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Transcriptome analysis of the copepod *Eurytemora affinis* upon exposure to endocrine disruptor pesticides: Focus on reproduction and development

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ABSTRACT

Copepods—which include freshwater and marine species—represent the most abundant group of aquatic invertebrates. Among them, the calanoid copepod *Eurytemora affinis* is widely represented in the northern hemisphere estuaries and has become a species of interest in ecotoxicology. Like other non-target organisms, *E. affinis* may be exposed to a wide range of chemicals such as endocrine disruptors (EDs). This study investigated the gene expression variation in *E. affinis* after exposure to ED pesticides—chosen as model EDs—in order to (i) improve the knowledge on their effects in crustaceans, and (ii) highlight relevant transcripts for further development of potential biomarkers of ED exposure/effect. The study focused on the reproduction function in response to ED. Copepods were exposed to sublethal concentrations of pyriproxyfen (PXF) and chlordecone (CLD) separately. After 48 h, males and females (400 individuals each) were sorted for RNA extraction. Their transcriptome was pyrosequenced using the Illumina® technology. Contigs were blasted and functionally annotated using Blast2GO®. The differential expression analysis between ED- and acetone-exposed organisms was performed according to sexes and contaminants. Half of the 19,721 contigs provided by pyrosequencing were annotated, mostly (80%) from arthropod sequences. Overall, 2,566 different genes were differentially expressed after ED exposures in comparison with controls. As many genes were differentially expressed after PXF exposure as after CLD exposure. In contrast, more genes were differentially expressed in males than in females after both exposures. Ninety-seven genes overlapped in all conditions. Finally, 31 transcripts involved in reproduction, growth and development, and changed in both chemical exposures were selected as potential candidates for future development of biomarkers.

1. Introduction

Copepods, which include 14,500 freshwater and marine species, represent the most abundant group of aquatic invertebrates (Huys and Boxshall, 1991; Humes, 1994; Boxshall, 2011). Because of (i) their place as major secondary consumers in aquatic food webs and (ii), their ability to bio-accumulate contaminants, they contribute to the transfer of pollutants to higher trophic levels (DiPinto, 1996; Fisk et al., 2001; Hoekstra et al., 2003; Stewart and Fisher, 2003; Covaci et al., 2006; Cailleaud et al., 2007a; Cailleaud et al., 2007b). Copepods are widely used for chemical testing because of their relatively small size and short life cycle. They represent promising model organisms for ecotoxicology (Raisuddin et al., 2007; Kulkarni et al., 2013).

Among the copepod taxon is the order Calanoida, which includes around 5,000 species most of which are planktonic (Boxshall, 2011; Bron et al., 2011). The calanoid copepod *Eurytemora affinis* is widely represented in the northern hemisphere estuaries and dominates the zooplankton population in the Seine estuary (France; (Mouny

Abbreviations: ED, endocrine disruptor; PXF, pyriproxyfen; CLD, chlordecone; PPDE, posterior probability differentially expressed.

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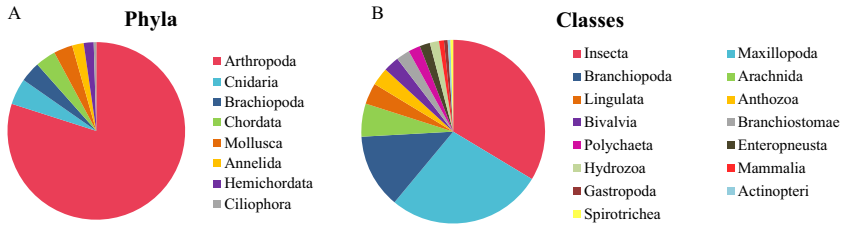

Fig. 1. Taxonomic distribution of top BLAST hit species. A, phylum-based assignment; B, class-based assignment. Species representative of at least five BLAST top hits in protein ortholog searching (nr base, BLASTx, Blast2GO®) were considered.

and Dauvin, 2002; Devreker et al., 2010). Despite the fact that its use in ecotoxicological studies has increased during the past decade (Forget-Leray et al., 2005; Lesueur et al., 2013; Boulangé-Lecomte et al., 2014; Lesueur et al., 2015), a lack of knowledge on gene expression in *E. affinis* remains. Until recently, sequencing was an expensive technique, unsuitable for non-model organisms. However, advances in next-generation sequencing (NGS) techniques such as RNA-seq will allow the production of whole genome and transcriptome sequences at a cheaper cost for diversified applications (Ekblom and Galindo, 2011). This evolution of technology has led to the genome and transcriptome exploration in non-model organisms such as copepods (Ekblom and Galindo, 2011; Riesgo et al., 2012; Valenzuela-Muñoz et al., 2015). Furthermore, in the field of ecotoxicology, sequencing progress has enhanced research into sub-individual effects of chemical compounds, and allows improved understanding of organism responses at a molecular level.

