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Impact of ecological doses of the most widespread phthalate on a terrestrial species, the ant *Lasius niger*

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ABSTRACT

Phthalates are synthetic contaminants released into the environment notably by plastic waste. Semi-volatile, they adsorb to atmospheric particles and get distributed in all ecosystems. Effects of this major anthropogenic pollution in economical species in aquatic habitats have attracted large interest. On the contrary, very few studies have focused on wild terrestrial species. Yet, these lipophilic molecules are easily trapped by insect cuticle; ants and other insects have been shown to permanently bear among their cuticular components a non-negligible proportion of phthalates, meaning that they suffer from chronic exposure to these pollutants. Oral route could also be an additional way of contamination, as phthalates tend to stick to any organic particle. We show here via a food choice experiment that *Lasius niger* workers can detect, and avoid feeding on, food contaminated with DEHP (DiEthyl Hexyl Phthalate), the most widespread phthalate found in nature. This suggests that the main source of contamination for ants is atmosphere and that doses measured on the cuticle correspond to the chronic exposure levels for these animals. Such an ecologically relevant dose of DEHP was used to contaminate ants in lab and to investigate their physiological impact. Over a chronic exposure (1 dose per week for 5 weeks), the egg-laying rate of queens was significantly reduced lending credence to endocrine disruptive properties of such a pollutant, as also described for aquatic invertebrates. On the contrary, short term exposure (24 h) to a single dose of DEHP does not induce oxidative stress in ant workers as expected, but leads to activation of the immune system. Because of their very large distribution, their presence in virtually all terrestrial ecosystems and their representation at all trophic levels, ants could be useful indicators of contamination by phthalates, especially via monitoring the level of activation of their immune state.
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1. Introduction

As the footprint of human activities on ecosystems becomes more obvious, understanding its impact on ecology and physiology of organisms is turning increasingly imperative. Among most widespread contaminants, plastic derivatives have attracted considerable attention over past decades. Phthalates belong to a growing family of plasticizers used to soften and increase flexibility of many manufactured products (food wrapping, cosmetics, inks, paints, medical devices, construction materials, etc.) that generate a large part of our waste. Since phthalates are not covalently bound to plastic grids, they can leach out of materials and contaminate the external environment. Despite poor volatility and solubility of phthalates, massive and ubiquitous natures of plastic pollution generate permanent release of these substances so that all ecosystems seem contaminated to various degrees by several phthalates permanently present in mixtures (Cole et al., 2011; Fromme et al., 2002; Teuten et al., 2009). DEHP (di-ethylhexyl phthalate) is the phthalate most commonly used by the plastic industry and represents most of the leakage into the wild (Bauer and Herrmann, 1997; Fromme et al., 2002). It is considered as highly toxic for the environment, so with four other phthalates, this molecule has been classified as a priority substance by the European Commission and is subjected to an environmental risk assessment (Oehlmann et al., 2008). Their suspected action as endocrine disruptors has promoted many in-depth studies of effects of phthalates on humans and model mammals. In parallel, the awareness of the massive contamination of aquatic and especially marine habitats by micro-particles of plastic (Law et al., 2010) has brought about toxicological studies on aquatic species, especially on those of agronomical interest. These studies have mainly

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pinpointed negative effects on reproduction and creation of an oxidative stress at the tissue level (e.g. Kovacic, 2010; Mankidy et al., 2013). On the contrary, very few studies have analyzed the impact of phthalates on wild terrestrial species and especially on insects. The phthalate exposure of the terrestrial insects is however not negligible. It proceeds mainly through atmospheric particles onto which phthalates are adsorbed (Teil et al., 2006). We have previously demonstrated that the lipidic nature of the insect cuticle makes it a good trap for these lipophilic compounds. In this way, DEHP has been commonly detected on the cuticle of wild ants, so that ants can be considered as chronically exposed to this chemical component (Lenoir et al., 2012). Ants thus appear well suited to serve as bioindicators for this kind of pollutant. Indeed, they occupy all terrestrial ecosystems, present a great variety of diets, have an ecological role of prime importance and are generally considered as ecosystem engineers and good indicators of ecosystem health (Abril and Gomez, 2013).

