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Phenotypic defects in newborn *Gammarus fossarum* (Amphipoda) following embryonic exposure to fenoxycarb

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ABSTRACT

During morphogenesis numerous morphogenetic factors ensure the production of a target phenotype. By disrupting these processes, a toxic exposure during this period could cause an increase of phenotypic defects. In the present study, embryos of the freshwater amphipod *Gammarus fossarum* were exposed throughout the embryogenesis to increasing concentrations of fenoxycarb (0, 0.5 µg L^{-1}, 5 µg L^{-1} and 50 µg L^{-1}), a growth regulator insecticide analog of the insect juvenile hormone. In addition, to identify morphogenesis' sensitive period, embryos were exposed during either early or late embryonic development to 5 µg L^{-1} of fenoxycarb. In newborn individuals from exposed embryos, three phenotypes were investigated: i) eye pigmentation, ii) length of the antenna and gnathopod of both left and right sides and iii) midgut tissue state. Developmental homeostasis was assessed by measuring fluctuating asymmetry and inter-individual variance of both the antenna and gnathopod. Exposure to 5 µg L^{-1} and 50 µg L^{-1} fenoxycarb throughout the embryonic development induced a delayed hatching and altered appendages size. Moreover, exposure to 5 µg L^{-1} throughout the embryogenesis and during the gastrulation phase impaired eye pigmentation, while exposure to 50 µg L^{-1} resulted in increased tissue damages of the midgut. No significant increase of fluctuating asymmetry was observed in exposed individuals, neither for the antenna nor for the gnathopod. These results demonstrate that fenoxycarb can alter embryonic development of *G. fossarum* without disrupting developmental homeostasis.

1. Introduction

Given that embryos are often considered the most vulnerable stages within the life of an organism (Scholz et al., 2008), their use in ecotoxicology is not only relevant for understanding the mode of action of toxic chemical compounds but also for assessing subtle ecologically-relevant toxic effects. Despite these advantages, ecotoxicological studies dealing with embryos of aquatic arthropods - the dominant taxon in freshwater animal communities - remain rare (but see LeBlanc et al., 2000; Lawrence and Poulter, 2001; Geffard et al., 2010), and most studies have considered hatching success as the sole endpoint of interest (Weis, 2014). However, understanding the toxicity of environmental chemical compounds in early life stages is important since embryonic toxicity has been shown to be implicated in population decline (Hopkins et al., 2006).

During embryonic development numerous morphogenetic factors and endocrine signaling pathways ensure the production of a target phenotype (Davidson, 1991; Fingerman et al., 1998). Phenotypic variation results from the interplay between several sources of variations – including genetic mutations, environmental influences and developmental stochastic errors – and regulating processes – including canalization, phenotypic plasticity and developmental stability (e.g. Debat and David, 2001). Developmental stability is the process buffering developmental errors, thereby ensuring phenotypic consistency in given environmental and genetic conditions, while canalization ensures such consistency under different genetic and environmental conditions (Nijhout and Davidowitz, 2003). As both sides of a bilaterally symmetrical organism are influenced by the same genes and environment, they only differ by random developmental errors, and fluctuating asymmetry (FA) has been proposed as a measure of developmental instability. Canalization (or a lack thereof), in turn, is commonly estimated by the inter-individual variance (e.g. Debat et al., 2009). Phenotypic plasticity describes the phenotypic change produced by a genotype in response to an environmental change. It has been suggested that extreme adverse

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environmental conditions (like extreme temperatures or chemical treatments) can alter developmental control mechanisms and induce a burst of phenotypic variation either within (e.g. Clarke, 1993; Chang et al., 2007) or among individuals (see Badyaev, 2005 for review). Such effects of stress upon phenotypic variation are however not systematic, and various studies have failed to detect them (e.g. Arambourou et al., 2012, 2014; see Hoffmann and Merilä, 1999 for a review).

Beyond size-related traits, pigmentation is also hormonally-regulated in crustaceans. For that reason, Fingerman et al. (1998) have proposed to use pigmentation defects as a sensitive marker of endocrine disruption in this taxon. In line with this, the pigmentation of the crustacean *Daphnia magna* has been shown to be disrupted by an exposure to the juvenile hormone analog pyriproxyfen (Rider et al., 2005).

In the present study, we aim to characterize the phenotypic consequences of endocrine disruption in a common crustacean, the amphipod *Gammarus fossarum*, and identify the most sensitive developmental stage in this species. We hypothesized that an exposure to an endocrine disruptor might interfere with processes involved not only in morphogenesis (developmental stability and canalization) causing an increase of FA and inter-individual variance, but also in pigmentation. At a finer scale, we hypothesized that phenotypic defects could translate into an increase of tissues damages.

