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Effect of psychiatric drugs on *Daphnia magna* oxylipin profiles

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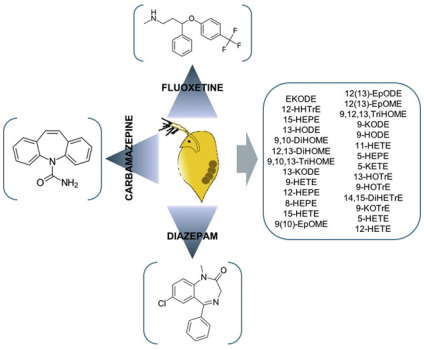
HIGHLIGHTS

- Oxylipin profiles in *D. magna* adults upon exposure to low concentrations of psychiatric drugs were studied.
- Up to 28 different oxylipins belonging to arachidonic, linoleic and eicosapentaenoic acid metabolic pathways were altered.
- Fluoxetine increased the accumulation of the prostaglandin product 12-HHTrE.
- Diazepam increased the concentration of 12 eicosanoids and other oxylipins.
- Carbamazepine had little effects only effecting one oxylipin from the linoleic acid metabolism.

GRAPHICAL ABSTRACT



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ABSTRACT

Neuro-active pharmaceuticals have been reported to act as endocrine disruptors enhancing reproduction in the model crustacean *Daphnia manga* at environmental concentrations of ng/L. Oxylipins and more specifically eicosanoids, which are lipid mediators formed from polyunsaturated fatty acids (PUFAs), are known to regulate reproduction together with other physiological processes in insects. In *D. magna*, the biosynthesis of eicosanoids and their putative role in the regulation of reproduction has been studied using transcriptomics, genomics and exposures to cyclooxygenase inhibitors. Quantification of eicosanoids and oxylipins derived from PUFAs upon exposure to pharmaceuticals is therefore crucial for a better understanding of the mode of action of neuro-active pharmaceuticals on aquatic invertebrates. The aim of this study was to investigate shifts in the oxylipin profile in *D. magna* adults upon exposure to environmental concentrations of the three psychiatric drugs, fluoxetine, diazepam and carbamazepine, with known effects of enhancing offspring production. Oxylipin profiles were determined in whole organism tissues using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Up to 28 different oxylipins belonging to arachidonic (AA), linoleic acid (LA), α-linoleic acid (α-LA) and eicosapentaenoic acid (EPA) pathways were detected and quantified in *D. magna* adults. Exposure to the selected psychiatric drugs showed that fluoxetine enhanced the accumulation of the cyclooxygenase (COX) product 12-

Abbreviations: AA, arachidonic acid; COX, cyclooxygenases; CYP, cytochrome 450; DiHETrE, dihydroxy-eicosatrienoic acids; DiHOMEs, dihydroxy-octadecenoic acids; EKODEs, epoxy-keto-octadecenoic acids; EPA, eicosapentaenoic acid; EpETrEs, epoxy-eicosatrienoic acids; EpODEs, epoxy-octadecadienoic acids; EpOMEs, epoxy-octadecenoic acids; ESI, electrospray ion source; HEPEs, hydroxyl-pentaenoic acids; HETEs, hydroxyl-eicosatetraenoic acids; HODEs, hydroxyl-octadecadienoic acids; HOTrEs, hydroxyl-octadecatrienoic acids; KODEs, oxo-octadecadienoic acids; KOTrEs, oxo-octadecatrienoic acids; LA, linoleic acid; LOX, lipoxygenases; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; PGH_2, prostaglandin H_2; PGs, prostaglandins; PUFA, polyunsaturated fatty acid; QC, quality control; SPE, solid phase extraction; SSRI, selective serotonin re-uptake inhibitor; TriHOMEs, trihydroxy-octadecenoic acids; TXs, thromboxanes; α-LA, alpha-linoleic acid.

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hydroxyheptadecatrienoic acid (12-HHTrE), whereas diazepam increased the concentration of eicosanoids belonging to the lipoxygenase (LOX) and cytochrome P450 (CYP) pathways (HETEs, EpOMEs, HODEs, HOTrEs and HEPEs) from the AA, LA, αLA and EPA pathways. Carbamazepine had little effect and only one LA-derived compound from the LOX pathway (13-HODE) increased significantly. This means that despite having different modes of action in humans, fluoxetine and diazepam up-regulated eicosanoid pathways in *D. magna*, closely related to known biologically active products that regulate reproduction in insects.