In this paper, we examine the effect of DEHP on several key aspects of the ecophysiology of the European black garden ant, *Lasius niger*. Ants were exposed to environmentally relevant doses of DEHP, that is to say the equivalent of the mean amount of phthalates detected on the cuticle of *L. niger* captured in the field (Lenoir et al., 2012). We then investigated disorders caused by DEHP using an integrative approach (behavioral until gene expression). First, phthalates have been proved repellent to insects in general and also to the red imported fire ants (Chen, 2005). We evaluated the *Lasius* workers' ability to avoid DEHP via a food-choice experiment with contaminated food. If workers are able to differentiate the pollutant and reject contaminated food, we will consider that the phthalate contamination found on their cuticle and due to transfer from the atmosphere constitute the main source of contamination for the ants. It will validate the use of this measured quantity as a dose of reference to evaluate the impact of environmental phthalate contamination on ants' physiology. As alteration of reproduction and oxidative stress are the most common effects of phthalates reported on aquatic invertebrates, we looked for alteration of these functions in ants. To address these points, the fertility of *Lasius* queens chronically exposed to DEHP for 5 weeks was assessed and compared to control queens; meanwhile the level of oxidative stress of *Lasius* workers exposed to DEHP was estimated by the level of lipid peroxidation measured 24 h and 48 h after exposure. Finally, we investigated the impact of DEHP exposure on ants' immune activity. Due to their great reactivity as a part of an alert system, immune-related genes and proteins are increasingly considered as good biomarkers for early monitoring of environmental perturbations (Lu et al., 2013). Nevertheless, only few studies have addressed immune effects of phthalates' exposure. We measured the level of expression of several marker genes involved in immune functions 24 h, 3 days and 7 days after DEHP exposure. If the synthetic molecule affects the physical integrity of the ant in any way, this will translate in a rapid activation of the immune system. For comparative purpose, a similar experiment was conducted with BBP (butyl benzyl phthalate), which is not found on *Lasius*' cuticle. Thus, its application corresponds more to an acute contamination rather than to a chronic exposure. If the toxicity per se of both molecules is similar (as suggested in rodents; Foster, 2005), we expect a higher/longer effect of DEHP compared to BBP because of a persistent effect of the former.

2. Material and methods

2.1. Insects and chemicals

Six colonies of *L. niger* were collected in an orchard near Tours in June 2011 and June 2012 (Azay sur Cher, FR; 47°19′38″N/0°48′35″E). Colonies were kept in laboratory (temperature 25 °C, natural daylight) in plastic boxes checked for the absence of DEHP and BBP (Lenoir et al., 2012). 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).

DEHP (CAS 117-81-7) and BBP (CAS 85-68-7) were purchased as pure chemicals from Sigma-Aldrich and diluted in methanol. Phthalates were applied at a dose of 2 ng onto the gaster of individual ants. This quantity reflects the global quantity of phthalates extracted from the cuticle of individual ants collected in the field, as we previously measured it (Lenoir et al., 2012). Thus our treatments approach the situation encountered by ants in nature.

2.2. Food choice experiment

A food choice was proposed to 30 workers of *L. niger* from 3 independent colonies (10 workers per colony). Ants were chosen among foragers and individually tested in a glass Petri dish that was previously cleaned with water, methanol and acetone, and then autoclaved. Pieces of food were prepared by mixing apples and honey with hot agar. The mixture was cooled at 4 °C and then diced in identical cubes of 200 mg that were frozen until used. Half an hour before each experiment, 2 cubes of food were defrosted and the excess of liquid was absorbed on a Whatman paper. One of the cube received on its top side 2 ng of DEHP diluted in 2 μl of methanol; the other cube received 2 μl of pure methanol (control). Five minutes later, the two cubes were placed in a clean Petri dish covered with Whatman paper; each cube was arranged to lie at an equal distance of the point of introduction of the tested ant and of the other cube. Once introduced in the study arena, each ant was observed for 30 min and the incidence of the following behaviors was recorded: antennation (number of occurrences when the ant came closer and contacted the cube of food with its antennae); stand on (each time the ant climbed onto the cube); eat (each time the ant opened its mandibles and crouched to eat food). We made the distinction between scenarios when the ants ate on the top (where the substances were deposited) and on the side of the cube.

2.3. Queen egg-laying rate

Mated queens were collected during their swarming flight in July 2011. They were kept individually in similar lab devices as described in Section 2.1, until colonies reached 20–30 workers. Experimentations were carried out during spring 2012. First, 30 queens were individually followed for their egg-laying activity over a period of 5 weeks. All eggs were retrieved from nests one week before the beginning of the experiment and then on checked and counted each week from T0 and for 5 weeks. In a second period, the queens were arbitrarily allocated to one of the three groups of treatment (10 queens per group). The first group received each week 1 μl of DEHP (2 ng/μl diluted in methanol) onto its gaster, the second group received 1 μl of pure methanol and the third group received nothing (control) but queens were submitted to the stress of handling as in the other two groups. At the end of the 5th week, eggs were retrieved from the nest and counted; the queens received their specific treatment and were put back into their nest. From then for 5 weeks, eggs were counted and queens received their treatment once a week. Results are given as the mean cumulated number of eggs recorded over time in nests of queens that received the same treatment.