To test these hypotheses, embryos were first directly exposed throughout the entire embryonic development to increasing fenoxycarb concentrations. Fenoxycarb is a growth regulator insecticide, analog of the arthropod juvenile hormone, used for controlling pest insect populations. Due to their very selective and unique modes of action (Sial and Brunner, 2010), growth regulator insecticides are being used increasingly (Nauen and Bretschneider, 2002), and they have great potential for replacing broad-spectrum insecticides. Furthermore, given that fenoxycarb is an analog of insect juvenile hormone, it has the potential to interact with hormonally-regulated morphogenetic processes, resulting in phenotypical defects. Indeed, several evidences supported the idea that methyl farnesoate pathways in crustaceans act as juvenile hormone pathways in insects, and can be disrupted by juvenile hormone analogs, including fenoxycarb (Oda et al., 2011; Miyakawa et al., 2013). Second, to identify critical periods of development, embryos were exposed to fenoxycarb during specific time windows of development (gastrulation, organogenesis or hatching). Three types of phenotypes were measured in newly hatched individuals: i) eye pigmentation, ii) length of antenna and gnathopod of both left and right sides and iii) midgut tissue state. The midgut tissue of amphipoda has been shown to be particularly sensitive to insecticide exposure (Doughtie and Rao, 1983; Saravana Bhavan and Geraldine, 2000). Moreover, due to the central role of this organ in food assimilation, an alteration of the epithelial cells could have severe consequences on the growth of newly hatched individuals. For each of the study traits we specifically investigated the effects of fenoxycarb on the frequency of abnormal phenotypes. FA was used as a measure of developmental instability while inter-individual variance was used as a measure of canalization.

2. Materials and methods

2.1. Fenoxycarb solutions

Fenoxycarb (pestanal, Sigma-Aldrich) was dissolved in acetone (for HPLC, Carbo-Elba) and added to the embryo media. Three fenoxycarb concentrations were tested: 0.5 $\mu g\,L^{-1}$, 5 $\mu g\,L^{-1}$ and 50 $\mu g\,L^{-1}$. The concentration of acetone (0.005%, v/v) was the same in all treatment solutions and control. For logistic reasons, solvent-free control was not considered. Note that previous studies carried out in the same species did not reveal any difference between solvent-free control and solvent control (acetone) conditions, neither for reproductive endpoints (Xuereb et al., 2011; Trapp et al., 2015) nor for embryonic development (Geffard et al., 2010). Fenoxycarb concentrations were checked at the beginning of the exposure and after 3 days of exposure, just before the renewal of the test media. Fenoxycarb analysis was performed by LC-MS/MS on an H-Class UPLC system (Waters) coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters). The chromatographic column was a Kinetex 1.7 µm EVO C18 100A (Phenomenex). The mobile phase (A) was composed of 0.4 mM ammonium acetate + 0.01% acetic acid in water and the mobile phase (B) was methanol. The injection volume was set at 2 µL. MS/MS detection was performed in multiple reaction monitoring (MRM) mode with the electrospray ionization source operating in the positive mode (ESI+). The MRM transitions were 302 → 116 (quantification transition) and 302 → 88 (confirmation transition). The quantification limit for fenoxycarb was 0.04 $\mu g\,L^{-1}$. At the beginning of the exposure, the measured concentrations ranged from 120% to 125% from the nominal values. After 3 days of exposure, the measured concentrations ranged from 98% to 114% of the nominal values.

2.2. Gammarid population

Specimens of *Gammarus fossarum* were sampled from a source population - used in our previous studies (Vigneron et al., 2015; Arambourou et al., in press) - in La Bourbre stream (near Lyon, France), and collected using a hand net. The organisms were quickly transported in plastic vessels to the laboratory, where they were kept at 14 °C with a 16:8-h light: dark photoperiod in synthetic water for 10 days before being used in experiments. As described by Moritt and Spicer (1996) and Nyman et al. (2013), synthetic water used in the present study is composed of (in mM): 2 ($CaCl_2{\cdot}H_2O$), 0.5 ($MgSO_4{\cdot}7H_2O$), 0.77 $NaHCO_3$ and 0.077 KCl (electrical conductivity = 600 µS/cm). Animals were fed ad libitum on pre-conditioned alder leaves (*Alnus glutinosa*).

