Effects of DEHP on the ecdysteroid pathway, sexual behavior and offspring of the moth Spodoptera littoralis

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Abstract:

Bis(2-ethylhexyl) phthalate (DEHP) is a widely produced plasticizer that is considered to act as an endocrine-disrupting chemical in vertebrates and invertebrates. Indeed, many studies have shown that DEHP alters hormonal levels, reproduction and behavior in vertebrates. Few studies have focused on the effects of DEHP on insects, although DEHP is found almost everywhere in their natural habitats, particularly in soils and plants. Here, we investigated the effects of DEHP on the sexual behavior and physiology of a pest insect, the noctuid moth *Spodoptera littoralis*. In this nocturnal species, olfaction is crucial for sexual behavior, and ecdysteroids at the antennal level have been shown to modulate sex pheromone detection by males. In the present study, larvae were fed food containing different DEHP concentrations, and DEHP concentrations were then measured in the adults (males and females). Hemolymphatic ecdysteroid concentrations, the antennal expression of genes involved in the ecdysteroid pathway (nuclear receptors *EcR, USP, E75,* and *E78* and *calmodulin*) and sexual behavior were then investigated in adult males. The success and latency of mating as well as the hatching success were also studied in pairs consisting of one DEHP male and one uncontaminated female or one DEHP female and one uncontaminated male. We also studied the offspring produced from pairs involving contaminated females to test the transgenerational effect of DEHP. Our results showed the general downregulation of nuclear receptors and calmodulin gene expression associated with the higher concentrations of DEHP, suggesting peripheral olfactory disruption. We found some effects on male behavior but without an alteration of the mating rate. Effects on offspring mortality and developmental rates in the N+1 generation were also found at the higher doses of DEHP. Taken together, the results of the study show for the first time that larval exposure to DEHP can induce delayed endocrine-disruptive effects in the adults of a terrestrial insect as well as effects on the next generation. To date, our study is also the first description of an impact of endocrine disrupter on olfaction in insects.

Keywords: Bis(2-ethylhexyl) phthalate, insect, *Spodoptera littoralis*, sexual behavior, hormone, nuclear receptors, olfaction, fecundity, mortality, stage duration, transgenerational effect

Introduction

Bis (2-ethylhexyl) phthalate (DEHP) is a widespread plasticizer (Bergé et al., 2013) that is mainly used to increase the flexibility of plastics, particularly polyvinyl chloride (PVC) products (EU, 2008). DEHP can easily be released into the environment since it is not covalently bound to plastic and can thus be found anywhere, mainly in soils. Most measurements in soils have been performed in agricultural and urban soils, with DEHP concentrations in the range from µg/kg to mg/kg.(Magdouli et al., 2013; Kim et al., 2019). In addition, plants can bioaccumulate DEHP (Sun et al., 2015) after exposure through multiple pathways via air (Du et al., 2010), soil and water runoff (Sun et al., 2015), leading to the possibility of phytophagous species contamination. Several studies highlighted DEHP accumulation in cultivated plants with a range of concentration going from 0.1 to 4150 µg of DEHP per gram of plant or leaf (i.e. dry weight) and from 26.5 to 362 µg of DEHP per gram of peel and flesh of three fruits (Aviles et al., 2019). Herbivorous insects are likely to ingest DEHP when feeding on cultivated plants.

Numerous studies have investigated the effects of DEHP on vertebrate species, including humans, in which it is now considered an antiandrogenic chemical (Moore et al., 2001). These endocrine disruptions were thus observed for various doses. Indeed, reproductive effects such as abnormal testis development or a decreased sperm count have been shown for example in rats exposed several days to DEHP at 500 mg/kg/day (Wu et al., 2010) and on zebrafish exposed ten days to DEHP from 0.5 to 5000 mg/kg (Uren-Webster et al., 2010). The disruption of female fecundity has also been reported in rodents exposed to DEHP at 20-200 µg/kg/day and 500-750 mg/kg/day and in fishes exposed to DEHP at various range of concentration from 100µg/L to 0.5 mg/L (Ye et al., 2014; Rattan et al., 2018, Adeogun et al., 2018). DEHP can act via multiple pathways and alter various biological systems, such as development, immunity, and sugar and lipid metabolism (reviewed in Mankidy et al., 2013). DEHP can alter reproductive physiology and social and sexual behavior in rodents (Lin et al., 2015; Quinnies et al., 2015; Hatcher et al., 2019). These studies suggest that the cognitive and behavioral deficits induced by DEHP could be indirectly due to its effects on the endocrine system.

The potential effects of DEHP on invertebrate species and, more specifically, on terrestrial insects have received less attention, despite the main role of lipidic hormones in their development and physiology. In particular, ecdysteroids and juvenile hormone are well known to play a crucial role in insect post-embryonic development (reviewed in Lafont, 2000), but they are also involved in adult reproduction and behavior (reviewed by Uryu et al., 2015). Interestingly, DEHP interferes with processes that are finely regulated by the endocrine system; for example, it alters development in the midge *Chironomus riparius* (Kwak and Lee, 2005) and decreases female fecundity in the ant *Lasius niger* (Cuvillier-Hot et al., 2014). Moreover, at the molecular level, DEHP was shown to interact with the ecdysteroid pathway by downregulating the expression of the ecdysteroid receptor (*EcR*) and its

partner ultraspiracle (*USP*) in *C. riparius* (Herrero et al., 2017; Planelló et al., 2011). As observed in vertebrates, DEHP seems to interfere with various physiological pathways in insects. Indeed, it modulates genes involved in stress responses such as heat shock protein genes in *C. riparius* (Herrero et al., 2017) as well as genes involved in reproduction (vitellogenin) and immunity (defensin) in *L. niger* (Cuvillier-Hot et al., 2014) and genes involved in metabolism (insulin-like peptide) in *Drosophila melanogaster* (Cao et al., 2016).

Interestingly, one study reported that DEHP exposure decreased the efficiency of prey catching by aquatic dragonfly larvae (Woin and Larsson, 1987), showing that DEHP can also affect insect behavior. More recently, Chen et al. (2018) showed that DEHP disrupts vision and courtship behaviors in *Drosophila melanogaster*. However, the underlying mechanisms of these behavioral effects were not investigated, but they appear to be essential since insects represent the major component of animal biodiversity and play a crucial role in ecosystem functioning (Obrist and Duelli, 2010). Insects develop complex behaviors that can be triggered by the integration of various signaling cues collected from their environment by sensory systems. Among these systems, olfaction is crucial in many species, especially nocturnal species, to locate food resources and mating partners or avoid predators. In several moth species, the response to odorant cues, especially sex pheromones, has been shown to be modulated by juvenile hormone (Anton et al., 2007) or ecdysteroids (Bigot et al., 2012). As hormones play a key role in the plasticity of insect odorant detection (Gadenne et al., 2016), it can be expected that any endocrine perturbation will affect the functioning of sensory systems from signal reception to olfactory-driven behaviors. In males of *Spodoptera littoralis,* we have shown that disturbing ecdysteroid hemolymphatic levels via the injection of the ecdysteroid 20-hydroxyecdysone (20E) alters the detection of the female-released sex pheromones by the antennae (olfactory organ) and reduces the associated behavioral response (Bigot et al., 2012). The transcription levels of ecdysteroid nuclear receptors and several olfactory actors, such as calmodulin *(Calm)* (Bahk and Jones, 2016), are also altered in the antennae (Bigot et al., 2012). All of these results show that ecdysteroids are involved in sex pheromone reception and modulate *S. littoralis* male sexual communication. This species thus represents a good model for investigating the effect of endocrine disruptors such as DEHP on the olfactory system and behavior of a terrestrial insect.

To test the effect of DEHP on *S. littoralis*, we fed larvae DEHP-contaminated food until pupation, testing a wide range of DEHP concentrations measured in environment or tested in previous studies (from 100 pg/g to 5 mg/g). The DEHP content in the diet was measured after mixture preparation to determine the precise amount in contaminated food and to take account of possible environmental unexpected contamination. We also measured the DEHP content remaining in adults (males and females). The antennal expression of nuclear ecdysteroid receptors (*EcR*, *USP*), early and early-late genes (*E75* and *E78*) and *Calm* as well as ecdysteroid hemolymphatic levels were analyzed

in DEHP-contaminated males and controls to detect which treatment could induce an endocrine disruption at the molecular level. Then, we analyzed the courtship behavior of treated males, the mating rate and fecundity of pairs in which either the female or male was treated with DEHP and the putative effects on the offspring of treated females to study the transgenerational effects of DEHP.

Material and methods

Insect rearing, treatment and tissue collection

S. littoralis larvae were reared on a semi-artificial diet (Hinks and Byers, 1976) at 23°C and 60%-80% relative humidity under a 16:8 light:dark cycle until emergence. From the third larval instar until pupation (*i.e.,* ~ 15 days), larvae were fed a semi-artificial diet containing different concentrations of DEHP (DEHP, PESTANAL® 36735, Sigma, France) as described in Aviles et al. 2019. We tested six environmentally relevant concentrations (100 pg, 1 ng, 10 ng, 100 ng, 1 µg and 10 µg per gram of food) and two higher concentrations (500 µg and 5 mg per gram of food), with DEHP dissolved in ethanol (ethanol, absolute, PROLABO®, VWR, France). The treated/control diet was stocked in a glass box until use to avoid direct contamination by the chemicals found in plastic containers. Male and female pupae were reared separately, and adults were fed sugar water (20 g/L). All adult males and females were exposed during larval stages as previously described in Aviles et al. (2019). Control larvae were reared with food containing only the solvent (500 µL of ethanol in 100 g of semi-solid food). Experiments on adults were performed during scotophase, with three-day-old males and females. Adult males and females were allowed to acclimate for at least one hour at room temperature before behavioral experiments or hemolymph and antenna collection. For each of the five replicates of each DEHP treatment, approximately 20 males were dissected to collect 40 antennae. Then the antennae were frozen in liquid nitrogen and stored at -80°C until the qPCR experiment.