2.4. Measure of lipid peroxidation in ants contaminated with DEHP (TBARS assay)

900 workers per colony were removed from 3 independent colonies and split into 3 groups of 300 workers each. The first group of 300 workers served as control; workers were just handled but received no treatment. In the second group, workers received 1 μl of methanol spread onto the gaster. In the third group, workers had their gaster applied with 2 ng of DEHP diluted in 1 μl of methanol. After treatments, each group of 300 workers was kept in the laboratory (temperature 25 °C, natural daylight) in glass tubes half filled with water. After 24 h, 10 batches of 15 ants were retrieved from each group, killed in liquid nitrogen and kept at −80 °C until used. 150 workers left were kept until 48 h and then split into 10 batches of 15 ants that were processed as above. Each condition was thus tested on 30 samples, each sample corresponding to 15 pooled ants and coming from 3 different colonies (10 replicates per colony).

Thiobarbituric acid reactive substances are an index of lipid peroxidation and oxidative stress. Pools of 15 frozen ants were homogenized with Kontes® Microtube Pellet Pestle® in a buffer containing 100 mM KH_2PO_4, 0.05% BSA, 10 mM EDTA, 0.13 mM butylated hydroxytoluene and 0.13 mM deferoxamine (pH 7.4). Ant lipid peroxides content was assessed spectrophotometrically at 540 nm, measuring the malondialdehyde (MDA) as an index of thiobarbituric acid-reactive products (TBARs) following the method described by Ohkawa et al. (1979). Results are expressed in nmol MDA/mg protein of ants. Protein concentrations in pools of ants were measured according to Bradford method.

2.5. Immune gene expression

From a pool of 240 workers from the same colony, 80 workers received 1 μl of DEHP (2 ng/μl diluted in methanol) applied onto their gaster; 80 received 1 μl of

BBP (2 ng/μl diluted in methanol); 80 received 1 μl of methanol as control. After application, ants that had received the same treatment were gathered in glass tubes half filled with water. 15–20 workers were removed from each batch at the times 24 h, 3 days and 7 days post-treatment. They were rapidly cooled for anesthesia, pooled by type and time of treatment in a cap tube and then put in liquid nitrogen. The experiment was carried out three times on 3 independent colonies. In one of the colonies an additional batch of 20 workers was retrieved 24 h after treatment; each worker was cooled on ice, dissected under binoculars and fat body with ovaries taken off and pooled in a cap tube containing 500 μl of Qiazol ("fat body" samples – Qiagen). All samples were kept at −80 °C until used.

For RNA extraction, entire bodies ("whole body" samples) were crushed in liquid nitrogen using mortar and pestle. The powder obtained was put in 1 ml of Qiazol (Qiagen) and processed according to the manufacturer's instructions, as were the "fat body" samples. After RNA extraction, genomic DNA was removed by DNase I treatment (RQ1 RNase-free DNase, Promega). For each condition, cDNA was produced from 1.2 μg of total RNA using the Superscript III enzyme (Invitrogen) according to the manufacturer's instruction, and lastly subjected to RNase H treatment (Promega). RT-experiments were carried out on each RNA sample to check for the absence of genomic DNA. PCR reactions were performed under all conditions with similar protocols, adapted for each gene considered. They had basically the same steps: 2 min at 95 °C, *n* cycles of 30 s at 95 °C/30 s at T_m/30 s at 72 °C, and 5 min at 72 °C. Table 1 shows for each target gene, sequences of the primers used, the T_m and the number of cycles performed in the PCR reactions. For technical reasons, the number of cycles sometimes varies between the different time-points studied so that, for each gene, comparisons are made only within and not between time-points.

Primers were designed against published sequences for the *defensin* gene (GenBank accession no. **EU401743.1**), *vitellogenin* (**DY543805.1**), *histone-2A* (**DY543813.1**), *superoxide dismutase* 1 (SOD1: **AY309973.1**) and 16*S* gene (**GQ503244.1**). Primers that target peptidoglycan-recognition protein (PGRP) were designed from the alignment of 3 PGRP sequences characterized in close ant species (*Myrmica rubra*: **ACT66864.1**; *Harpegnathos saltator* **EFN81745.1**; *Camponotus floridanus*: **EFN73970.1**). The primers selected enabled us to amplify a band of the expected size (150 bp), which when sequenced best matched with an ant PGRP (**ACT66863.1**; query coverage: 98%, *E* value: 5e−31). This sequence was thus further considered as a partial sequence of a *L. niger* PGRP and was deposited in Genbank under accession no. **KF384640**.