2.3. Embryo exposure

Embryos were gently extracted from 2-days fertilized females as described by Rehm (2009) and 175 embryos per condition were transferred into 25 ceramic well plates, containing 1 ml of UV-sterilized solution at the desired fenoxycarb concentration. Embryos were exposed at 14 ± 1 °C with a 16:8-h light: dark photoperiod for c. 23 days. 50% of the medium were renewed every 3 days. The embryo media consisted of NaCl-enriched synthetic water (8.8 mM NaCl, electrical conductivity = 1600 µS/cm) because preliminary tests (data not shown) showed a better survival of gammarid embryos in this medium. Chorion breakage was considered indicative of the day of hatching. Newborn individuals were removed from theirs wells for phenotypic analysis 2 days after chorion breakage in order to allow for the sclerotization of their exoskeletons.

2.3.1. Experiment 1

Embryos were exposed to increasing fenoxycarb concentrations (0, 0.5, 5 and 50 $\mu g\,L^{-1}$) throughout the entire embryo cycle (from 2 days post-fertilization to 2 days post-hatching).

2.3.2. Experiment 2

To pinpoint sensitive periods of development, we exposed embryos to 5 $\mu g\,L^{-1}$ of fenoxycarb for shorter periods of time: during gastrulation (from 2 days post-fertilization to 13 days post-fertilization), during organogenesis (from 13 days post-fertilization to 22 days post-fertilization) and during hatching (from 22 days post-fertilization to 2 days post-hatching). The hatching period might be particularly sensitive because the chorion may prevent toxic uptake (Hamdoun and Epel, 2007).

2.4. Eye pigmentation

After hatching, 23 individuals per condition were photographed

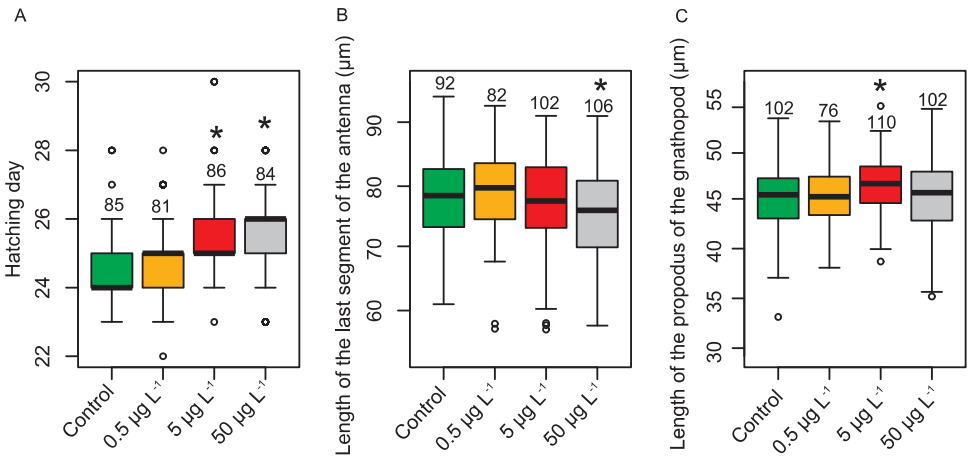

Fig. 1. Day of hatching (A), length of the last segment of antenna 1 (both right and left sides) (B) and length of the propodus of the gnathopod 2 (both right and left sides) (C) in newborn individuals of embryos exposed to increasing concentrations of fenoxycarb. Numbers above the bars represent the sample size. *: significant difference with the control group (Mann Whitney Wilcoxon test, $p < 0.05$).

under a stereomicroscope (Leica, MZ16), equipped with a video camera (Leica, MC 170 HD). To block out ambient light and illuminate the organisms with minimal reflections, the stereomicroscope was equipped with an illuminator dome (LED5000 HDI™). To immobilize the living newborn individuals, they were placed on a microscope slide covered with a coverslip. A photo of the left eye was taken at a 10 magnification. On the eye, several parameters were measured as described by Flores and Chien (2011): the total area (A), the average shape circle factor (Fk), the chroma and the hue values. The Fk characterizes the proximity of the object to a circle (an ideal circle has *Fk = 1*). It was calculated as:

$$Fk = \frac{4\pi A}{p^2}$$

with A = eye area and p = eye perimeter.

