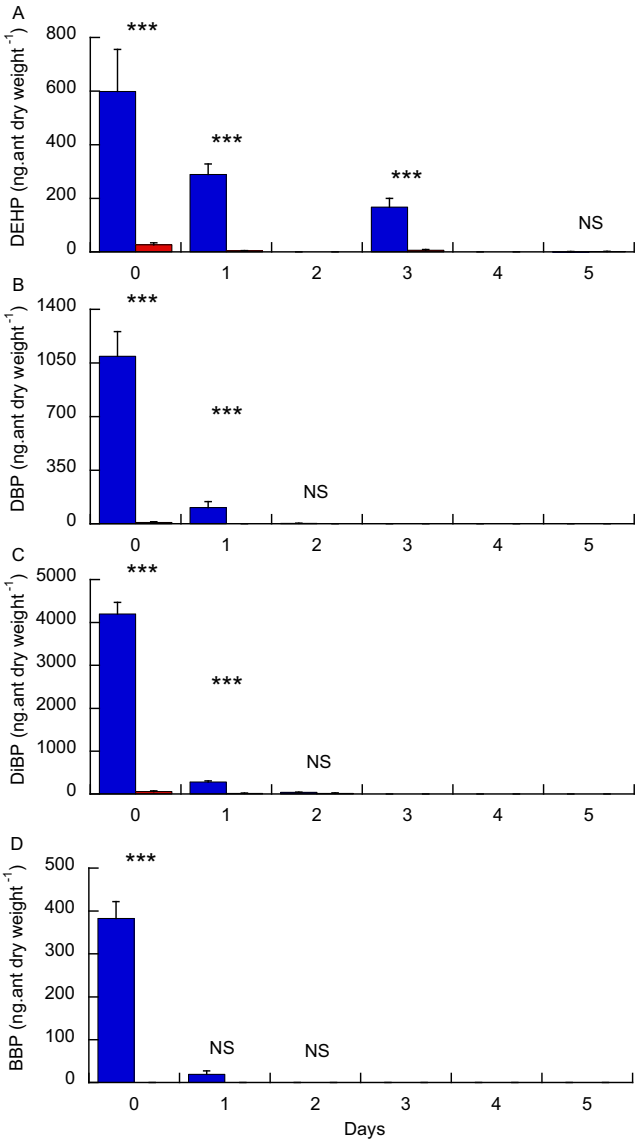

Fig. 1 Quantities of phthalates remaining on ants over time after the deposition of 2,000 ng compared to controls (ng/mg DW) (control in *red*, treated in *blue*). Phthalates are the following: DEHP (**a**), DBP (**b**), DiBP (**c**), and BBP (**d**). *Stars* or *letters* denote significant differences between treated ants (that received phthalates) and controls (that received the methanol). **$P<0.01$, ***$P<0.001$; *NS* nonsignificant. For DEHP treatment $n=45$; control $n=39$; for DBP treatment $n=30$; control $n=30$; for DiBP treatment $n=28$; control $n=27$ and for BBP treatment $n=30$; control $n=30$ (total numbers of ants)

ants, it almost totally disappeared in 24 h from the cuticles of live ants. The quantities of eicosane retrieved on the cuticles of dead and live ants was not significantly different on day 0 ($P=0.66$). In live ants, the mean quantity dropped down after 24 h (between day 0 and day 1, $P<0.00001$). Between day 1 and day 2, the difference was not significant ($P=0.85$). In contrast, the quantity of eicosane recovered on corpse cuticles did not vary significantly between day 0 and day 2.

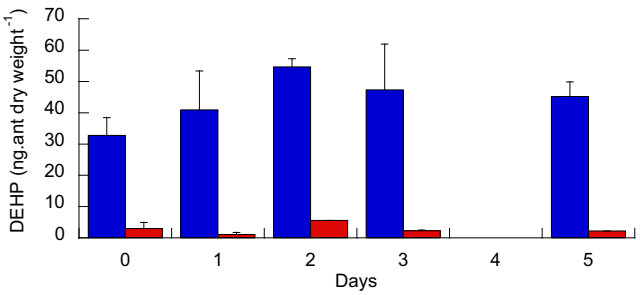

Fig. 2 Quantity of DEHP remaining on ant corpses (*blue*, ng/mg DW) after 1, 2, 3, and 5 days compared to controls (*red*) (treatment $n=25$; control $n=23$; total number of ants). No significant differences between treated ants and control ants according time; for each day, all the differences between treated and control are significant

Discussion

All the phthalates, including BBP, which has not been found on Touraine ants, remained trapped on the cuticles of live ants in quantifiable quantities. This result confirms that ants can be considered as good bioindicators of the presence of phthalate pollution. All the phthalates that were deposited on the cuticle of live ants in large quantities declined rapidly to control levels. The speed at which the compounds disappeared was generally related to molecular weight and alkyl chain length: DBP and DiBP (mw 278.4; 4C alkyl chains) were absorbed in 2 days, while the process took 5 days for DEHP (mw 390.6; 8C alkyl chain). BBP (mw 312.4; 4C and 6C alkyl chains) disappeared more rapidly, in only 1 day; this result may explain why it is generally not found on ant cuticles. These differences are also probably due to the physic chemical properties and mainly related to their hydrophobicity, volatility.