Equal volumes of PCR reactions (typically 20 μl) were loaded on 1.5% agarose gels stained with BET. Results were visualized on a UV-transilluminator and acquired as images with the BioCapt software (Vilber Lourmat).

2.6. Statistical analyses

For the food-choice experiment, behavioral data were compared using permutation tests under StatXact 9 (Cytel Studio). For comparison of queen-egg-laying rates, the statistical approach was as follows. In order to test if the treatment had a significant effect on the number of queen-laid eggs, we performed a Generalized Linear Model with a Poisson distribution for the response variable, i.e. the number of eggs, in a given week, with the treatment as the dependent variable (Crawley, 2007). The questions were 1) whether the treatment had a significant effect on the number of eggs present in the nest in a given week and if so, 2) which was the treatment with lesser number of eggs. To test for the first question, we performed a likelihood ratio test between two alternative GLMs: with or without the treatment as a dependent variable. If this test was significant, then we conducted pairwise comparisons among the three treatments (control, methanol and DEHP). For this, we looked at the estimated coefficients by the GLM model for each pair and if it was significantly higher than zero we concluded that one treatment shows a higher number of eggs than others. We only report this in results when two treatments show a significantly different number of eggs in a given week. The statistical tests were conducted using the procedure GLM in the R software (R Development Core Team, 2011). Finally, different measures of lipid peroxidation at 24 h and 48 h were compared with ANOVA with general scores tests. For all statistical tests and unless specified, the level of significance was set at 0.05.

3. Results

3.1. Worker ants detected and avoided feeding on food contaminated with DEHP

Behavioral responses in the food choice experiment clearly indicated that ants were able to discriminate between food contaminated with DEHP and food spiked with solvent only (Fig. 1). Workers inspected equally both kinds of food, as suggested by equal amounts of antennation. They even climbed preferably on the phthalate-contaminated cubes ("Stand on"), but they clearly refrained from eating food contaminated with DEHP as compared with methanol only ("Eat (total)"). Interestingly, we observed that ants preferred to feed on the side of the block rather than on top of it, whatever was poured on it ("Eat (side)" vs. "Eat (top)"), showing that the solvent alone was not neutral to them.

3.2. DEHP treatment reduced the egg-laying rate of queens

The egg-laying rates of three groups of *Lasius* queens were followed, first, in the absence of any treatment (referred to as "pre-treatment phase"). After five weeks, the "treatment phase" began

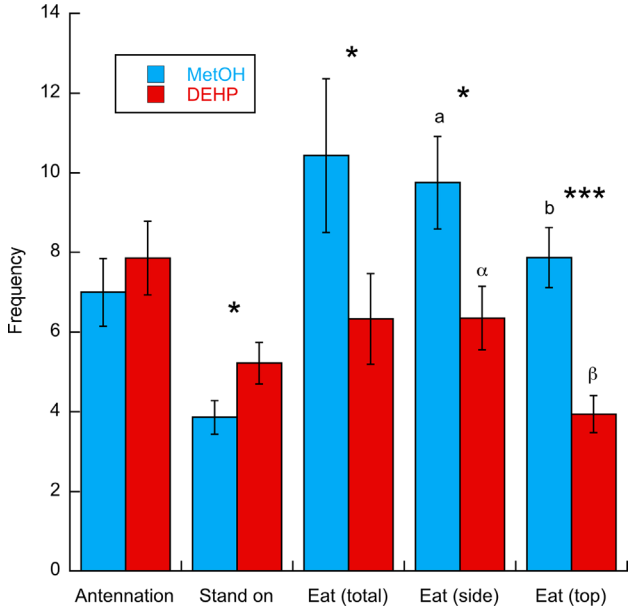

Fig. 1. *Behavioral response of Lasius niger workers facing contaminated food.* Individual workers were proposed a choice test between food spiked with methanol and food contaminated with DEHP diluted in methanol. The behavior of each worker ($n=30$) was observed for 30 min and the number of antennations, the number of times the individual climbed on the cube ('stand on'), the number of times it eats the food, anywhere on the cube ('total'), on one side of the cube ('side'), or on the top of the cube ('top') were counted. Results are given as mean numbers. Asterisks indicate significant differences for comparisons across treatment (*$p<0.05$, ***$p<0.0001$); letters refer to comparisons across behaviors (a vs. b: $p<0.001$; α vs. β: $p<0.0001$).