Red, green and blue values were obtained from the plugin RGB measure (ImageJ software, Schneider et al., 2012) and converted into the CIELab color space, a color space that has been shown more suitable for human eye color perception (Stevens et al., 2007). The a chromaticity coordinate (+a is defined as red, -a as green) and the b chromaticity coordinate (+b is defined as yellow, –b as bleu) were used to calculate the chroma value, which defined the color intensity:

$$Chroma = \sqrt{a^2 + b^2}$$

The color appearance was described thanks to the hue value:

$$Hue = a \tan 2(b, a)$$

2.5. Histology

Ten new-born individuals were fixed in 4% formaldehyde solution, then washed and desiccated using baths of increasing alcohol concentrations (starting with 70%). Afterwards, the individuals were embedded in paraffin. Serial cross sections of 5 µm were cut and coloured with hematoxylin–eosin.

2.6. FA and inter-individual variance

After fixation in 4% formaldehyde solution, approximately 65 individuals per condition were dissected for morphometric measurements of the antenna and the gnathopod. The length of both the last segment of antenna 1 and the propodus of gnathopod 2 were measured (Appendices, Fig. A.1.). The gnathopod 2 is a sexually dimorphic appendage, required for successful copulation in *Gammarus* spp. (Hume et al., 2005). Analyses of size FA were performed as recommended by Palmer and Strobeck (2003) using a two-way mixed model ANOVA with side (fixed) and individual (random) as factors. This ANOVA also provides a test for directional asymmetry (side effect) and for inter-individual variation (individual effect). Following Palmer and Strobeck (2003), to test for allometric effect, we assessed the relationship between absolute asymmetry and trait size by nonparametric Spearman correlation test. To assess measurement error, all measurements were taken twice. ME5 was used as a measurement error descriptor and FA10b was used as a FA index (Palmer and Strobeck, 2003). FA10b is an index ruling out measurement error and correcting for trait size effect (allometry) by expressing deviation from symmetry as a proportion of the trait mean. Inter-individual variation was measured as the individual mean square (MS) in the ANOVA. Because both FA10b and the individual MS are variance estimates, we used F-test to compare differences in FA and individual variation between the control and the fenoxycarb-exposed groups. Morphometric analyses were carried out with R (R Development Core Team, 2014).

2.7. Statistical analysis

Mortality was analysed with Cox proportional hazards model with right-censoring (meaning that some individuals were still alive at the end of the experiment). Day of hatching was analysed using a mixed effects model with fenoxycarb treatment as fixed factor and well-plates within treatment as random factor. Differences in eye phenotypes and both antenna and gnathopod length were analysed with a non-parametric Mann Whitney Wilcoxon test.

3. Results

3.1. Experiment 1

According to our previous observations (Arambourou et al., in press), the median duration of embryonic development was approximately 24 days in the control group. We observed a significant delay of one day and two days in the 5 µg L^{-1} and 50 µg L^{-1} exposed groups, respectively (ANOVA, 5 µg L^{-1}: $t = 2.1$, $p = 0.03$ and 50 µg L^{-1}: $t = 2.3$, $p = 0.02$) (Fig. 1A). As observed in our previous study (Arambourou et al., in press), the embryo survival cultured ex-marsupium was rather low (approximately 50%) but no significant difference of mortality between the control group and the fenoxycarb-exposed groups was observed (Cox proportional hazard model, all $p \geq 0.05$) (Appendices, Fig. A.2. A).

Despite the variability among individuals of the length of the last antennal segment, a significant reduction was detected at 50 µg L^{-1} (Mann Whitney Wilcoxon test, $W = 5754$, $p = 0.029$) (Fig. 1B) while the length of the propodus of the gnathopod was significantly increased at 5 µg L^{-1} (Mann Whitney Wilcoxon test, $W = 4340$, $p = 0.004$) (Fig. 1C).

Neither the antenna nor the gnathopod exhibited directional asymmetry (F test, all $p \geq 0.05$). Repeatability, expressed as the ME5

Table 1
Results of the two ways ANOVA (side × individual) for the length of the last segment of the antenna and the propodus of the gnathopod: measurement error descriptor (ME5), individual MS and FA level in newborn individuals of embryos exposed to increasing concentrations of fenoxycarb. In bold: significant difference by comparison with the control group (F test, $p < 0.05$).