DEHP content analysis in adults

Sample Treatment

DEHP content was measured in males and females that developed from DEHP-contaminated larvae or control larvae as described in Aviles et al. (2019). The insects were stored at -18°C, lyophilized and crushed in a mortar. Samples of 0.2 g dw were spiked with an internal standard (IS) (DEHP-d4: 2 µg) in a 40 mL glass centrifuge tube overnight at 4°C. The next day, the samples were extracted with 15 mL of hexane/acetone (50/50 [v/v]) in a Bransonic ultrasonic bath for 20 min (US-EPA method 3550B for ultrasonic extraction 1996). The extracts were centrifuged (2 min at 4500 rpm), and the supernatants were retained. This procedure was performed twice, and the two extracts were combined before being concentrated with an EZ-2 system and finally under a nitrogen stream.

The clean-up procedure, derived from USEPA method 3620B (for phthalate ester and PCB fraction clean-up), consisted of partitioning in a Florisil cartridge (Supelco [1 g]). After conditioning, loading the extract and washing with 10 mL of hexane, DEHP was eluted with 10 mL of hexane/acetone (90/10 [v/v]). The extract was concentrated (400 µL) under a nitrogen stream and deposited in an injection vial before analysis.

Analytical Conditions

Analyses were performed by gas chromatography–mass spectrometry (GC–MS) with a 7890 A gas chromatograph coupled to a 5975 A mass spectrometer (Agilent Technologies, Les Ulis, France). The analytical conditions are summarized in Table S1.

Validation method

The instrument detection limit (IDL; the concentration of DEHP in the standard solution corresponding to a signal-to-noise ratio (SNR; peak-to-peak) of 3) was 2 pg DEHP. The method detection limit (MDL; corresponding to the concentration of a spiked sample for an SNR (peak to peak) of 9) was 46 ng DEHP/g (dw). Finally, recoveries for the entire procedure were estimated via the determination of the recovery rate (RR), which was 84% in our experiment. Simultaneously, for each set of analyses (n = 10), method blanks were included.

Hemolymph collection and ecdysteroid titration

Hemolymph collection and enzyme immunoassay (EIA) titrations were performed as described in Bigot et al. (2012), except that we centrifuged adult males at 100 g for 5.5 min to collect the hemolymph. For each experimental condition, two to five pools of hemolymph from 7 to 15 individuals (approximately 50 µL) were collected. The hemolymph was suspended in 250 µL of methanol (purity 99.9%, MERCK, France) and centrifuged at 9,500 g for 10 min. The supernatant was collected, and the last two steps were repeated once. The supernatant was then dried under vacuum using a SpeedVac Concentrator (Eppendorf, France). The samples were dissolved in buffer for an enzyme immunoassay (EIA) adapted from Porcheron et al. (1989). EIA titration was performed using polyclonal anti-20E antiserum L2 (dilution 1:62,500) and 2-succinyl-20-hydroxyecdysone coupled to peroxidase (dilution 1:100,000). O-Phenylenediamine dihydrochloride, OPD and hydrogen peroxide solution tablets (OPD, Sigma, France) were used for the chromogenic reaction, and the optical density (OD) was measured at 450 nm. Calibration curves were produced for L2 antiserum using 20E (range from 16 - 2000 fmol; gift from René Lafont, Sorbonne University, Paris, France), and the data are expressed as pg 20E equivalents/µL of hemolymph. Ten to seventeen biological replicates were performed for each experimental condition.

RNA isolation, cDNA synthesis and qPCR

Antennae were homogenized with a Polytron (Fisher Scientific, France). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the RNAeasy kit (Qiagen, USA) following the manufacturer's instructions. RNA quality and quantity were checked by spectrophotometry at 260 nm and 280 nm (BioPhotometer, Eppendorf, Hamburg, Germany). Treatment with DNase I (Roche, USA) was performed in accordance with the manufacturer's instructions, and single-stranded cDNAs were synthesized from 5 µg RNA with Superscript II reverse transcriptase (Invitrogen) based on the manufacturer's protocol. For each experimental condition, four or five biological replicates were performed. Five genes were first tested as putative housekeeping genes (*actin*, *ATPase, Rpl13, tubulin and ubiquitin*) via Bestkeeper analysis (Pfaffl et al. 2004). *Rpl13* (GenBank FJ979921) was chosen as the reference gene because it demonstrated the most stable expression levels between sample groups. Forward and reverse primers for housekeeping and candidate genes (*ECR, USP, E75, E78* and *Calm*) (Table S2) were designed from EST library sequences using EPRIMER 3 software as described in Bigot et al. (2012).

PCR was performed in a LightCycler 480 Real-Time PCR Detection System (Roche Applied Science, France) according to a method adapted from Bigot et al. (2012). Each 10 µL reaction consisted of 5 µL of Absolute Blue SYBR Green Fluor (Thermo Scientific, Waltham, MA, USA), 4 µL cDNA (6.25 ng/µL) and 0.5 µL of each primer (10 µM). The PCR program consisted of an initial step at 95°C for 5 min, then 50 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Under these conditions, a single, discrete peak was detected for all primers tested, and all primers showed efficiencies of 90-100%. Each run included the fivefold dilution series, the candidate genes, the reference gene, and negative controls. The average Ct value of each triplicate reaction was used to normalize the candidate gene expression level to the geometric mean of the *Rpl13* level in Q-Gene (Simon, 2003).

Behavioral experiments

We used two behavioral setups, one to test the response of a single male to pheromonal stimulation and another to perform a competitive test between treated and control males in the presence of a female. Experiments were conducted as described in Lalouette et al. (2015). Individuals were maintained in the dark and acclimated until the experiment was initiated in mid-scotophase. Assays were performed under red light and standardized conditions (T = 22 ± 1°C, RH = 70 ± 10%). We used QuickCam® Pro 9000 to record tests with pheromonal stimulation over a 4 min period and competitive tests over 15 min.

The first setup allowed us to follow the response of a single male placed in a square Petri dish (12x12 cm). Pheromonal stimulation was performed under a constant stream of air (0.6 m/min) supplied via a Pasteur pipette with the thinner end inserted through the side of the Petri dish (Fig S1A). The Pasteur pipette contained filter paper loaded with either 10 µl of the *S. littoralis* main pheromone compound, (Z9,E11)-tetradecadienyl acetate (Z9,E11-14:Ac), diluted in hexane or 10 µl of hexane as a control. Three doses of the pheromone were tested: 10 ng/µl and two-fold and ten-fold dilutions. The filter paper was replaced every 15 min. The latencies of several stereotyped behavioral events were then recorded: antennal erect (AE), wing fanning (WF), extrusion of genitalia (EG), and movements towards the pheromonal stimulus (M). The time spent reaching the pheromonal source was also measured. Insects were considered inactive when none the stereotypic behavioral sequence described above was observed.

In the second setup, a control and a treated male were placed in the same square Petri dish (Fig S1A). To distinguish them, a very short piece of the right (treated male) or left wing (control male) was cut one day before the experiment. A female was then introduced into the arena, and male sexual behavior was recorded for 15 min with a CC infrared light-sensitive camera (QuickCam® Pro 9000, Logitech). Several stereotyped behavioral events described above (genitalia extrusion, wing fanning, move towards the female and abdomen curvature) were analyzed. The latency, duration and number of iterations were then compared between the two males. In the control experiment, two untreated males were in competition for the female.

Mating tests

Mating tests were performed during scotophase under dim red light in round plastic boxes (8 cm in diameter) containing a strip of filter paper serving as laying medium for the female and a food source (sugar water into the plastic cap of a 1.5 mL Eppendorf tube). Three combinations were tested: a control female placed with either a control or a treated male and a contaminated female with a control male. The occurrence of mating was checked every 30 min, and the onset and the end of mating were then recorded, allowing us to estimate the average mating time and the mean latency before mating (duration between the introduction of the female and mating).

Transgenerational effects on the N + 1 generation

Since insect hormones are crucial for egg production in females and the quality of offspring (Meiselman et al., 2017), we decided to focus on the fate of the eggs produced by DEHP-contaminated females. Eggs obtained from females contaminated by DEHP and non-contaminated females (controls) mated with control males were placed in incubation chambers at 23°C on a semi-artificial diet. Numbers of hatchings were counted to establish the success of hatching (in %). The

development of fifty (N+1) larvae was followed from L3 to the adult stage to determine the duration of larval and pupal stages, larval and pupal mortality rates, and the longevity of adults after emergence.

Statistical analyses

Statistical analyses were performed using R 3.2.2/R Studio 1.0 (R Core Team, 2015) or JMP-Pro 13 software (SAS Institute Inc. 2016). P-values lower than 0.05 were considered significant. For discrete dependent variables (binomial variables for larval mortality and pupal mortality), contingency tables for the DEHP treatments were analyzed by Chi square tests. For continuous dependent variables, we checked for normality and homoscedasticity to perform one-way analysis of variance. When at least one of these conditions was not satisfied (and after attempting the logarithmic transformation of the dependent variable), we tested the effect of the different DEHP treatments via the nonparametric Kruskal-Wallis test. When we identified a significant ($p < 0.05$) effect of the overall effect of DEHP treatment (for all experimental groups), we performed partial tests comparing each DEHP concentration to the control group. The partial test of continuous dependent variables was Student's test or Dunnet's test or, when normality or homoscedasticity was not satisfied, the Wilcoxon test or linear regression with permutations test (lmperm package in R).

Results

1. DEHP content after larval treatments in adult males and females

As shown in Fig 1A, we observed an unexpected contamination of the artificial diet (**Fig 1A**), reflected in a discrepancy between the expected and measured amounts of DEHP in food. This contamination was also observed in our previous study (Aviles et al., 2019). We thus completed the previous analysis by measuring the DEHP content in more adults, including individuals that developed from larvae exposed to 443 ng/g and 676 ng/g DEHP (**Fig 1A**). Under the 443 ng/g condition, the DEHP contents of the contaminated males and females were similar to those of the controls (392 ng/g) (**Fig 1B**). However, compared to the respective controls, DEPH contents were higher in males exposed during larval stages at higher doses. In females, DEHP contents were also higher under all of these treatments except for the 19.7 µg/g condition. Differences between males/females were observed at 1.1 µg/g, 19.7 µg/g and 4.3 mg/g.

2. DEHP reduces the transcription levels of calmodulin and genes involved in the ecdysteroid pathway in adult male antennae

EcR expression was significantly downregulated by 676 ng/g, 19.7 µg/g and 4.3 mg/g DEHP but not by 443 ng/g or 1.1 µg/g DEHP. *USP* transcription levels were also lower at the same

concentrations, but these differences were statistically significant only under treatment with 676 ng DEHP/g. *E75, E78 and Calm* expression was downregulated by every tested DEHP concentration, with the exception of the control conditions (control and 443 ng/g). Under the 443 ng/g condition, as the DEHP contents and gene expression levels of contaminated males were similar to those of the controls (392 ng/g), we decided to focus on the higher concentrations of DEHP.