Table 1

Gene	F primer	R primer	T_m (°C)	Number of cycles (*n*)		
				24 h	3 days	7 days
PGRPaia	GAAAGGAATACAGAACTATCATATG	CCAATGCTCTTGTTGTTGAA	55.5	32	39	39
Defensin	CGCAGAAGGAAGACGAATCT	TTCCTCTGAGAAGGCAGTGA	58.5	36	38	38
Vitellogenin	CTTAAACGAGGCTCCGAACA	GCATCTAAGCCCTGGAGAAA	59.5	33	36	36
Histone-2 A	GAATACCTCGCCGCTGAA	GCCATTTGTGATACTTTGTGGA	59.5	32	35	35
Superoxide dismutase 1	TGGTTCAAGTGCAGTGAAGG	ACCAGCTTCCACGTTTCCTA	59.5	27	30	30
16S	TTTGAAATTAAATAGCCGCAGT	AGGGTCTTCTCGTCCCATAA	58	40	40	40

and each batch received DEHP, solvent (methanol; one queen died during the experiment, so that only 9 queens were used for statistical analyses) or nothing (control; enduring just the manipulation).

Fig. 2 shows that during the pre-treatment phase, number of eggs counted in "DEHP nests" were not significantly different from that in "control" and "methanol" nests, suggesting that the three batches of queens collectively displayed the same egg-laying rate over the 5-week period. On the contrary, two weeks after the beginning of the treatment phase, and then in each week until the end of the experiment, the number of eggs counted in the nests was statistically smaller for DEHP treated-queens compared to control and methanol-treated queens (Fig. 2). The difference in the number of eggs produced even increased with time and with chronicle exposure to the phthalate.

3.3. The classic effect of DEHP on oxidative stress was not observed in ants

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxides form consequently to cellular injury and are often used as indicators of oxidative stress in cells and tissues.

Ants that received DEHP showed no elevation of MDA level in their tissues 24 h or 48 h after application, compared to control ants (Fig. 3). Contrarily to what has been described for several aquatic invertebrates, ants apparently suffered faint oxidative stress after DEHP treatment, at least in the short term ($p=0.06$ and 0.37 respectively). Note that the nearly significative difference at 24 h is mainly explained by the effect of methanol (Student's t-test: control vs. methanol, $p=0.07$; methanol vs. DEHP, $p=0.70$). In line with this unexpected result, the level of expression of SOD1 (superoxide dismutase 1) was only slightly affected by the

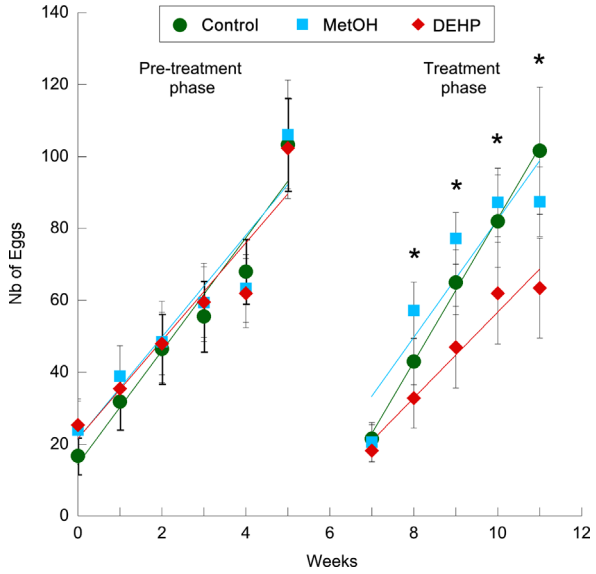

Fig. 2. *Influence of DEHP contamination on the egg-laying rate of Lasius niger queens.* In a first control period ("pre-treatment phase", week 0–5) the different queens received no treatment. One week before the beginning of the experiment, all eggs in nests were retrieved. In week 0, eggs laid in the week were counted and then each week the number of eggs in nests were reported until week 5. In week 6, the eggs were again retrieved from nests. From then on and for 5 weeks, the queens were applied once a week with DEHP diluted in methanol (red diamonds, $n=10$), with methanol only (blue squares, $n=9$) or were only handled every week but received no treatment ('Control', green circles, $n=10$). As for the first period, eggs were counted in each nest every week for a period of 5 weeks ("Treatment phase", week 7–11). Results are given as means ± SD for each batch. Asterisks notify a number of eggs statistically lower for DEHP-treated queens compared to control and methanol-treated queens ($p<0.05$).