Last segment of the antenna	ME5 descriptor (%)	Individual MS. 10^{-2} (F test, p value)	FA. 10^{-2} (F test, p value)
Control	99	2.9	5.3
0.5 µg L^{-1}	95	3.1 (0.412)	3.6 (0.111)
5 µg L^{-1}	98	3.5 (0.262)	5.6 (0.423)
50 µg L^{-1}	97	3.2 (0.370)	4.5 (0.282)
Propodus of the gnathopod	ME5 descriptor (%)	Individual MS. 10^{-2} (F test, p value)	FA. 10^{-2} (F test, p value)
Control	93	1.9	5.7
0.5 µg L^{-1}	91	1.5 (0.228)	6.1 (0.410)
5 µg L^{-1}	93	**1.2 (0.037)**	5.5 (0.449)
50 µg L^{-1}	90	3.0 (0.059)	7.7 (0.156)

index, was greater than 90% (Table 1). No difference in FA was detected between the control group and the fenoxycarb-exposed groups neither for the last segment of the antenna nor for the gnathopod propodus (F tests, all $p \geq 0.05$) (Table 1). A significant reduction of inter-individual variability of the gnathopod length was observed in the 5 µg L^{-1} exposed group (F test, $F_{50, 54} = 1.65$, $p = 0.037$; Table 1).

By comparison with the control group, both chroma and hue values were significantly impaired in the 5 µg L^{-1} exposed group (Mann Whitney Wilcoxon test, chroma: $p = 0.02$ and hue: $p = 0.007$) (Fig. 2C and D). In other words, eyes were redder and exhibited a weaker light intensity in this group. No significant difference was observed for eye shape circle factor and eye area between the control group and the fenoxycarb-exposed groups (Pairwise Mann Whitney Wilcoxon tests, all

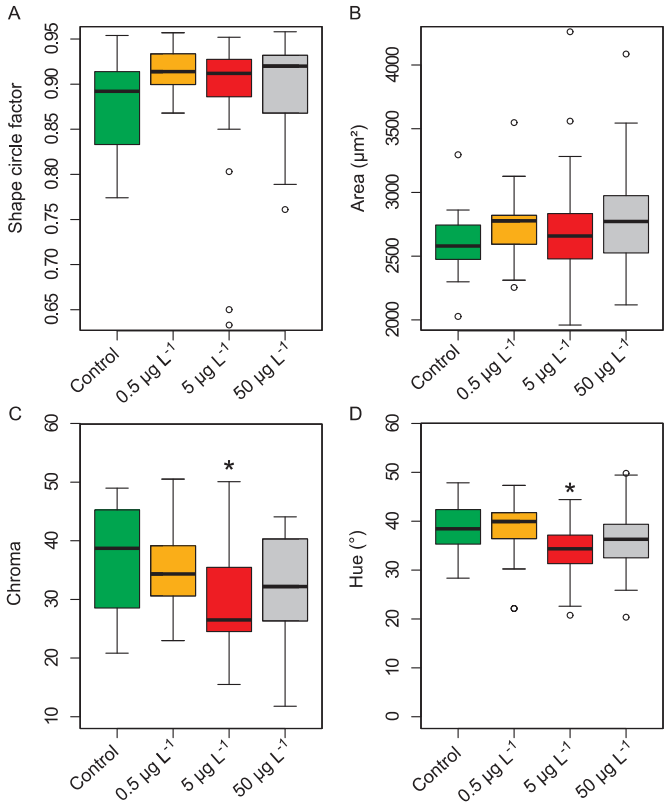

Fig. 2. Eye shape circle factor (A), eye area (B), eye chroma (C) and eye hue (D) in newborn individuals of embryos exposed to increasing fenoxycarb concentrations. *: significant difference with the control group (Mann Whitney Wilcoxon test, $p < 0.05$). Twenty-three individuals per conditions were analysed.

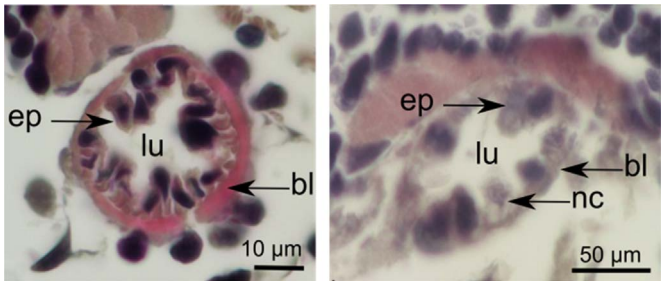

Fig. 3. Midgut with a single-cell layer epithelium (ep) surrounding the lumen (lu) and lined by the basal lamina (bl) in an individual from the control group (A). In the 50 µg L^{-1} exposed group, necrosis (nc) of the epithelial cells and thinning of the basal lamina were observed.

$p \geq 0.05$).

In the control group and in both the 0.5 µg L^{-1} and 5 µg L^{-1} treated groups, the midgut exhibited a well-organised structure with an inner layer of epithelial cells surrounded by a basal lamina. In the 50 µg L^{-1} treated group, necrosis of the epithelial cells and thinning of the basal lamina were observed (Fig. 3).