3. A high concentration of DEHP increases hemolymphatic ecdysteroid levels in adult males

The hemolymphatic ecdysteroid levels of three-day-old males were thus measured during scotophase at three different times: 12 h, 15 h and 18 h. The profile of ecdysteroid variation in control insects confirmed previous observations (Polanska et al., 2009): from the beginning of scotophase (*i.e.* 12 h), 20E levels increased to reach a maximum in the middle of scotophase (*i.e.*, 15 h) and then decreased rapidly at the end of scotophase (*i.e.*, 18 h) to a lower level than at the beginning of scotophase (**Fig. 3**). The peak at 15 h was observed only in controls and males contaminated by DEHP at 19.7 µg/g. Hemolymphatic ecdysteroid concentrations measured at 18 h were significantly lower than those measured at 12 h in control males but also in males contaminated by DEHP at 1.1 µg/g and 4.3 mg/g (linear regression with permutations, $p<0.05$). No statistically significant difference was observed between the various DEHP treatments and the control except at the highest concentration, which led to a globally significantly higher concentration of ecdysteroids than in the controls (linear regression with permutations, $p<0.001$).

4. Effects of DEHP on the sexual behavior of males in response to female main pheromonal compound

To test whether the variation in the transcript levels of *calm* and ecdysteroid nuclear receptors in the antennae of treated males could be linked to an alteration of olfactory functioning extending up to the behavioral level, we investigated the sexual behavior of males in response to the main pheromonal component: Z9,E11-14:Ac (**Fig 4**). We used the pheromonal component at a concentration equivalent to that in one female and two dilutions to test the sensitivity of males to lower concentrations of the pheromone. We observed significant differences between control and treated males for several behavioral events, which differed according to the dose of DEHP ingested during the larval stage. For example, after stimulation by one female equivalent of the pheromonal compound (**Fig 4**), there was a faster time to the observation of the first wing fanning in 19.7 µg/g-contaminated males (**Fig 4C**). We also observed a difference in the response depending on the quantity of pheromonal compound used to stimulate the males. While the first wing fanning appeared earlier in the 19.7 µg/g-contaminated males stimulated by one female equivalent of the pheromone (**Fig 4D**), we observed a significant increase in time to the observation of the movement of antennae,

wing fanning and the first behavioral events under the same DEHP treatment with stimulation with half of one female equivalent of the pheromone (**Fig 4G, H and I**). We also observed a significant faster time to the observation of the first movement of body in males contaminated with 676 ng/g (Fig 4K) stimulated with half of one female equivalent of pheromone. No difference was observed between DEHP treated males and controls stimulated with 1/10 equivalent female of pheromone (Fig 4 M to R).

5. Effects of DEHP on the sexual behavior of males competing for a female

We investigated the sexual behavior of males using a competitive assay described in Lalouette et al. (2015), allowing the direct comparison of the behavioral responses of control males and males that developed from DEHP-contaminated larvae. When the courtship behavior of contaminated and control males exhibiting successful mating with females were compared, effects were mostly observed for males from larvae fed with 676 ng DEHP/g of diet (**Fig. 5**). We thus observed a significant increase in the duration and number of moves towards the female (**Fig. 5A**), the number of abdomen curvatures (**Fig. 5D**) and the duration of genitalia extrusion (**Fig. 5B**) in comparison to the corresponding control. The 1.1 µg/g DEHP males took longer to show the first abdomen curvature. Interestingly, under these two DEHP conditions, we also observed that the 1.1 µg/g DEHP males tended to mate more often than the controls (**Fig. S2**), whereas no effect was observed at the others concentrations.

6. Effect of DEHP on mating

Considering the effect of DEHP on certain sexual behavioral events, we expected an impact of larval contamination on the mating rates of pairs consisting of either a DEHP-contaminated male and a control female or a control male and a treated female (**Fig. 6**). The mating duration of pairs consisting of DEHP-treated males and control females was similar to that of the controls (data not shown, p=0.2), whereas the duration was significantly shortened for pairs including DEHP-treated females for all the treatments except for 676 ng/g (**Fig 6A**). Interestingly, the latency before mating (**Fig 6B**) was also significantly increased for these mating pairs as well as for pairs including treated males (**Fig 6A**), at least under two conditions. Nevertheless, mating rates (**Fig S3)** appeared globally unchanged, at approximately 80% for the majority of the conditions, with the exception of the mating rates of contaminated females treated with 676 ng/g and 1.1 µg/g (74% and 77%, respectively), which tended to be lower than that of the control (i.e., 89%, **Fig S3**).

7- Effect of DEHP on fertility

The number of females that laid eggs and the number of egg packs per female did not significantly differ between any of the tested conditions (p=0.42 for the laying rate, p=0.12 for egg packs). The hatching success of eggs was not-altered except for the eggs laid from control females mated with contaminated males that developed from larvae treated with 4.3 mg/g DEHP (**Fig 6D**). No effect on egg hatching was observed for pairs including contaminated females (**Fig 6E**).

8- Transgenerational effects of DEHP

The eggs of females were kept to follow the post-embryonic development of the next generation until emergence. Larvae were fed a control diet. Larval stages appeared to be affected (**Fig 7A**), with a significant decrease in mortality being observed during the early larval stage (L1 to L3) under the 1.1 µg/g and 4.3 mg/g conditions and during the late larval stages (L4 to L7) under the 1.1 µg/g and 19.7 µg/g conditions. Pupal mortality was also significantly reduced under the 19.7 µg/g DEHP treatment (**Fig 7A**). In addition, the duration of larval stages (**Fig 7B**) was significantly shortened under the 1.1 µg/g and 19.7 µg/g conditions. As expected in *S. littoralis,* the pupal stage in females was shorter than that in males (**Fig 7B**). However, for both sexes, DEHP seemed to induce a lengthening of the pupal stage, particularly in females (**Fig 7B**). Finally, females of the N+1 generation whose mothers came from larvae fed with 4.3 mg DEHP/g of diet presented a significant decrease in longevity (10 days for controls *versus* 6.5 days for 4.3 mg/g-contaminated females) (**Fig 7B**).

Discussion

Our study provides new description of the impact of endocrine disruptors in insects. Thus, larval exposure to DEHP induced delayed effects on adults and the next generation of *S. littoralis*. DEHP reduced the expression of ecdysteroid responsive genes in male antennae, altered some male sexual behavior's items, but not the mating rate. Finally, the more important effect of DEHP was on the duration of larval stages and the adult lifespan of offspring.

As reported in Aviles et al. (2019), we previously studied the quantity of DEHP found in female and male adults *of S. littoralis* contaminated during the larval stage. We did not observe any significant difference in the quantity of DEHP between control larvae and larvae exposed to DEHP concentrations below 1 ng/g (although a tendency of an increase was observed at this concentration of DEHP). Moreover, in this previous study, we observed a significant increase in DEHP content in pupae and adults in comparison to larvae (Aviles et al., 2019). These new measurements completed this previous analysis by providing information on DEHP contents in more adults, including individuals that developed from larvae exposed to 443 ng/g and 676 ng/g DEHP (**Fig 1B**). Similar

DEHP contents were found in adults in comparison to the results reported in Aviles et al. (2019), and these concentrations were higher than those observed in larvae, including those in adults that developed from larvae exposed to 443 ng/g and 676 ng/g of DEHP. However, we observed that the DEHP contents of contaminated males and females that developed from larvae treated with 443 ng/g DEHP were similar to those of the controls (392 ng/g). Interestingly, under this DEHP condition, we did not observe changes in gene expression in comparison to other DEHP conditions, indicating that the 443 ng/g condition can be considered similar to a control. On the other hand, whereas we did not observe a difference in the DEHP content of larvae exposed to the 676 ng/g concentration, we found a significant difference between the controls and contaminated adults for this DEHP condition, probably due to bioaccumulation during post-embryonic development (Aviles et al., 2019). Finally, we observed a higher DEPH content in both sexes in comparison to the control adults under all conditions except in 19.7 µg/g DEHP-contaminated females, which did not differ from the control. This result coupled with the observed variation in DEHP contents between males and females suggests a sex-specific difference in DEHP metabolism. This effect depending of the sex was rarely reported and currently mainly in vertebrates. Thus, a difference of DEHP effect administrated at 3 and 30 mg/kg/day during 15 days was observed on hypothalamic-pituitary axis of prepubertal offspring rats (Carone et al., 2012). In mice, trait anxiety induced by DEHP at 20 to 540 mg/kg was different between male and female (Park et al., 2015). In African sharptooth catfish (*Clarias gariepinus*), histological examination of the gonads showed that females responded stronger, compared to males, to 2 weeks of DEHP exposure with concentrations from 10 to 400 µg/L, and each sex presented different malformation of gonads (Adeogun et al., 2018). These previous results are consistent with our observations of sex specific difference in DEHP effects.

In previous study (Bigot et al. 2012), we demonstrated that the antennae of males are under ecdysteroid endocrine control and that a change in 20E hemolymphatic levels can induce changes in the ecdysteroid signaling pathway and *calmodulin* (*Calm*) transcription levels in antennae. We thus used real-time polymerase chain reaction (RT-qPCR) to quantify the effects of DEHP treatments during larval instars on the transcript levels of several nuclear receptors (*i.e.*, *EcR, USP, E75* and *E78*) and *alm* in the antennae of three-day-old adult males (**Fig. 2**). With the exception of the 443 ng/g condition, we highlight in this study that after oral contamination during the larval stage, DEHP can induce the downregulation of the expression of the ecdysteroid nuclear receptor (*EcR-USP*, *E75*, *E78*) and *Calm* genes in the antennae of adult males, even at low doses of DEHP (676 ng/g of DEHP in food). Our results confirm and complete the data obtained previously in larvae of the aquatic midge *Chironomus riparius*. In this species, Planelló et al. (2011) demonstrated that *EcR* and *USP* transcription could be downregulated in larvae after 24 h of exposure to DEHP dissolved in water at

0.1 g/L. This downregulation was also observed after 48 h, 72 h and 96 h of exposure to DEHP concentrations between 1 ng/L and 1 µg/L.(Herrero et al., 2017)

DEHP can also interfere with steroid production or metabolism (Borch et al., 2006; Zhou et al., 2013). To confirm this potential effect of DEHP, we replicated the hemolymphatic analysis during scotophase (Bigot et al. 2012) to correlate the above results with ecdysteroid titrations in adult males that developed from contaminated larvae. Under some DEHP conditions, ecdysteroid titers can also be affected both qualitatively (*i.e.*, regarding the form of the ecdysteroid curve during scotophase) and quantitatively (*i.e.*, regarding the quantity of ecdysteroid measured in hemolymph).