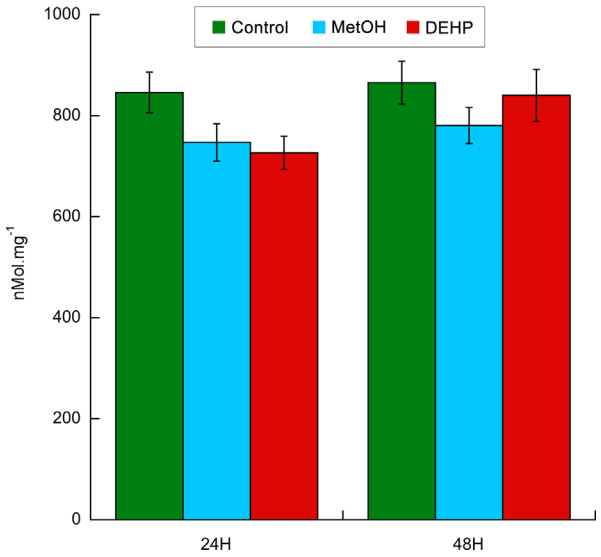

Fig. 3. *Measure of the accumulation of oxidative damage in ants treated with DEHP.* Lipid peroxidation was monitored by the dosage of malondialdehyde (MDA) in pools of 15 ants, 24 h and 48 h after treatment. MDA is currently used as an indicator of oxidative stress in cells and tissues. Results are given as means of 30 measures ± SD (left='control', middle=methanol treatment, right=DEHP treatment; 10 measures per colony, with 3 different colonies; ANOVA with general scores tests at 24 h and 48 h, ns).

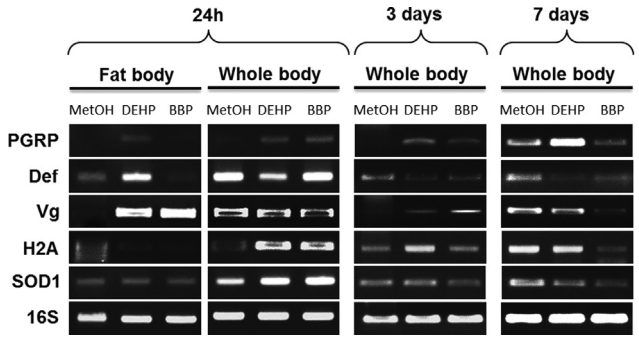

Fig. 4. *Differential expression of several markers involved in the animal defense in response to phthalate treatments. Lasius* workers received a single application of methanol ('MetOH', solvent), DEHP (2 ng) or BBP (2 ng). Levels of expression of selected genes were estimated by semi-quantitative RT-PCR. Measures were realized on whole insects ('whole body') or specifically in the abdominal fat body and ovaries ('fat body'). The expression of the 16S ribosomal gene was used as a reference. Levels of expression were estimated at 24 h, 3 days and 7 days after contamination.

phthalate treatment in the short term (24 h; Fig. 4) and mid-term (3-7d; Fig. 4). SOD1 is an intracellular copper–zinc superoxide dismutase that plays a crucial role in the control of oxidative stress in eukaryotic cells.

3.4. DEHP and BBP treatments selectively affected the expression of defense genes

Four immune genes were selected to serve as markers of immune activation in ants exposed to phthalates. PGRP (Peptidoglycan-recognition protein) are immune receptors able to activate the Toll or the immune deficiency (Imd) signal transduction pathways, or induce proteolytic cascades that generate antimicrobial products, induce phagocytosis, hydrolyze peptidoglycan, and protect insects against infections. Defensin is an important immune effector in insects; positively charged, the small peptide attacks negatively charged bacterial cytoplasmic membrane and leads to its disruption. Vitellogenin (vg) has a dual role in

reproduction and in immune defense in social insects (Amdam et al., 2004). Finally, the histone-2A gene (H2A) is suspected of playing an evolutionarily conserved role in innate immune defense, with sensing and/or microbicidal capabilities (Augusto et al., 2003; Cho et al., 2009; Wang et al., 2011).

Fig. 4 displays modulations of expressions of these immune genes in response to DEHP or BBP treatments. Several immune genes appear as up regulated in response to phthalate treatment in the short term (24 h); the expression of the bacterial sensor PGRP appears slightly enhanced in the fat body and at the scale of the entire body, while the antibacterial peptide Defensin is clearly up-regulated in the fat body (the main source of biosynthesis of AMP in insects). Note that the down-regulation of defensin expression visible in the whole body is not supported by the other two colonies tested. Vg is clearly induced in the fat body (the main site of biosynthesis of vg) 24 h after DEHP treatment. H2A is also induced in response to both phthalates in the whole body. On the mid-term, only the PGRP gene still shows an up-regulation, mainly in response to DEHP, 3 and 7 days after treatment, underlining the long-lasting impact of this specific phthalate. Other modulations observed on the figure are not consistent among the three colonies tested and are, thus, ignored.