As non-target organisms, copepods are exposed—like other invertebrates—to a large range of chemical compounds. Agriculture products, e.g. pesticides, can particularly impact estuaries by contaminating and potentially damaging species such as *E. affinis* (Cailleaud et al., 2009; Tan et al., 2009; Cailleaud et al., 2011; Ernst et al., 2014). Some pesticides are known to act as endocrine disruptors (ED). EDs are defined as 'exogenous substances that cause adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function' (WHO and UNEP, 2013) and can cause adverse effects on reproduction, growth and development, either through a perturbation of production or biotransformation of a hormone, or by affecting the binding of a hormone to its receptor (Rodríguez et al., 2007). Among ED pesticides, pyriproxyfen (PXF) acts as a juvenile hormone agonist (Sullivan and Goh, 2008), whereas chlordecone (CLD) is an oestrogen agonist (Okubo et al., 2004). In the United States, PXF has been the most used insecticide for controlling California red scale (*Aonidiella aurantii*), the silverleaf whitefly (*Bemisia argentifolii*) and the red imported fire ant (*Solenopsis invicta*) in citrus, cotton, vegetable and peanut crops (Sullivan and Goh, 2008). CLD was widely used as an insecticide against leaf-eating insects, ants, and cockroaches, and as a larvicide for flies in citrus trees, tobacco plants and lawns. Despite its prohibition in 1976 in the United States, it was extensively applied against the banana weevil in the French Indies from 1972 to 1993 due to dispensations (Dromard et al., 2016). Biomarkers are interesting tools to study the ED mechanism of action and to detect early responses of organisms. They have been widely investigated in vertebrate species, particularly by using the vitellogenin measurement (Scholz and Mayer, 2008). The relevance of such biomarkers has been measured in invertebrate species (Jubeaux et al., 2012). However, to date, the panel of ED biomarkers remains poor in invertebrates.

We investigated the sequencing of the whole transcriptome of *E. affinis* after exposures to model pesticides i.e. PXF and CLD using the Illumina® technology in order to 1) examine the mode of action of ED pesticides on both reproduction and development and 2) identify candidate transcripts impacted by EDs which could *in fine* constitute potential molecular biomarkers. As sex-related analysis appears to be a major concern in transcriptomics (Farlora et al., 2014), the transcriptomes of both sexes were sequenced. Differential expression analyses made it possible to identify sex-specific and compound-specific patterns.

2. Materials and methods

2.1. *E. affinis* sampling and stalling

E. affinis copepods were sampled in the oligo-mesohaline zone of the Seine estuary (49°28′19.24″/0°27′55.303′; Haute-Normandie, France). As previously described (Lesueur et al., 2015), copepods were collected with a WP2 plankton net (200 μm mesh size; 1 m diameter) in the sub-surface part of the water column. Samples were directly passed through two sieves (500- and 100 μm mesh size) to eliminate large particles and predators. The collected copepods were kept in estuarine water in isotherm containers and brought back to the laboratory. To eliminate any remaining fragments, the copepods were sieved again (100 μm sieve) and washed in artificial brackish water (a mixture of UV-treated filtered (1 μm) sea water and deionized water; salinity 10). Then, they were maintained in a culture chamber under optimal conditions (aeration, salinity 15, 15 °C, 12:12 light: dark photoperiod (Devreker et al., 2009)). They were fed ad libitum with *Isochrysis galbana* algae (approximately 15,000 cells/mL; Aquacaux, Octeville sur Mer, France). Before any experiments were performed, the copepods were depurated for at least 3 days in artificial brackish water (Cailleaud et al., 2011).

2.2. Chemical exposure of *E. affinis*

Pyriproxyfen (PXF, CAS No. 95737-68-1)—also referred to as Pestanal®—and chlordecone (CLD, CAS No. 143-50-0)—also referred to as Kepone—were purchased as analytical standards from Sigma-Aldrich (Saint-Quentin Fallavier, France). Stock solutions were prepared in acetone (Chromasolv®, Sigma-Aldrich) in such a way that the final solvent concentration in ED exposures was 0.005% w/v. The solvent control exposure was conducted under the same conditions as ED exposures, with a final acetone concentration of 0.005% w/v. All glass tanks were saturated with artificial brackish contaminated water for a week to avoid contaminant adsorption during further experiments. After depuration, the copepods were divided into three 10 L tanks of fresh contaminated water. The copepods were exposed in optimal conditions (aeration, salinity 15, 15 °C, 12:12 light:dark photoperiod) to sub-lethal concentrations of pesticides. The sub-lethal concentrations (i.e. 10 μg/L) were pinpointed from acute toxicity tests performed according to the International Organization for Standards (ISO14669, 1999); data not shown). After 48 h, for each condition, 400 adult females (stage 1, (Boulangé-Lecomte et al., 2014) and 400 adult males were pooled from the three replicates (135 individuals per replicate). The animals were then quickly washed in distilled water and stored at −80 °C until used. A pool