1. Introduction

There is an urgent need for assessing the risks of long-term exposure to low doses of human prescribed pharmaceuticals (Fent et al., 2006). Waste water treated and untreated effluents are the main source of pharmaceuticals and/or their metabolites to water. Consequently, pharmaceuticals are continuously released into the environment and thus their negative effects are independent from their persistence in the environment (Fent et al., 2006; Petrović et al., 2003).

Antidepressants and anxiolytic drugs deserve special attention due to their usage and environmental persistence. Surveys in US have reported levels of 12–540 ng/L of fluoxetine, the active ingredient of Prozac, in surface waters and effluents (Kolpin et al., 2002) and total concentrations of serotonin reuptake inhibitors in aquatic systems were measured in the range of 840 ng/L to 3.2 μg/L (Metcalfe et al., 2010; Vasskog et al., 2008). Diazepam, is widely used to treat anxiety (Riss et al., 2008). Concentrations of diazepam ranging from 4 to 40 ng/L have been found in Spanish urban rivers (Valcárcel et al., 2012). Carbamazepine is a medication used primarily in the treatment of epilepsy and neuropathic pain (Ambrósio et al., 2002). Carbamazepine is fairly persistent in water and hence can be found at concentrations ranging from 1 to up to 3000 ng/L in rivers receiving waste water treatment effluents (Muñoz et al., 2009; Tixier et al., 2003).

Recent studies have reported that, at environmentally relevant concentrations, antidepressants and anxiolytic drugs alter the behavior of fish, mollusks and crustaceans (Ford and Fong, 2016). The targets of these compounds are highly conserved across vertebrates and they are also found in the invertebrate crustacean *D. magna* (Gunnarsson et al., 2008). Previous studies have reported that the selective serotonin re-uptake inhibitor fluoxetine increased brain serotonin activity in *D. magna*, and increased reproduction and positive phototactic behavior at as low concentrations as 100 ng/ L (Garreta-Lara et al., 2016; Rivetti et al., 2016). There is a cross-talk between serotonin receptors and arachidonic/prostaglandin (AA/PGs) metabolism (Tournois et al., 1998), which regulate reproduction in insects (Spracklen and Tootle, 2015). Thus, it is possible that fluoxetine, by increasing serotonin activity up-regulates AA/PGs metabolism, and hence increases reproduction. In decapod crustaceans, exposure to serotonin or serotonergic drugs increased the levels of the crustacean hyperglycemic hormone, which regulates energy metabolism (Santos et al., 2001), anxiety-like and aggressive behavior (Fossat et al., 2014; Kravitz, 2000) and stimulated ovarian maturation (Sarojini et al., 1995). Furthermore, it has been recently reported that the psychiatric drugs carbamazepine and diazepam at low concentrations (100 ng/ L) also increased reproduction and alter phototactic behavior in *D. magna* (Rivetti et al., 2016). The main target of carbamazepine is blocking voltage-dependent sodium channels (Ambrósio et al., 2002), but there is reported information indicating that carbamazepine also causes increases in extracellular serotonin levels (Dailey et al., 1997). Accordingly, carbamazepine may also act like fluoxetine increasing serotonin activity and hence PGs synthesis and reproduction. Diazepam acts at the GABA signaling pathway, which has been related to the expression of predatory induced life-history defenses in *D. magna* like growth, timing and reproductive output (Weiss et al., 2012). In rats, diazepam alters dopamine (Biswas and Carlsson, 1978), which also modulates reproduction and behavior in crustacean (Tierney et al., 2003), and PGs (Labhsetwar and Zolovick, 1973).