4. Discussion

The current study investigates, for the first time, the impact of environmentally relevant doses of phthalates on the physiology of a terrestrial arthropod, the ant *L. niger*. We show that foragers detect and avoid DEHP, the most widespread phthalate found in nature. Besides, we evidence that, at actual dose, DEHP acts as an endocrine disruptor for ants also, inducing a decrease in the egg-laying rate of queens. In parallel, the workers do not seem to suffer oxidative stress as shown in other invertebrates, but clearly have their immune system activated.

A repellent effect of several phthalates had already been described towards the red fire ants (Chen, 2005), and sensilla of *Bombyx mori* were proved to be sensitive to dimethyl phthalate (Ziesmann et al., 2000). Besides, some phthalates are commonly included in the formulation of mosquito repellents (Karunamoorthi and Sabesan, 2010; Khoobdel et al., 2007). Accordingly and considering our behavioral results, it is probable that *Lasius* foragers avoid the most contaminated substrates and evict the most contaminated food from the diet of their colony. Yet, phthalates are found on their cuticle, as they are found on the cuticle of ants living in areas clean of plastic waste (Lenoir et al., 2012). It confirms that the atmosphere is the main source of phthalate contamination for ants. Indeed, phthalic acid esters are semi-volatile organic compounds that are distributed in the atmosphere between vapor and particulate phases. We have previously shown that the lipidic layer that covers the cuticle of insects constitutes a trap for these highly hydrophobic compounds (Lenoir et al., 2012). Consequently, we formulate the hypothesis that the dose measured onto the cuticle corresponds to the actual dose to which the individual is exposed, notably without any additional internal contamination coming from food. It is therefore legitimate to consider the mean measure of 2 ng per ant previously established (Lenoir et al., 2012) as a realistic dose to evaluate the impact of phthalate contamination on wild ants. To fully analyze the results, it appears that workers prefer to feed themselves on the side rather than on the top of the cubes, also when methanol only is applied (Fig. 1, side vs. top). Given that the drop of liquid was systematically deposited on the top center of the cube, this result might suggest that ants are also able to detect methanol. This specific behavior might either reflect their preference to eat clean nutrients or just highlight their tendency to make it simple and stay on the soil.

One of the most documented detrimental effects of phthalates on wildlife concerns reproductive disturbance. Most of them, including DEHP, display estrogen-like properties and directly modulate various parameters linked to fertility (egg production, vitellogenin synthesis, sperm quantity and motility, viability of embryos; see review in Maradonna et al. (2013) and Oehlmann et al. (2009)). DEHP applied in an environmental dose onto the cuticle of ants also impacts their fertility, as revealed here by contaminated *Lasius* queens that produce fewer eggs. Note that the effect is significant, but appears weak, compared to the impact described in some models (worms— Dixon et al., 1999; Wilson et al., 2002); (fishes: Carnevali et al., 2010; Kim et al., 2002; Shioda and Wakabayashi, 2000; Uren-Webster et al., 2010); (tadpoles: Larsson and Thurén, 1987; Lee et al., 2005); (mice: Schmidt et al., 2012). However, in nature, these molecules are maintained permanently on the cuticle at a low level (Lenoir et al., 2012) so that the effect observed on the queens' fertility may finally lead to a negative impact on the yield of colonies in the wild, threatening ants' biodiversity. The mechanism that goes from phthalate exposure to reduced fertility is far less understood in invertebrates than in vertebrates. In vertebrates, phthalates often impair reproduction through an estrogenic and anti-androgenic effect that arises either through the targeting of steroid biosynthesis pathways resulting in greater production of E2 with a concurrent reduction in concentration of testosterone (for DEHP) or through estrogen mimicking potency and transactivation of the endogenous receptor (for BBP), both ways affecting multiple steps of egg production (Jobling et al., 1995; Mankidy et al., 2013; Maradonna et al., 2013). In some models, phthalate treatments were shown to lead to the induction of vitellogenin (vg) production in males (Barse et al., 2007) and in females (Carnevali et al., 2010; Maradonna et al., 2013). Vgs are major precursors of the egg-yolk proteins which provide energy reserves for embryonic development in oviparous organisms. They are mainly produced by the liver in vertebrates, and by its equivalent, the fat body, in insects. We show here that DEHP and BBP treatments also induce the expression of a *vg* gene in *Lasius* workers (Fig. 4). Linking the two results – egg-laying rate reduction and induction of vg – appears quite counterintuitive. Nevertheless, such a double effect has already been documented in female zebrafish (Carnevali et al., 2010). In this vertebrate, environmentally relevant doses of DEHP inhibit the expression of receptors involved in oocyte maturation (LHR and mPRβ), and also cause a significant dose-dependent decrease in the expression of the cyclooxygenase 2 (ptgs2) gene, which codes for an enzyme essential for the ovulation process. Such combined effects lead to both a higher plasmatic level of vg which is no longer loaded in oocytes and a dramatic reduction of embryo production, as observed in our model. However, the induction of the *vg* gene in *Lasius* workers does not preclude other modes of action in queens, since the regulation of gene expression is highly caste-dependent in social insects (Graff et al., 2007).