3.2. Experiment 2

The day of hatching was not significantly different between the control group and the groups exposed to 5 µg L^{-1} of fenoxycarb during specific time windows (ANOVA, all $p \geq 0.05$) (Fig. 4A). The survival rate in the control group was higher than 60%. No significant difference in mortality was observed among groups (Cox proportional hazard model, all $p \geq 0.05$) (Appendices, Fig. A.2. B).

No significant difference in antenna length was detected among groups (Pairwise Mann Whitney Wilcoxon test, all $p \geq 0.05$) (Fig. 4B) while a slight but significant increase in the propodus length was observed in the group exposed to 5 µg L^{-1} during the gastrulation phase (Mann Whitney Wilcoxon test, $W = 4722$, $p = 0.045$; Fig. 4C). A similar effect was observed in the individuals exposed to 5 µg L^{-1} fenoxycarb (experiment 1) throughout the embryonic development.

Neither the antenna nor the gnathopod exhibited directional asymmetry (F test, all $p > 0.05$). Repeatability was greater than 86% (Table 2). No difference in FA or inter-individual variation was detected between the control group and the fenoxycarb-exposed groups, neither for the last antennal segment nor for the propodus of the gnathopod (F tests, all $p \geq 0.05$; Table 2).

By comparison with the control group, chroma was significantly reduced in the group exposed to fenoxycarb during the gastrulation phase (Mann Whitney Wilcoxon test, Chroma: $p = 0.02$) (Fig. 5). In other words, the individuals from this condition exhibited eyes with a weaker light intensity. No significant difference between the control and the fenoxycarb-exposed groups was detected for eye shape circle factor, eye area and eye hue value (Mann Whitney Wilcoxon tests, all $p \geq 0.05$).

4. Discussion

Fenoxycarb exposure delayed the hatching, altered appendages length, induced tissue damages and impaired eye pigmentation in newborn individuals from exposed embryos.

We observed a delayed hatching in both groups exposed to 5 and 50 µg L^{-1} of fenoxycarb throughout embryonic development. This result is consistent with previous studies reporting delayed hatching in other estuarine and marine crustacean species exposed as embryos to juvenile hormone analogs (McKenney and Celestial, 1996; McKenney, 1999; Ghekiere et al., 2007). Delayed hatching might have been caused by the interference of fenoxycarb with hormonal pathways.

Deleterious effects - both reduced antenna length and midgut tissue damages - were observed in the group exposed to 50 µg L^{-1} throughout

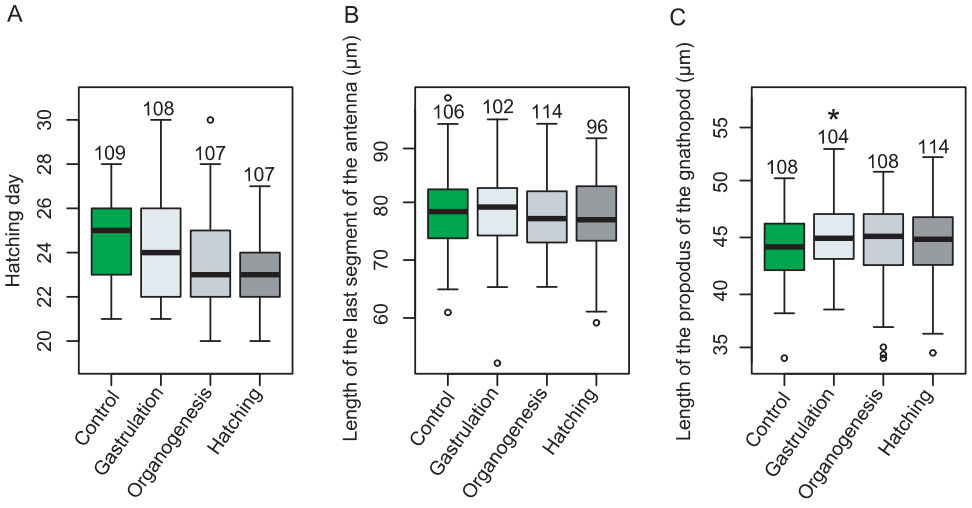

Fig. 4. Day of hatching (A), length of the last segment of antenna 1 (both right and left sides) (B) and length of the propodus of the gnathopod 2 (both right and left sides) (C) in newborn individuals of embryos exposed to 5 µg L^{-1} fenoxycarb during specific time windows. Numbers above the bars represent the sample sizes. *: significant difference with the control group (Mann Whitney Wilcoxon test, $p < 0.05$).