Here, we can hypothesize that DEHP that is still present in adults of *S. littoralis* due to bioaccumulation could directly act at this stage and/or induce delayed effects in adults by altering larval processes, as shown in vertebrates (Ljungvall et al., 2005; Norman et al., 2007). Considering the potential mechanisms of action, DEHP could induce either an indirect effect or a direct effect. The indirect effect could be due to an effect on *EcR* gene expression by inducing a general stress response, as observed under different physical or chemical stresses. For example, Gong et al. (2015) observed that, in the early juvenile *Scylla parmamosain* mud crabs, *EcR* expression was downregulated at low temperature (14°C) or high salinity. A direct effect could be due to the binding of DEHP at a nuclear receptor of the ecdysteroid signaling pathway. Currently, no study has demonstrated the ability of DEHP to bind to an *EcR* receptor in invertebrates. However, phthalates have been observed to be possible ecdysteroid receptor agonists, as observed for benzyl butyl phthalate (BBP) (Planello et al., 2011; Herrero et al., 2015), or antagonists, as observed for DEHP (Herrero et al., 2017) and diethylphthalate (DEP) (Dinan et al., 2001), consequently inducing an increase or decrease in *EcR* gene expression, respectively. In vertebrates, whereas some phthalates are able to interact with nuclear receptors (Takeuchi et al., 2005), an interaction of DEHP with estrogen and androgen receptors is not clearly established (Kim et al., 2010; Sun et al., 2012). In fact, DEHP is a known ligand of the *thyroid receptor* (*TR*) (Ishihara et al., 2003) and the peroxisome proliferator activated receptor (*PPAR*) (Singh and Li, 2011; Maradonna et al., 2013), which is a nuclear receptor involved in glucose and lipid metabolism (Grün and Blumberg, 2009; Hong and Park, 2010). Interestingly, *E75* and *E78* in insects are closely related to *PPAR* (Bridgham et al., 2010; Hong and Park, 2010) and could be targets of DEHP.

The change in *Calm* antennal expression in *S. littoralis*-contaminated males suggests that DEHP could also alter the function of the olfactory peripheral system via the ecdysteroid pathway. *Calm* is a ubiquitous protein that acts as a sensor of Ca^{2+} (Andruss et al., 1997; Bahk and Jones, 2016) and plays a crucial role in olfactory processing (Xu et al., 2017). For example, *Calm* is involved in olfactory receptor trafficking to ciliated dendrites and in maintaining olfactory receptor neuron sensitivity in cases of prolonged odorant exposure (in particular pheromone receptors) (Bahk and

Jones, 2016). Thus, a decrease in *Calm* could imply the presence of fewer pheromone receptors at the dendrite surface. In addition, *Calm* plays an important role in genomic and nongenomic ecdysteroidogenic signal transduction (Di Cara and King-Jones, 2013; Jing et al., 2015). Altogether, our results are consistent with the hypothesis of Herrero et al. (2017) that DEHP could function as an ecdysone antagonist. Indeed, in a previous study in adult males of *S. littoralis*, we reported an increase in the antennal expression of the three nuclear receptors (*EcR*, *USP* and *E75)* and *Calm* that was dependent on the quantity of 20E injected at the beginning of scotophase. Even if feedback regulation from the early and early-late genes to their own expression and the expression of *EcR* and *USP* can take place in the ecdysteroid pathways (Huet et al., 1995), DEHP treatment induced the downregulation of all of these genes in the present study, strongly suggesting that this antagonist effect of DEHP occurs at the molecular level.

The change in ecdysteroid levels observed here at the highest dose could also be considered a general stress response. Some studies have highlighted that the stress response in insects involves an increase in the production of ecdysteroids, similar to what is observed for glucocorticoids in vertebrates (Peric-Mataruga et al., 2006). Indeed, ecdysteroids can induce the synthesis of stress-protective enzymes or change the stress-induced activity of antioxidative protection enzymes (Krishnan et al., 2007; Peric-Mataruga et al., 2006). In male *Drosophila virilis*, exposure to short- and long-term heat stress increases 20E levels in the whole body (Hirashima et al., 2000). In female *D. virilis* under heat and nutritional stress exposure, octopamine levels increase, leading to an increase in juvenile hormone levels, which in turn increases 20E levels (Gruntenko and Rauschenbach, 2008). An ecdysteroid increase was also observed in first-instar larvae of the decapod *Homarus americanus* exposed to the pesticide heptachlor, a known environmental endocrine disruptor (Snyder and Mulder, 2001).

DEHP altered several behavioral events observed in response to pheromonal stimulation. In males, the alterations were quite heterogeneous. In the first behavioral bioassay allowing the control of the quantity of the pheromonal component used to stimulate one male, we observed that DEHP induced mainly changes at two concentrations (i.e. 676 ng/g and 19.7 µg/g) (**Fig 4 and 5**). We also identified different responses for some behavioral events when the amount of the pheromonal component was reduced. For example, we observed a shorter time to the first observed movement of wing fanning in 19.7 µg/g-contaminated males in comparison to the control after stimulation with one female equivalent of pheromone, whereas a later appearance of this event was recorded in the same contaminated males after stimulation with half of one equivalent of the pheromone. The reduction in the quantity of pheromone used to stimulate the males is a strategy used to avoid saturation of the pheromonal receptors of the olfactory neurons. Indeed, with saturated conditions, it is statistically more likely that a pheromone molecule binds to a receptor and induce a behavioral

response, even in the event of disturbance of the olfactory system or the central nervous system by DEHP. In addition, females during the courtship process do not continuously produce sex pheromone. Males are therefore attracted by a pheromonal plume with varying concentrations of pheromones. Thus, a lower quantity of pheromone to stimulate males avoid this saturation phenomenon and could potentially allow to easily detect disturbances of the olfactory system and behavioral responses. Our results showed that there is a difference in responses between the two highest concentrations of pheromones. The first is difference is for the condition 19.7 µg/g for which, in saturated condition, we were able to observe faster appearance of wing fanning in comparison to controls while longer time was need to observe the appearance of wing fanning and two others behavioral items in males stimulated with less sex pheromone (Fig 4 C, G, H and I). The faster appearance of the first movement of body for the 676 ng/g condition (Fig 4K) is consistent with the increase of the duration and the number of several behavioral items of DEHP-contaminated males in competition with control males for a single female (Fig. 5A to B). The duration of mating was not altered when males were DEHP contaminated (data not shown), whereas we observed an increase in the latency of mating in males that developed from larvae exposed to 1.1 µg/g and 4.3 mg/g of DEHP. The response of the olfactory system and the associated behavior of males can be modulated by hormones in insects through the modulation of gene expression in antenna-related areas of the brain responsible for the processing of olfactory information (Gadenne et al., 2016, Bigot et al., 2012). Endocrine disruptors could potentially have an effect on the olfactory system through changes in circulating hormone titration, the hormone-receptor interaction, and the signaling pathways induced after receptor activation (Hanioka et al., 2008; Planello et al., 2011; Tarrant et al., 2011). Here, in *S. littoralis,* DEHP disruption was observed at several levels (*i.e.*, the ecdysteroid concentration in hemolymph and nuclear receptor expression in antennae), suggesting that peripheral and central disruption of olfactory processes could take place and may explain the change in behavioral responses. In any case, our results showed that there was no consequence for the abilities of males to reach pheromonal sources (**Fig 4F**). Even in the competitive behavioral assays, while the contaminated males exhibited more intense mating behavior than the controls (**Fig 5**), we observed a tendency of DEHP-contaminated males to exhibit an increased mating success under only one DEHP condition (**Fig S2**).

The effects of DEHP on the mating behavior of females were more homogenous than those found for males since we observed a reduction in the duration of mating coupled with an increase in the latency time of mating under the three higher concentrations of DEHP (**Fig 6B and C**). Several studies on females have reported an age modulation of the behavior and electrophysiological responses of antennae to nonpheromonal signals (Gadenne et al. 2016). For example, the responses to plant odors are often enhanced after mating, as females must find a suitable oviposition site (Gadenne et al. 2016). Even though the endocrine modulation of female behavior has been less

studied than that in males, there is a crucial role of hormones in regulating the production of pheromones, sexual attractiveness and the temporal organization of sexual behavior in female moths (Fedina et al., 2017; Bloch et al. 2013). DEHP could interact with this hormonal regulation in *S. littoralis* to induce the disruption of female behaviors. However, as observed for the males (with the exception of the 4.3 mg/g condition), no consequence of DEHP treatment on the mating rate and egg hatching was observed.

However, strong effects on the offspring were observed. The decrease in egg hatching associated with contaminated males (**Fig 6C**) suggested a disruption of the production of sperm in males. Some studies have reported that hormones play a crucial role in the formation of functional male accessory glands and the fertility of males (Sharma et al., 2017). Insecticides with ecdysteroid agonist effects in *Spodoptera litura* affect male reproductive tract development, testicular volume and the number of sperm transmitted to females (Seth et al., 2004). Similar effects have been observed in vertebrate models. For example, a reduction in egg hatching has been observed in *Danio rerio* that is due to the cessation of meiosis, making spermatozoa non-functional according to the authors (Uren-Webster et al., 2010). After egg hatching, DEHP appeared to have important effects on the percentage of mortality in the larvae and pupae as well as the duration of these stages. Overall, our results showed that offspring survival was better when eggs were produced by pairs involving females contaminated with the highest concentrations of DEHP, which resulted in accelerated larval development but a longer pupal stage and shorter adult longevity in females obtained from mothers subjected to the 4.3 mg/g condition. The surprising enhancement of offspring survival associated with the highest concentrations of DEHP might reveal a higher investment in offspring quality by stressed mothers, which could be a plastic response related to a process of terminal investment in reproduction (Stearns, 1992).