Unexpectedly, DEHP exposure causes no oxidative stress in *Lasius* workers, whereas it is one of the main effects observed in vertebrates and aquatic invertebrates (Ferguson et al., 2012; Ghosh et al., 2010; Lu et al., 2013; Wang et al., 2012). Indeed, phthalates were shown to disrupt oxidative balance by increasing the production of ROS (Lu et al., 2013), by inhibiting the production/activity of antioxidant enzymes (Manojkumar et al., 1998) and/or by enhancing lipid peroxidation (Mankidy et al., 2013; Santhosh et al., 1998). In our study and despite large sampling, we failed to evidence any increase in lipid peroxidation in *Lasius* ants treated with DEHP. This suggests either a level of exposition too low to show any damage in such a short exposition time or efficient mechanisms of protection against oxidative stress in ants.

The slight induction of the *sod*1 gene observed at 24 h in the whole body may contribute to such protection, but remains weak to explain the absence of lipid peroxidation. The cuticular blend is of special importance for the social organization of ants, since it is involved in many aspects of inter-individual recognition. Such a repertoire of information needs to be kept up to date, which calls for an efficient and rapid metabolism of the lipidic compounds that lie onto the cuticle. Maybe this efficient metabolism is able to rapidly inactivate the pollutant, limiting the negative effect on ants' physiology.

The ant immune system appears quickly ignited in response to an environmentally relevant dose of DEHP. PGRP receptors and the defensins are directly involved in the sensing and response to pathogens in many organisms. H2A is suspected to be a source of immune effectors and apart from their reproductive role, vg was shown to have direct immune functions in vertebrates (Li et al., 2008; Zhang et al., 2011) and to have functions in somatic maintenance in honey bees, notably affecting longevity (Amdam et al., 2004; Corona et al., 2007). More than a dysfunction, the rise in immune gene expression in response to DEHP may thus be part of a general mechanism of enhanced immune protection in response to phthalate exposure. This response may result from a chemical alteration of the cuticle due to phthalate deposition. Some physical damage may be perceived by the defense system of the animal, leading to an increase in barrier immune effectors. Alternatively, phthalates may directly target immune pathways. Indeed, DEHP and BBP can directly modulate the expression of specific genes through the modification of DNA methylation in their promoter region (Kang and Lee, 2005; Singh and Li, 2012; Wu et al., 2010). In hepatocytes from rats, Ghosh et al. (2010) demonstrated an induction of the NF-κB pathway in response to DEHP exposure, which could be consistent with an overexpression of antibacterial effectors, as observed in our study. According to our results, the induction of immune genes in *Lasius* workers is clear after 24 h. Except for the *pgrp* gene, consequences over several days are more difficult to interpret and seem more colony-specific. Nevertheless, given that ants bear phthalates on their cuticle at a constant rate (Lenoir et al., 2012), wild individuals probably suffer a chronic exposure that possibly leads to a permanent activation of their immune system. Moreover, in nature, they are exposed to a complex mixture of phthalates with possible cumulative effects. Indeed, each phthalate may have its own set of disturbed genes, as evidenced here with DEHP and BBP which do not modulate our immune markers in exactly the same way. Further studies would be necessary to determine if such a permanent state of immune alarm is detrimental to the ants' fitness.

5. Conclusion

The ant *L. niger* is chronically exposed to phthalates at a dose a priori low but sufficient to induce immune activation and a fertility decline, which may prove deleterious in the long-term. Previous studies had already pinpointed lack of investigation on biological effects of phthalates on invertebrate phyla, and especially on terrestrial species. Because of their very large distribution, their presence in virtually all non-polar terrestrial ecosystems and their representation at all trophic levels, ants could be useful indicators of this type of contamination in the terrestrial habitats, especially via monitoring of their immune state. Beyond laboratory experiments, ants also offer the opportunity to look into effects of plastic contamination on wild populations. Our future works will address the case of ants collected in pristine locations, far from any urban life in the primitive forest of French Guyana, in an attempt to find ants bearing no phthalate on their cuticle, which has been unsuccessful so far. Such control populations would be most useful to assess the effect of phthalates on exposed populations.

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