In contrast, when DEHP was deposited on the cuticles of dead ants, the remaining quantity was stable for 5 days.

Other possibilities can be invoked to explain the disappearance of the phthalates. First, as they are semivolatile, they can evaporate, become aerosolized, and subsequently be degraded

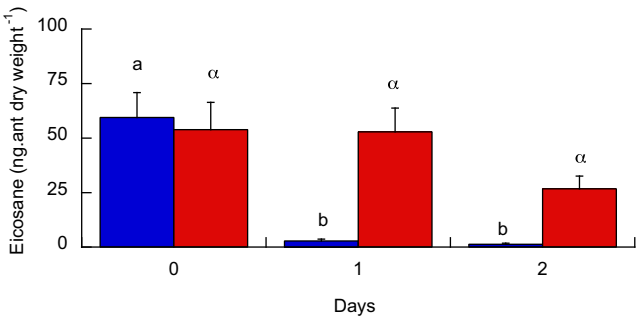

Fig. 3 Quantity of eisocane on live (*blue*) and dead (*red*) ants (ng/mg DW) after 1 and 2 days (live ants $n=59$; dead ants $n=58$; total number of ants). Differences between live ants *a* and *b*; between dead ants with α

by atmospheric photoxidation (half-life of 0.2–2 days for DEHP; 0.5 to 6 days for the others) (Staples et al. 1997). However, if evaporation were operating, we would expect the pattern to be the same for live and dead ants maintained under the same conditions. Second, it is known that phthalates are metabolized by microorganisms (Keyser et al. 1976; Staples et al. 1997). For example, aerobic bacteria in mangrove sediments in Taiwan were found to degrade phthalic acid in a few days (Yuan et al. 2010). This process may have operated in our study, but it seems to have been unimportant as it should have also affected compound disappearance from dead ants, unless cuticular bacteria died when the ants were freezer-killed which is unlikely for 2 h at −18 °C. Since DEHP on dead ants does not disappear, we suggest that phthalates are actively degraded inside the body by enzymes as it has been shown in earthworms (Albro et al. 1993). We cannot exclude a possibility of self-grooming as ants groom themselves regularly, especially when they are contaminated, and we showed that ants perceive the presence of phthalates (Cuvillier-Hot et al. 2014) which is confirmed by the antennal lobe response in *Drosophila* (Ran et al. 2012). But probably, the most fact is that phthalates are actively absorbed by the live cuticle. Insect cuticles have been shown to both excrete and absorb lipids, such as hydrocarbons (Meskali et al. 1995; Vauchot et al. 1998; Bagnères and Blomquist 2010) and enzymes (Semenova et al. 2011). Our results confirm that eicosane is also absorbed by the cuticle of live ants. In our previous study, we found phthalates mainly in ant fat bodies (Lenoir et al. 2012). This prior result indicates that all lipidic substances, including lipophilic phthalates, are probably metabolized in the ant's fat body, and it would be interesting to know how they are degraded. It would also be interesting to compare the absorption of phthalates in ants and in nonsocial insects. Absorption may simply be a generalized cuticular detoxification mechanism in insects or a response specific to social insects that serves to actively metabolize hydrocarbons used in nestmate recognition (a generalized mechanism in social insects (see Lenoir et al. 1999).

Phthalates generally make up less than 1 % of ant cuticular lipids (Lenoir et al. 2012). The results of this study show that they are internally absorbed without cuticular bioaccumulation. Bioaccumulation has been observed for some pollutants. For instance, polybrominated diphenyl esters (PBDE) accumulate in high concentrations on domestic cricket (*Acheta domestica*) cuticles and are 20 times higher in their lipids. They become further concentrated in frogs that eat these crickets (Hale et al. 2002; Gaylor et al. 2012). It is well known that some metals also become concentrated in ants (Migula et al. 1993; Gramigni et al. 2013). In contrast, no bioaccumulation of phthalates is observed in freshwater animals (Heudorf et al. 2007; Teil et al. 2012). In terrestrial food chains, bioaccumulation is supposed to be limited by biotransformation (Staples et al. 1997), although there is some indication that phthalates bioaccumulate in the annelid species *Eisenia foetida* (Hu et al. 2005). Our results confirm that terrestrial arthropods like ants capture, absorb, and degrade phthalates, which are released into the environment in great quantities on a regular basis. It is known that ants are ecologically important; they are considered to be ecosystem engineers and good bioindicators of ecosystem health (e.g., Abril and Gómez 2013). As a result, we suggest that ants in particular and terrestrial arthropods in general may contribute to the removal of phthalates from the local environment. If phthalates are being metabolized, it would also suggest that they are biologically active compounds as it has been shown on *L. niger* (Cuvillier-Hot et al. 2014). Future studies should therefore measure the effects of phthalates on reproduction, growth, immunity, and the survival of insects in general.

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