Table 2
Results of the two ways ANOVA (side × individual) for the length of the last segment of the antenna and the propodus of the gnathopod: measurement error descriptor (ME5), individual MS and FA level in newborn individuals of embryos exposed to 5 µg L^{-1} fenoxycarb during specific time windows.

Last segment of the antenna	ME5 descriptor (%)	Individual MS. 10^{-2} (F test, p value)	FA. 10^{-2} (F test, p value)
Control	98	2.5	5.4
Gastrulation	86	2.3 (0.384)	4.5 (0.259)
Organogenesis	98	1.7 (0.079)	4.6 (0.279)
Hatching	99	3.0 (0.253)	5.1 (0.424)
Propodus of the gnathopod	ME5 descriptor (%)	Individual MS. 10^{-2} (F test, p value)	FA. 10^{-2} (F test, p value)
Control	90	1.6	4.6
Gastrulation	92	1.4 (0.317)	5.5 (0.273)
Organogenesis	90	2.2 (0.125)	5.3 (0.314)
Hatching	90	1.7 (0.413)	4.8 (0.442)

the embryonic development. Concentrations lower than 50 µg L^{-1} might have not been sufficient to induce such effects. Reduced size and increased tissue damages have been widely associated with a toxic exposure (Lawrence and Poulter, 2001; Souza et al., 2011), as reported in the decapod *Lithodes Santolla* exposed to heavy metals as embryos (Rodríguez et al., 1998). Alterations of the epithelial cells of the midgut and the hepatopancreas have been observed in several crustaceans exposed to organic compounds (Doughtie and Rao, 1983; Saravana Bhavan and Geraldine, 2000; Bianchini and Monserrat, 2007). Such tissue damages, by hampering the efficiency of food assimilation in juveniles, might have long-term consequences on gammarid population dynamics.

An increase in the size of the propodus - a secondary sexual characteristic - was observed in the group exposed to 5 µg L^{-1} throughout the embryogenesis. A similar pattern was observed in the group exposed to similar concentration during the gastrulation phase, which suggests that this phase is particularly sensitive to fenoxycarb. Interestingly, a similar effect has been observed in the stag beetle: males exposed to fenoxycarb during the prepupal developmental period exhibited an increased length of their mandible, a sexually selected trait (Gotoh et al., 2011). Similarly, female daphnids exposed to the juvenile hormone analog methopren showed a stimulation of the development of a secondary sexual trait (abdominal process) but only at low concentration (Olmstead and LeBlanc, 2001), as was observed in the present study. Note that under fenoxycarb exposure, different patterns of length variation were observed for the last segment of the antenna and the propodus of the gnathopod, suggesting that the development of these two appendages is regulated independently.

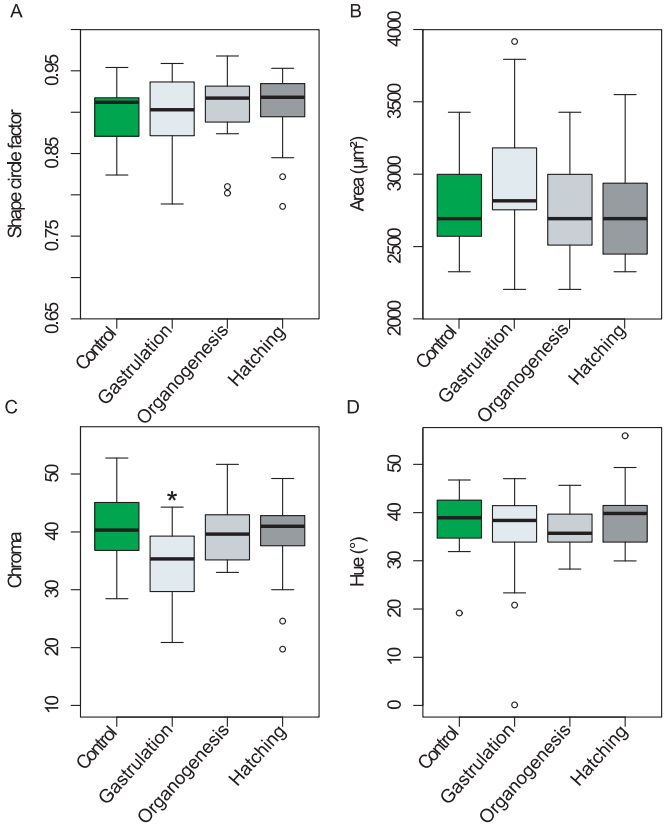

Fig. 5. Eye shape circle factor (A), eye area (B), eye chroma (C) and eye hue (D) in newborn individuals of embryos exposed to 5 µg L^{-1} fenoxycarb during specific time windows. *: significant difference with the control group (Mann Whitney Wilcoxon test, $p < 0.05$). Twenty-three individuals per conditions were analysed.