Oxygenated lipids, collectively known as oxylipins, are endogenous signaling molecules that are highly diverse in terms of their structure, metabolism and biological function, including cell proliferation, inflammation, immune actions and blood pressure regulation (Buczynski et al., 2009). Oxylipins are derived from polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), linoleic acid (LA), α-linoleic acid (α-LA) and eicosapentaenoic acid (EPA by COX (cyclooxygenases) and LOX enzymes (lipoxygenases) or by cytochrome P450 (CYP) forming a complex pool of bioactive components (Barquissau et al., 2017; Lundstrom et al., 2011; Zhao et al., 2014). Oxylipins derived from the different AA, LA, α-LA and EPA pathways are shown in Fig. 1. Prostanoids, including prostaglandins (PGs) and thromboxanes (TX), are generated from AA via the initial oxidation through COX pathways (Stanley, 2006). Leukotrienes are generated via the LOX pathways. LOX-dependent products include LOX hydroperoxidases families such as the AA-derived hydroxy-eicosatetraenoic acids (HETEs), as well as the LA-derived hydroxy-octadecadienoic acids (HODEs), α-LA-derived hydroxy-octadecatrienoic acids (HOTrEs) and EPA-derived hydroxy-pentaenoic acids (HEPEs) (Strassburg et al., 2012) (Fig. 1). CYP enzymes can convert AA and LA via epoxygenase activity into epoxy-eicosatrienoic acids (EpETrEs) and epoxy-octadecenoic acids (EpOMEs), respectively. These epoxygenase products from AA and LA are rapidly metabolized via soluble epoxide hydrolase (sEH) to their corresponding diols, dihydroxy-eicosatrienoic acids (DiHETrEs) and dihydroxy-octadecenoic acids (DiHOMEs) (Schmelzer et al., 2005). In addition, CYP epoxygenase can also metabolize α-LA-derived resulting in epoxy-octadecadienoic acids (EpODEs) (Gabbs et al., 2015). In *D. magna*, eicosanoids act as important lipid mediators in reproduction, the immune system and ion transport (Heckmann et al., 2008). In addition to the well-known eicosanoids derived from AA, LA together with EPA are essential fatty acids for growth and reproduction in *D. magna* (Martin-Creuzburg et al., 2010).

To date, few studies have investigated PUFA-derived *D.magna* oxylipins levels. The quantification of these chemically and structurally related oxylipins derived from different PUFAs has become a challenge, but the development of LC-MS/MS-based methods is enabling this possibility. Analytical methods were developed in order to detect and quantify a large number of eicosanoids and other oxylipins in several biological matrices (Checa et al., 2015; González-Peña et al., 2017; Lundström et al., 2011; Strassburg et al., 2012). Additional biological information can be obtained by performing chiral-based analyses on theS and R isoforms of oxylipids (Balgoma et al., 2013). Equalamounts of isoforms S and R or an excess of the former means, respectively, that oxylipins are produced enzymatically or by autooxidation (Willenberg et al., 2015). Until now, no mass spectrometric verification has been performed for eicosanoids and other oxylipins in *daphnids*. These lipid mediators are present at very low levels in endogenous systems (Balgoma et al., 2013) and therefore require targeted methods for