In a previous study on the effect of DEHP on the post-embryonic development of *S. littoralis* (Aviles et al., 2019), we showed that similar DEHP treatments did not induce significant mortality, except for the high non-environmental concentration of 4.3 mg/g. Moreover, when the duration of larval and pupal stages was considered together, we only observed longer development for insects exposed to 19.7 µg/g and 4.3 mg/g DEHP. Here, the delayed effects of the treatment on the N+1 generation appeared more clearly. Altogether, our results highlight the need to study the transgenerational effects of such pollutants. Transgenerational effects on endocrine disruptors have been shown in vertebrates such as in mice, in which low doses of DEHP can alter behaviors as well as the level of stress hormones in the N+1 generation (Quinnies et al., 2015, 2017). Most of these studies on vertebrates have addressed the effects of EDC on the genital system (ovaries, testes, etc.). In invertebrates and particularly insects, these putative transgenerational effects of DEHP have not yet been investigated. In *D. melanogaster,* a transgenerational impact of DEHP on the body weight

of offspring larvae has been shown recently (Chen et al., 2019), but the possible effects on their post-embryonic development and the longevity of the resulting N + 1 adults were not investigated. To our knowledge, our results demonstrate such effects in an insect or any invertebrate for the first time.

Conclusion

The modulation of the olfactory system by endocrine disruptors has rarely been studied in vertebrates (Jackman et al., 2018; Palouzier-Paulignan et al., 2012), and our study provides the first description of the impact of endocrine disruptors on the olfactory organ. Exposure to DEHP during larval stages appeared to induce delayed disruption in adults by altering some parameters of sexual behavior in adult males or females but ultimately with little effect on the ability of males to find a female for mating or the ability of females to produce viable eggs. Given the complexity of the underlying mechanisms of action of DEHP due to its multiple targets and potential effects, further studies are required to understand the mode(s) of action of this chemical on the olfactory system of insects. Electrophysiological studies would greatly increase our understanding of these DEHP effects on male olfactory-induced sexual behavior. Further investigations on the transgenerational effect of DEHP are also necessary. Indeed, we demonstrated here that contamination during the larval stage can influence the reproductive physiology of females or males and the production and quality of eggs and offspring. Chen et al. 2019 showed that DEHP is a promotor of obesity in the N+1 generation, since the contamination of *D melanogaster* males can affect the weight of newly produced larvae. An understanding of these effects appears crucial today, particularly in relation to the life cycle of insects as well as for the explanation of physiological disruptions that are currently complicated to study in vertebrate models.

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Legends

Figure 1. (A) Theoretical and measured DEHP contents in food. (B) Quantification of DEHP contents in adult males and females (in ng per g of individuals/n=3-4 biological replicates for each condition corresponding to a pool of 3 to 10 individuals). DEHP contents were quantified by GC-MS in three-day-old adults. For each condition and sex, the mean value ± s.e.m. is indicated. For the statistical analysis (linear regression with permutations test (lmperm package in R)), a significant difference is indicated by a different letter. The first uppercase letter, X in (X/y), represents the statistical comparison between the different DEHP conditions and controls. The second lowercase letter, y in (X/y), represents the statistical comparison performed for similar DEHP (or control) conditions between adult males and females.

Figure 2. Normalized expression of nuclear receptor genes: *Ecdysteroid Receptor* (*EcR*) and *Ultraspiracle* (*USP*), and ecdysteroid-induced genes: *E75*, *E78* and *calmodulin* (*Calm*), in the antennae of control or DEHP-contaminated adult males. The x axis represents "Measured quantity of DEHP per gram of food". Expression levels were quantified by quantitative PCR during the scotophase of three-day-old males (n=6 for each condition). Asterisks indicate significant differences between means and controls (* = $p < 0.05$, ** = $p<0.01$, *** = $p<0.001$, linear regression with permutations test (lmperm package in R)).

Figure 3. Quantification of the hemolymphatic concentration of ecdysteroids (in pg 20E eq/µL) in adult males. Ecdysteroid concentrations were quantified by EIA during the scotophase of three-day-old males at the beginning (*i.e.,* 12 h), middle (*i.e.,* 15 h) and end of scotophase (*i.e.,* 18 h) (n=10 to 17 biological samples, corresponding to the pool of 3 to 6 insects for each condition). The x axis represents "Measured quantity of DEHP per gram of food". Bars with asterisks indicate significant differences between means in comparison to the controls (* = $p< 0.05$, linear regression with permutations test (lmperm package in R)).

Figure 4. Behavioral responses of males to the main pheromonal compound. The left column corresponds to males stimulated with one female equivalent of pheromonal compound. The right column corresponds to males stimulated with half of one female equivalent of pheromonal compound. Values were determined by the analysis of video recordings of behavioral assays involving 1 male

(DEHP-contaminated or control) exposed to the pheromonal compound in an arena (n = 11 to 21 individuals per condition). The log of the mean of the measured values was used to represent the results for each condition. The x axis represents "Measured quantity of DEHP per gram of food". Asterisks indicate significant differences between means and controls (* $p < 0.05$).

Figure 5. Behavioral responses in competitive behavioral assays between 2 males (contaminated and control) *versus* 1 female. Values were determined by the video analysis of competitive behavioral assays (n= 10 to 17 individuals per condition). The mean of the values obtained in mated males (contaminated or uncontaminated) is reported in the graph. The statistical analysis was performed between the values obtained in contaminated males at one DEHP concentration that succeeded in mating with the female and those from uncontaminated males of the same DEHP condition that also succeeded in mating with the female. Bars with asterisks indicate significant differences between means and controls (* $p < 0.05$, Wilcoxon test).

Figure 6. Mating and egg hatching for *S. littoralis* pairs involving either a contaminated male or contaminated female. Values were determined by the direct analysis of pairs in round plastic boxes (n= 28 to 30 pairs per condition). The x axis represents "Measured quantity of DEHP per gram of food". Bars with asterisks indicate significant differences between means and controls (*= $p < 0.05$, Wilcoxon test or student test).

Figure 7. Effect of DEHP treatment on the N+1 generation. n = 28 to 30 pairs per condition. The x axis represents "Measured quantity of DEHP per gram of food". Bars with asterisks indicate significant differences between means and controls (* = $p< 0.05$, ** = $p<0.01$, *** = $p<0.001$, Wilcoxon test or student test).

Figure S1. Behavioral assays for studying the effect of DEHP. (A) Photograph of the arena for competitive bioassays between DEHP-contaminated and control males in the presence of a female. (B) Photograph of the arena with an experimental male in the presence of a pheromonal compound. (C) Summary of the observed behavioral events and measured parameters (latency, duration and number of occurrences). (D) Events quantified during the behavioral sequences.

Figure S2. Comparison of mated treated and control males in competitive behavioral assays with 1 female. Values were determined by the video analysis of competitive behavioral assays (n= 10 to 17 individuals per condition). The mean of the values obtained from mated males (contaminated or

uncontaminated) is reported in the graph. The x axis represents "Measured quantity of DEHP per gram of food". The statistical analysis was performed between the values obtained for contaminated males at one DEHP concentration that succeeded in mating with the female and those from uncontaminated males of the same DEHP condition that also succeeded in mating with the female.

Figure S3. Percentage of mating in *S. littoralis* pairs involving either a contaminated male or contaminated female. Values were determined by the direct analysis of pairs in round plastic boxes (n= 28 to 30 pairs per condition). The x axis represents "Measured quantity of DEHP per gram of food".

Table S1. Analytical conditions used for the determination of DEHP contents in *S. littoralis* adult males and females by GC-MS. IS: internal standard.

Table S2: Sequences of the primers used for RT-qPCR amplification in *S. littoralis* adult male antennae. F: forward primer; R: reverse primer.

Figure 1.

A

Amount of DEHP added in food (per g of fresh food)	0 (Control)	100 pg	1ng	100 ng	10µg	5 mg
Amount of DEHP measured in food (per g of fresh food)	392 ng	443 ng	676 ng	1.1 µg	19.7µg	4.3mg

B

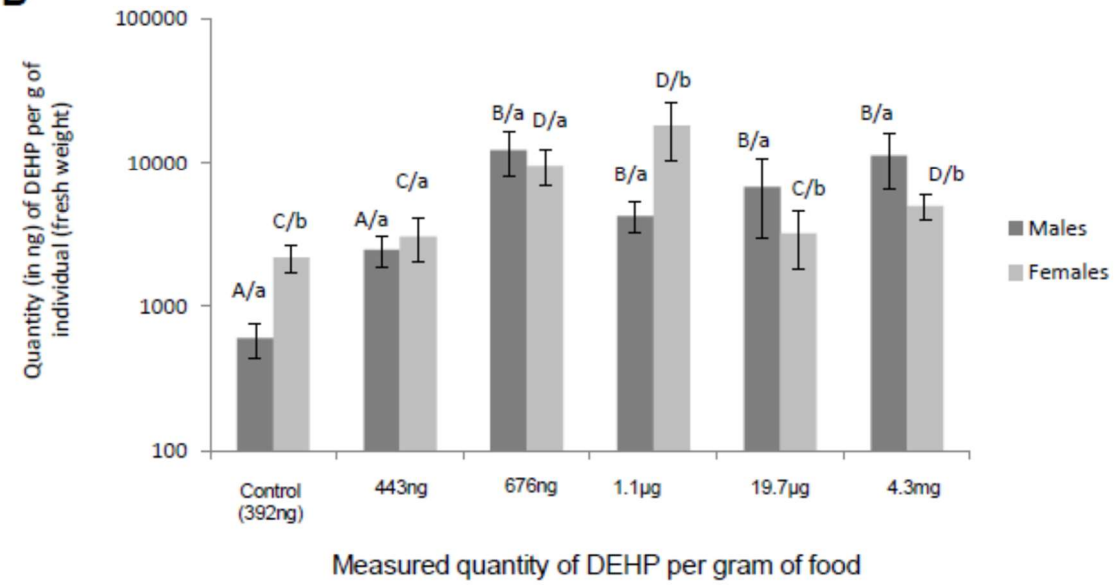


Figure 2.

