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

Hélène Arambourou[a,*], Arnaud Chaumot[a], Emmanuelle Vulliet[b], Gaëlle Daniele[b], Nicolas Delorme[a], Khédidja Abbaci[a], Vincent Debat[c]

[a] Irstea Lyon, Freshwater Ecosystem, Ecology and Pollution Research Unit, 5 rue de la Doua, 69616 Villeurbanne Cedex, France
[b] Univ Lyon, CNRS, Université Claude Bernard Lyon 1, ENS de Lyon, Institut des Sciences Analytiques, UMR 5280, Villeurbanne, France
[c] Institut de Systématique, Evolution, Biodiversité ISYEB – UMR 7205 – MNHN CNRS UPMC EPHE, Muséum National d'Histoire Naturelle, Sorbonne Universités, Paris, France

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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

* Corresponding author.
E-mail address: helene.arambourou@irstea.fr (H. Arambourou).

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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