Juvenile hormones play an important role in arthropod morphogenesis (e.g. Laufer et al., 1997; Rotllant et al., 2000; Nakamura et al., 2007). In line with this, morphological defects have been associated with exposure to juvenile hormone analogs in insects (Licht et al., 2004) and crustaceans (Oda et al., 2011). In the present study, no significant effect of fenoxycarb on antenna and gnathopod length FA was detected and only a limited effect was observed on inter-individual variation. These results suggest that, in *Gammarus fossarum*, fenoxycarb does not interfere with the processes involved in developmental stability and canalization. Several hypotheses can be proposed to explain such limited morphological response. First, it might be due to a low binding affinity of fenoxycarb to gammarid juvenile hormone receptors, as

suggested by a recent study of Miyakawa et al. (2013) in the crustaceans *Daphnia magna* and *Daphnia pulex*. Second, as stated by Hamdoum and Epel (2007), the lack of morphological defects could be partly explained by the protective role of the chorion, which may limit fenoxycarb uptake. Another explanation may be related to the method used in the present study, which might not be powerful enough for detecting subtle morphological changes in such small appendages. Precisely quantifying the shape of these structures - for example using a geometric morphometric analysis of microscopic computed tomography scans - might prove more sensitive than our traditional metric approach (Holdsworth and Thornton, 2002). This alternative – exploratory – analysis would however come at a prohibitive cost both in terms of time and money. Besides, the repeatability of our measurements was good and similar estimates of phenotypic variation have been successfully used in equivalently small traits (e.g. Peluffo et al., 2015), suggesting that the lack of effect reported here is unlikely due to a methodological limitation. Some morphological defects induced by embryonic exposure might be detected at later developmental stages: it would thus be interesting in future studies to assess phenotypic variation in juveniles and adults.

Newborn individuals exposed to 5 μg L^{-1} exhibited redder eye with a weaker color intensity. In line with this, a redder coloration of the cuticle of the white shrimp was observed by Martínez et al. (2014) after copper exposure. Similarly, both body and eye pigmentations were disrupted in the decapod *Chasmagnathus granulatus* exposed as embryos to heavy metals (Zapata et al., 2001; Lavolpe et al., 2004). Given that pigmentation is known to be under endocrine regulation in crustaceans (Fingerman et al., 1998), the eye pigmentation impairment observed at 5 μg L^{-1} might be related to the action of fenoxycarb on the endocrine regulation pathways. The gastrulation phase might be particularly critical for eye pigmentation, as newborn individuals of embryos exposed during this period also exhibited impaired eye pigmentation (weaker color intensity). In line with this, red-pigment-concentrating hormone was shown to be expressed at this stage in the crab *Carcinus Maenas* (Chung and Webster, 2004). In the present study, pigmentation disruption was only detected at 5 μg L^{-1}. Such a non-monotonic response has been widely observed in arthropods exposed to endocrine disruptors (Soin and Smagghe, 2007; Tatarazako and Oda, 2007). Further studies are required to determine whether the eye pigmentation impairment was due to the disruption of endocrine regulation pathways (Fingerman et al., 1998) or to an alteration of the retinoid metabolism which may be involved in eye pigmentation in crustaceans (Feldman et al., 2010). These results could open interesting perspectives for the use of eye pigmentation alteration – which is easy to measure – as a sensitive marker of crustacean endocrine disruption.

5. Conclusions

In conclusion, this study demonstrates that the juvenile hormone analog fenoxycarb is capable of interfering with gammarid embryogenesis (appendages size, eye pigmentation and tissue state) without disrupting developmental homeostasis (i.e. neither canalization nor developmental stability were strongly affected). Further studies are needed to determine whether the lack of severe morphological defects was specific to the juvenile hormone analog used (e.g. low binding affinity of fenoxycarb to crustacean hormonal receptors) or due to the study stage (embryo protected by the chorion). By bringing new insights into the mode of action of toxicants, the gammarid embryogenesis assay could provide a novel and interesting addition to existing bioassays for testing endocrine disruptors in crustaceans.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.06.017.

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