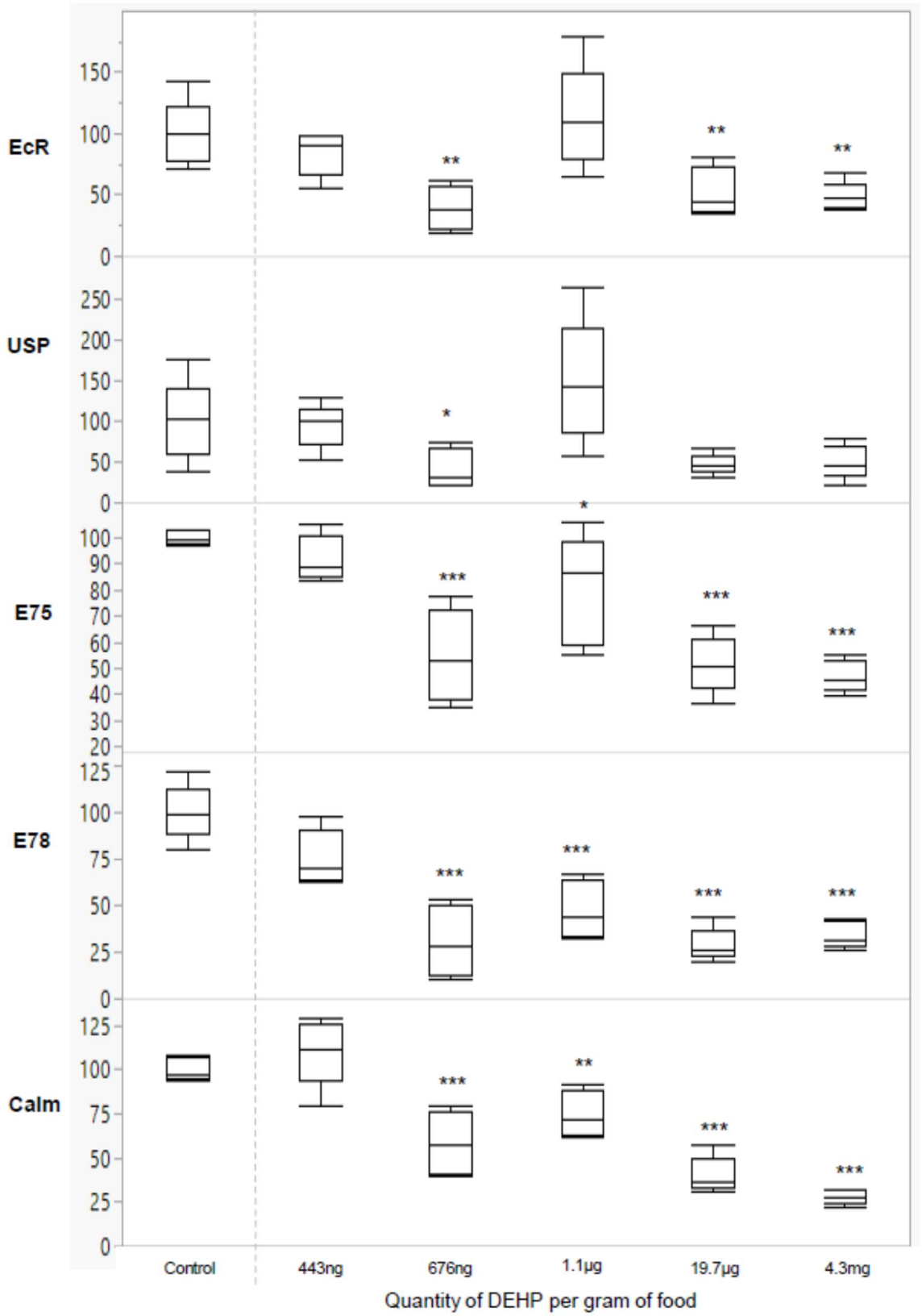

EcR
USP
E75
E78
Calm
Control
443ng
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food

Figure 3.

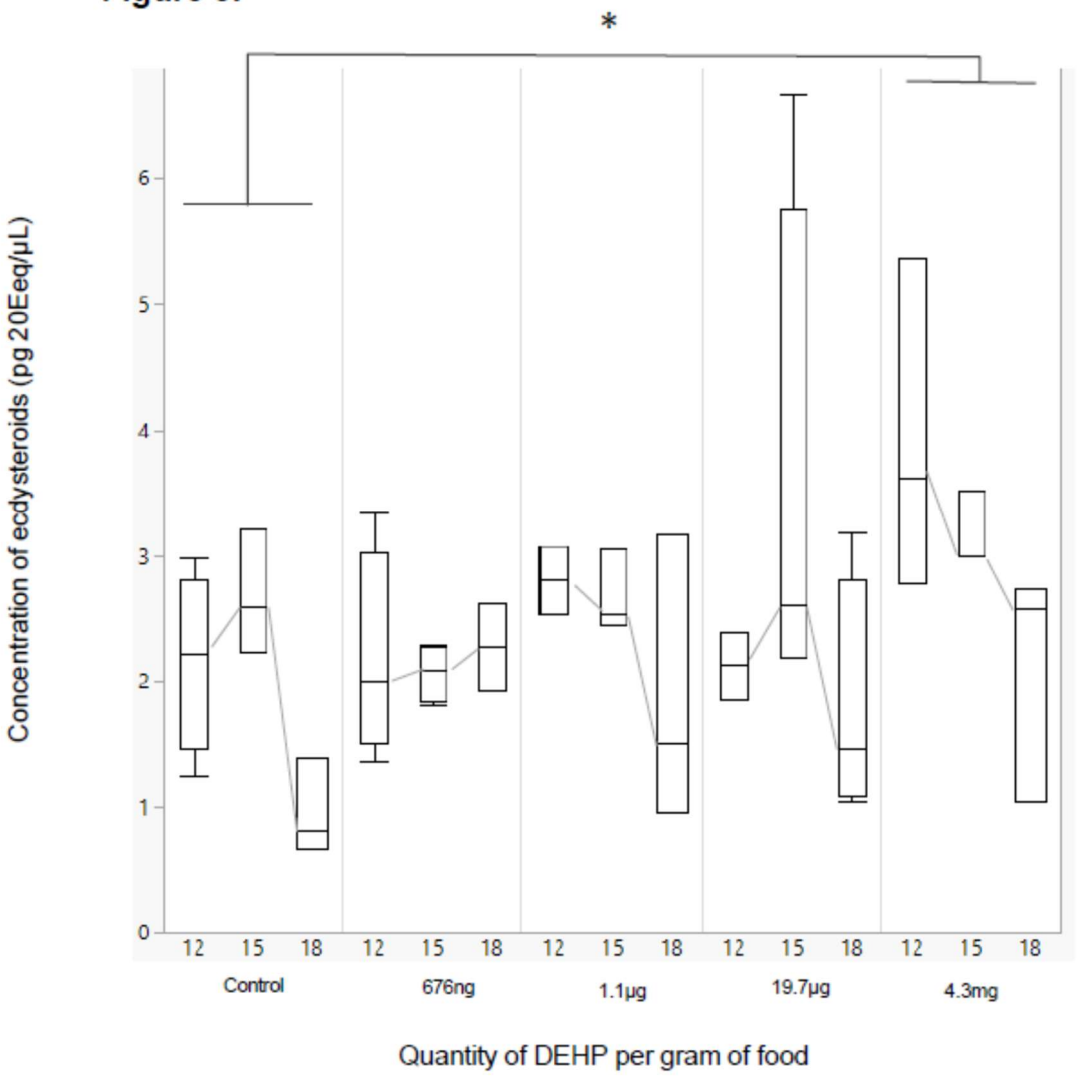

*
Concentration of ecdysteroids (pg 20Eeq/µL)
0
1
2
3
4
5
6
12 15 18 12 15 18 12 15 18 12 15 18 12 15 18
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food

Figure 4.

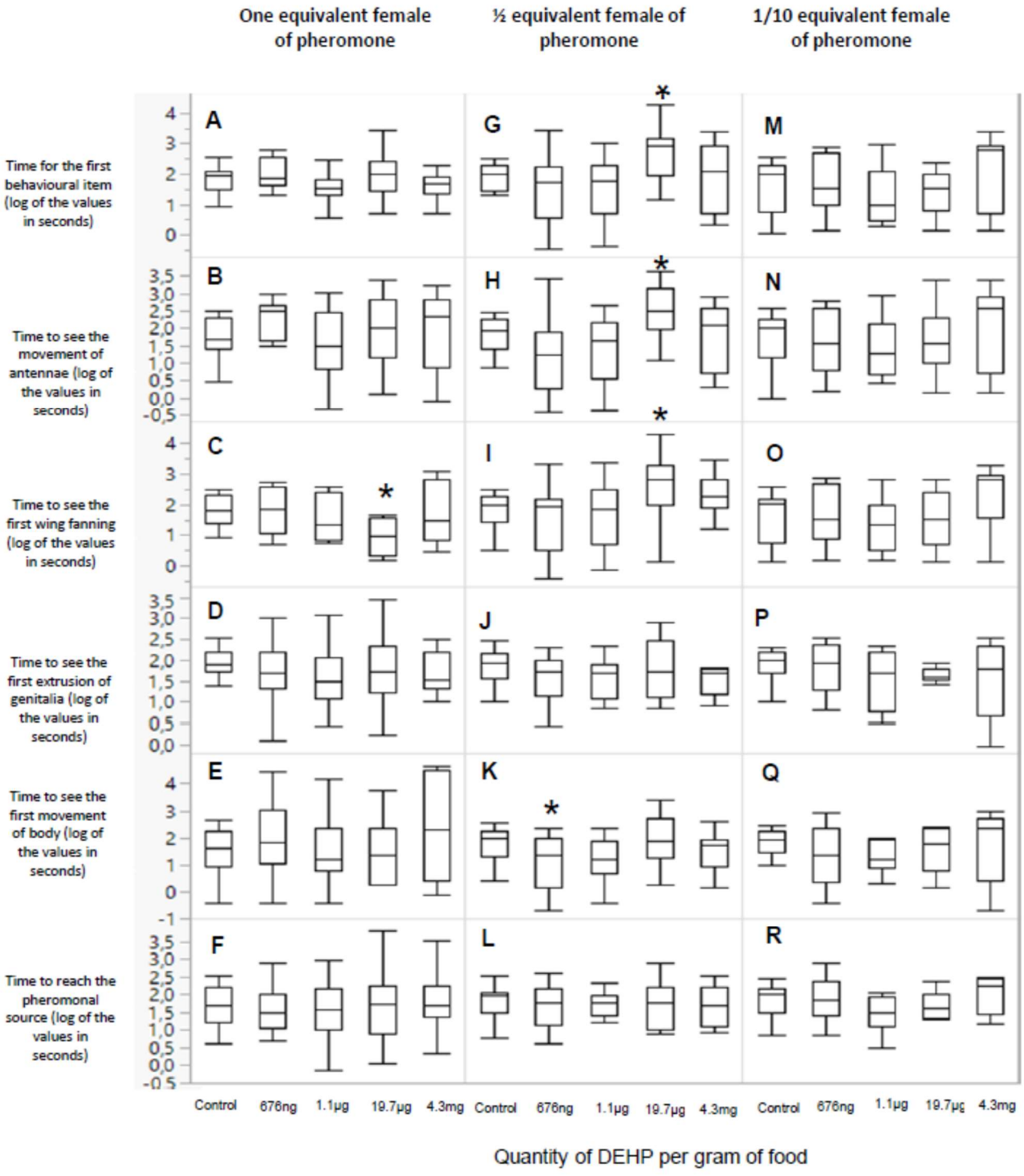

One equivalent female of pheromone
½ equivalent female of pheromone
1/10 equivalent female of pheromone
Time for the first behavioural item (log of the values in seconds)
Time to see the movement of antennae (log of the values in seconds)
Time to see the first wing fanning (log of the values in seconds)
Time to see the first extrusion of genitalia (log of the values in seconds)
Time to see the first movement of body (log of the values in seconds)
Time to reach the pheromonal source (log of the values in seconds)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food

Figure 5.