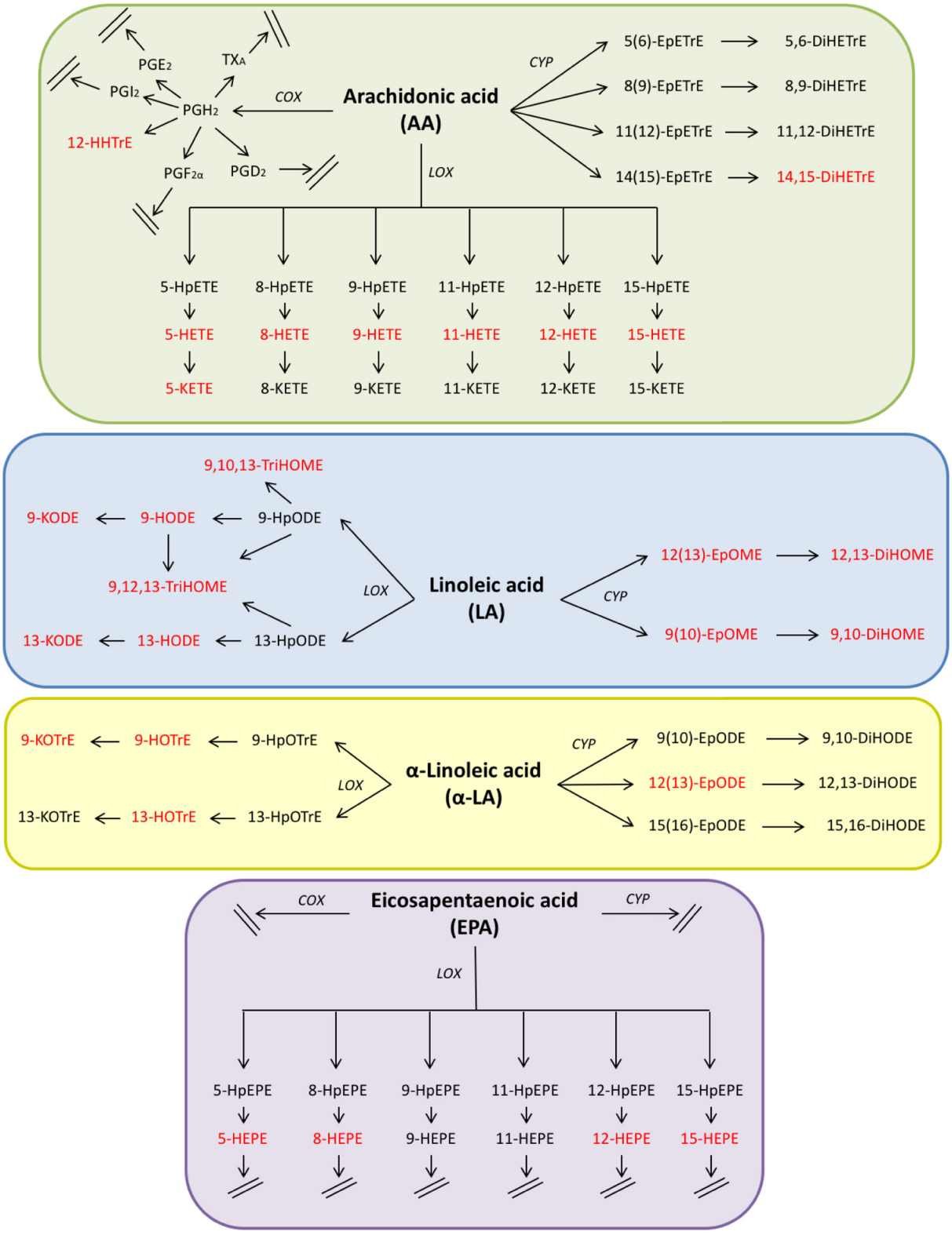

Fig. 1. Outline of the oxylipins biosynthesis pathways derived from (AA, *green*), linoleic acid (LA, *blue*), α-linoleic acid (α-LA, yellow) and eicosapentaenoic acid (EPA, *purple*) via cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome 450 (CYP) pathways. Oxylipins marked in red were detected and quantified in *D. magna*. Diagram modified from Gabbs et al. (2015). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

quantification. Determining their biological activities, as well as their profiles in *D. magna,* will increase our understanding and could have very important implications for several areas of ecological risk assessment.

Using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), the aim of the present study was to characterize the alterations induced by fluoxetine, carbamazepine and diazepam in the *D. magna* eicosanoid and other oxylipin profiles.

2. Materials and methods

2.1. D. magna growth conditions

All experiments were performed using the well-characterized clone F of *D. magna* maintained indefinitely as pure parthenogenetic cultures (Barata and Baird, 1998). Photoperiod was set to 16 h light: 8 h dark cycle and temperature at 20 ± 1 °C. The culture medium was changed every other day. Bulk cultures of 10 animals in 1 L were maintained in ASTM hard water at high food ration levels (5×10^5 cells mL^{-1} of *Chlorella vulgaris*), as described in Barata and Baird (1998).

2.2. Exposure assays

Exposure assays were performed using fluoxetine hydrochloride (CAS-No. 56296-78-7; analytical standard, purity 100%, Sigma-Aldrich), carbamazepine (CAS-No. 298-46-4; analytical standard, purity 99%, Sigma-Aldrich) and diazepam (CAS-No. 439-14-5; analytical standard, purity 99%, Sigma-Aldrich). Due to the high number of animals needed to carry out the tests (60 adults per treatment) two independent assays were performed, with one control group for each assay: (i) fluoxetine and carbamazepine and (ii) diazepam. Experiments were initiated with neonates <14 h old collected from third brood females from the bulk cultures described above. Animals were exposed to the studied drugs in triplicate in groups of 20 individuals in 2 L glass vessels at a concentration of 100 ng/ L in ASTM at high food. The studied drugs were added directly in water from stock solutions of 100 μg/L prepared in nanopure Milli Q water. An additional three 2 L glass vessels filled with 20 animals without drugs were used as control treatments. Media was changed every other day. Remaining culture conditions were similar to those depicted above. Animals were maintained until they released the first brood of eggs into their brood pouch (8–10 days). Then, animals were brooded by gently flushing water into their brood pouch, pooled into eppendorfs, snap-frozen in liquid N_2 and stored at −80 °C until extraction.