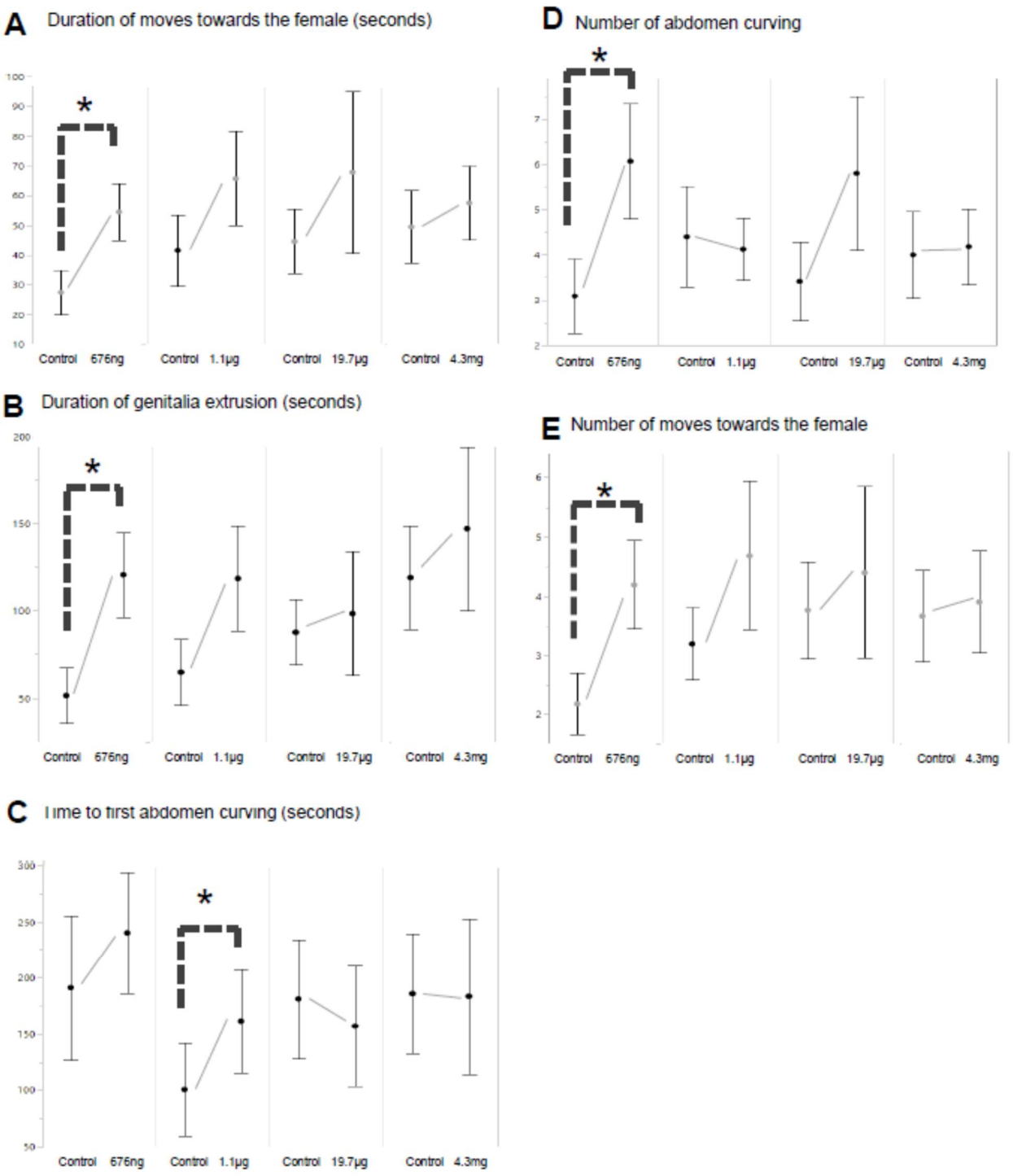

A Duration of moves towards the female (seconds)
D Number of abdomen curving
B Duration of genitalia extrusion (seconds)
E Number of moves towards the female
C Time to first abdomen curving (seconds)
Control 676ng
Control 1.1µg
Control 19.7µg
Control 4.3mg
*

Figure 6.

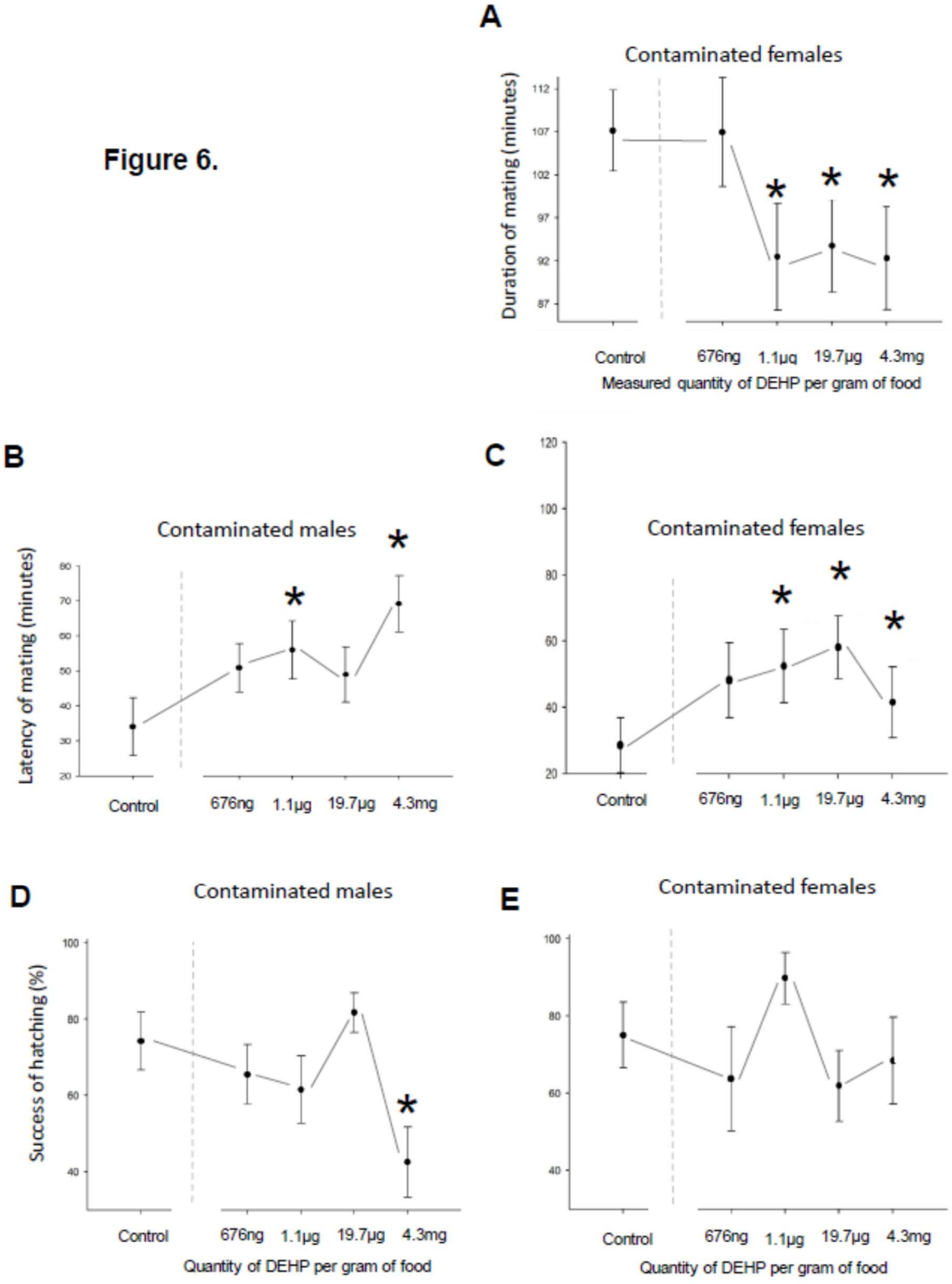

A
Contaminated females
Duration of mating (minutes)
112
107
102
97
92
87
*
*
*
Control
676ng
1.1µg
19.7µg
4.3mg
Measured quantity of DEHP per gram of food
B
Contaminated males
Latency of mating (minutes)
*
*
Control
676ng
1.1µg
19.7µg
4.3mg
C
Contaminated females
120
100
80
60
40
20
*
*
*
Control
676ng
1.1µg
19.7µg
4.3mg
D
Contaminated males
Success of hatching (%)
100
80
60
40
*
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food
E
Contaminated females
100
80
60
40
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food

Figure 7.

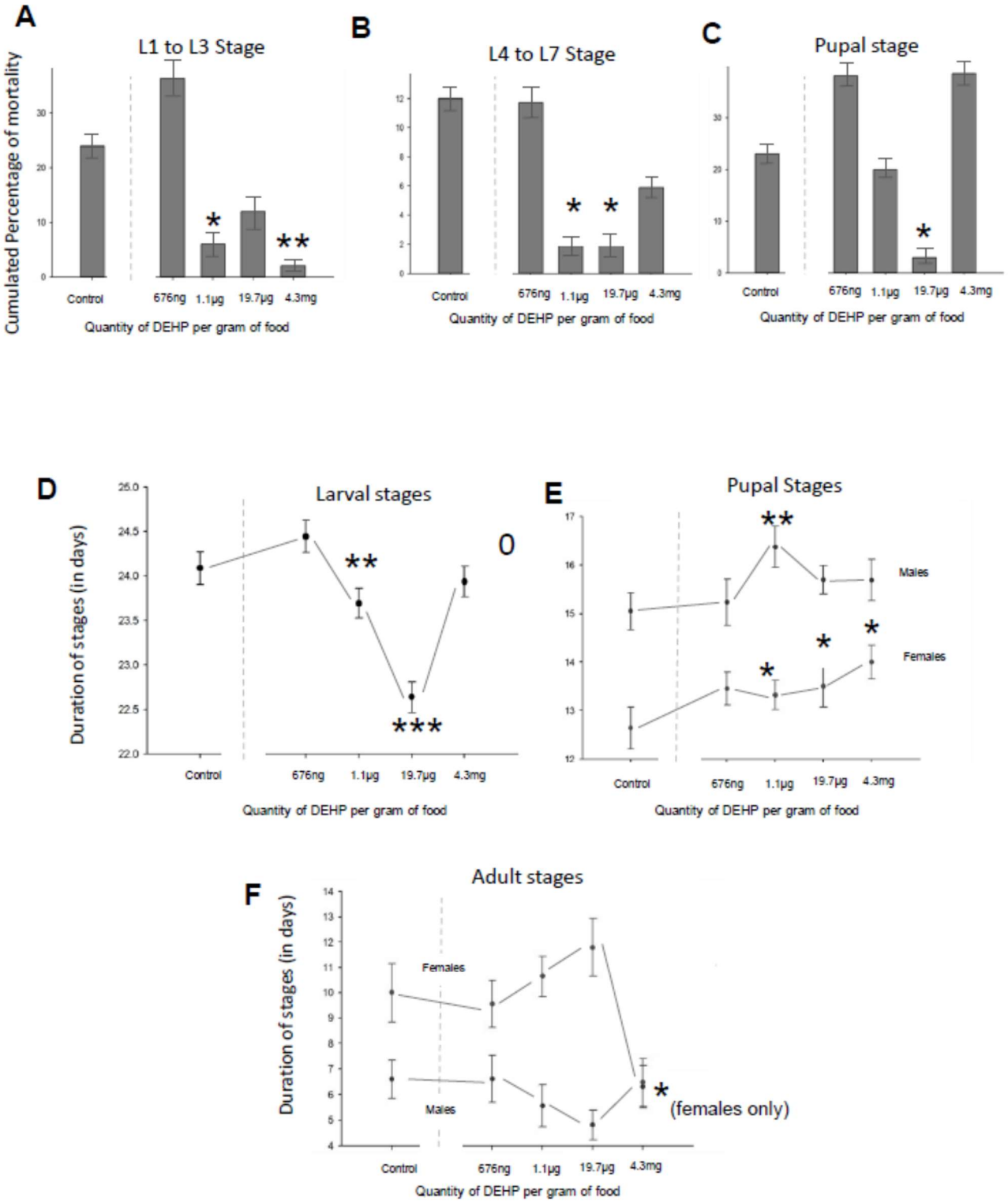
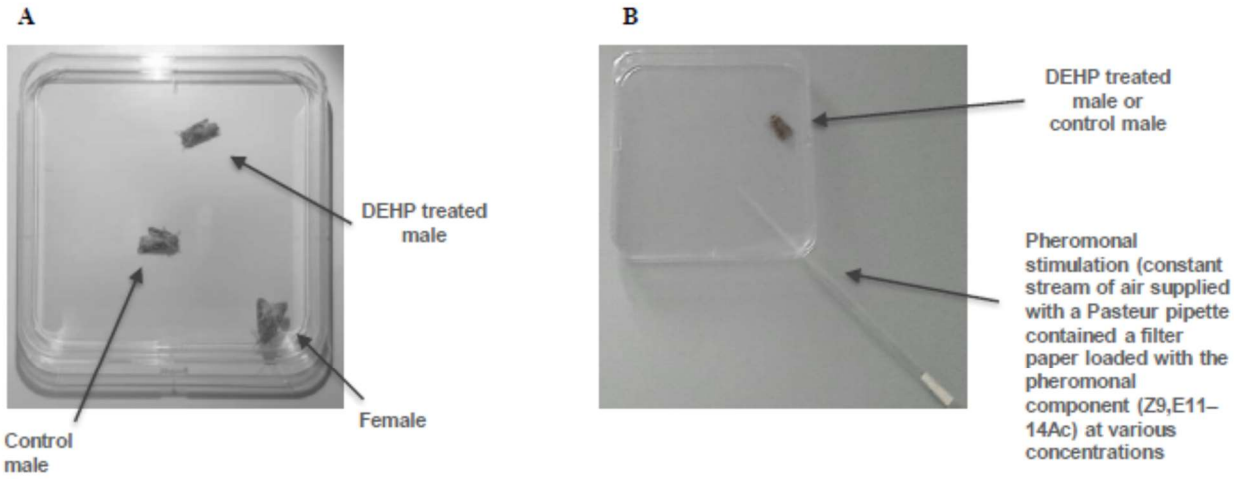

A
L1 to L3 Stage
Cumulated Percentage of mortality
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food
B
L4 to L7 Stage
Quantity of DEHP per gram of food
C
Pupal stage
Quantity of DEHP per gram of food
D
Larval stages
Duration of stages (in days)
Quantity of DEHP per gram of food
E
Pupal Stages
Males
Females
Quantity of DEHP per gram of food
F
Adult stages
Duration of stages (in days)
Females
Males
(females only)
Quantity of DEHP per gram of food

Figure S1.



D

	First movement	Antennal erect	Genitalia extrusion	Wing fanning	Move toward the female	Abdomen curving
Latency	X	X	X	X	X	X
Duration			X	X	X	
Number of occurrences				X	X	X

Figure S2.

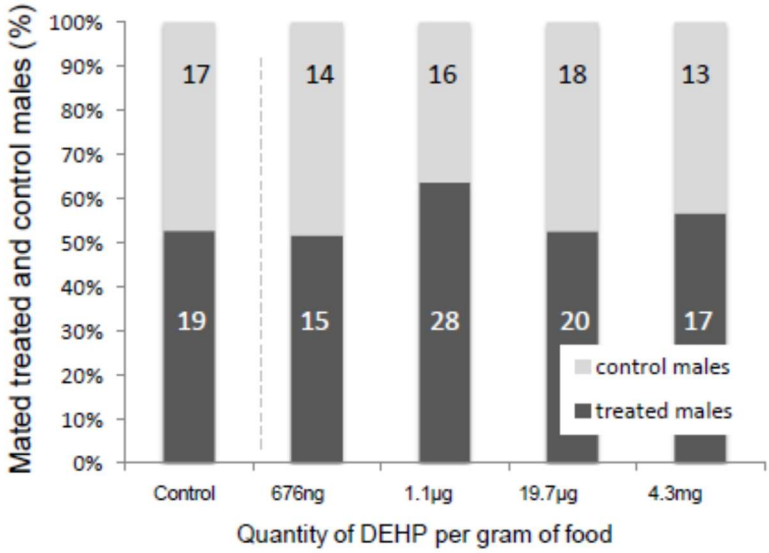

Mated treated and control males (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
17
14
16
18
13
19
15
28
20
17
control males
treated males
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food

Figure S3.

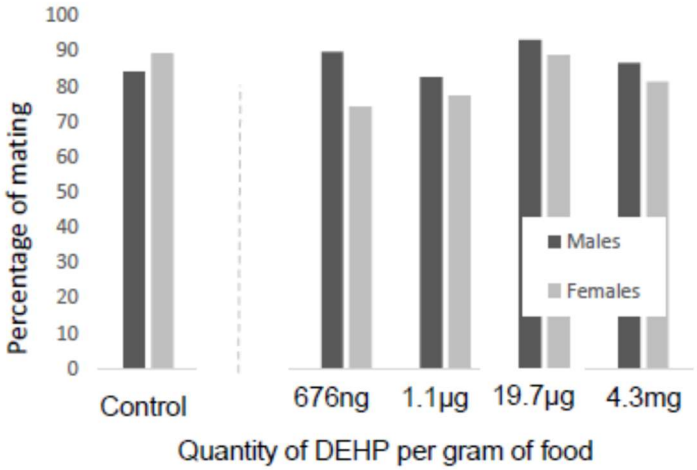

100
90
80
70
60
50
40
30
20
10
0
Percentage of mating
Males
Females
Control
676ng
1.1µg
19.7µg
4.3mg
Quantity of DEHP per gram of food

Table S1

Injector	Guard column	Column	Oven	Helium	Detector	Quantification (bold), qualification (normal), and IS (italic)	Reference
290°C, 1µL	Deactivated silica pre-column (1m; 0.25mm ID from Restek)	ZB-SemiVolatils (30m, 250µm film thickness from Phenomenex)	50°C (1 min), 30°C/min to 280°C then 15°C/min to 310°C, held for 4 min	1mL/min	EI 70eV	**149**, 279 *153*, *171*	(Teil et al., 2013)

Table S2.

Primer name	Description	DNA sequence (5' – 3')
EcR	Ecdysone receptor	F TGCGAGGAAAAAGTGAAGTG
		R TTCCGGGGACATTACCATAG
USP	Ultraspiracle	F CATGTCAGTGGCGAAGAAAG
		R CCAGCGAACAGTCAACAGTC
E75	Early response gene E75	F GACGCCCAAATCGGCCTCTTCTGTGCT
		R CTGCTGCCGCAGTAATTCCTTGTG
E78	Early late response gene E78	F AAAACATGCAGGCTGTGTCA
		R GTCCCCAGCATCTTCATCAT
Calm	Calmodulin	F GCGTTCTCACTGTTCGACAA
		R CTCACTGTCGGTGTCCTTCA