2.3. Extraction protocol

Oxylipins and their respective internal standards were purchased from Cayman (Ann Arbor, MI, USA). For the extraction of oxylipins, 10 μL of internal standards solution was spiked to the pools of 20 *daphnids* and dissolved in 400 μL methanol (MeOH). Homogenization was performed using stainless steel beads (0.9– 2.0 mm of diameter) added to each sample, and shaken for 6 min with a Digital Cell Disruptor supplied by Scientific Industries. Samples were sonicated in an ultrasound bath for 20 min, centrifuged at 10,000*g* for 5 min at 4 °C and the supernatant was withdrawn. *Daphnids* were then re-extracted with 350 μL of a mixture of MeOH:$CHCl_3$:H_2O (4:2:1), following the above described procedure. Finally, the supernatants were combined and evaporated to dryness under nitrogen gas. The sample extract was then purified using solid phase extraction as previously described by Balgoma et al. (2016) with some modifications. Briefly, 1 mL of extraction buffer (Na_2HPO_4:citric acid pH = 5.6) was added to the dry extracts and automated solid phase extracted (SPE) on an Extrahera™ automated extraction system (Biotage, Uppsala, Sweden) using ABN Biotage cartridge columns (60 mg, 3 mL, Biotage). The resulting fraction was concentrated under nitrogen gas and reconstituted with 70 μL of MeOH and 10 μL of double-deionized water. Extracts were filtered through 0.1 μm membrane spin filters (Merck Millipore, Darmstadt, Germany) before UPLC-MS/MS analysis. In addition, two quality controls (QC) were pooled from *daphnids* samples, extracted as the samples, and then used to control for quantification reproducibility and batch extraction effects.

2.4. UPLC-MS/MS quantification of oxylipins

The chromatographic separation was performed on an Acquity UPLC BEH C18 column equipped with a pre-column (ACQUITY UPLC BEH C18 VanGuard Pre-column, 1.7 μm, 2.1 mm × 5 mm) (Mildford, US). Oxylipins were determined using a Xevo TQ-S mass spectrometer (Waters) equipped with an Electrospray Ion Source (ESI) operated in negative ionization mode. The details of the chromatographic and MS conditions have been previously published (Balgoma et al., 2016). For quantification purposes, a calibration curve was prepared by spiking 10 μL of the internal standard mixture to 10 calibration levels. A calibration curve was injected at the beginning and at the end of the sequence. Linear regression curves with 1/x weighting were built using the ratio between each compound and its respective internal standard. A list of detected oxylipins and their selected reaction monitoring (SRM) transitions is provided in Table SI1.

A chiral LC-MS/MS method was used for the separation and identification of the 9-hydroxy-octadecadienoic acid (9-HODE) and 13-hydroxy-octadecadienoic acid (13-HODE) enantiomers to determine if the origin was enzymatic (*S*) or non-enzymatic (*R*) in the samples analyzed. For chirality determination, separation was carried out on an Acquity UPLC separation system using a Chiralpak® AD-RH column (150 × 2.1 mm, 5 μm) from Daicel Corporation (France). A linear gradient was set between 0 and 6.3 min from 40% of mobile phase A (0.1% of acetic acid in water) to 98% of mobile phase B (Acetonitrile:Isopropanol, 90/10 (v/v)). The flow rate and column oven were set to 0.6 mL min^{-1} and 40 °C, respectively. The MS was operated in the negative ionization mode. The SRM transitions for the compounds measured are reported in Table SI1.

2.5. Statistical methods

Effects of fluoxetine and carbamazepine treatments relative to unexposed controls on concentration levels of oxylipins were assessed by one-way ANOVA analyses followed by Dunnett's post-hoc tests. For diazepam Student's *t*-test were used to compare treatment effects. Hierarchical clustering of fold changes relative to controls of oxylipins across treatments was performed using the Euclidean distance. The software used in this work includes MassLynx, TargetLynx (Waters, Milford, MA) and Graph Pad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA). ANOVA, Student's *t*-test and clustering analyses were performed using the Statistics toolbox under MATLAB 2015a computer and visualization environment (The Mathworks Inc. Natick, MA, USA).

3. Results

3.1. Detection of oxylipins in D. magna

A total of 28 oxylipins derived from different PUFAs were detected and quantified in *D. magna* controls as well as in samples exposed to fluoxetine, carbamazepine and diazepam (Table 1). Most oxylipins detected and quantified were biosynthesized via the COX, LOX and CYP enzymatic pathways (Fig. 1). These included seven oxylipins from the AA: 12-hydroxy-heptadecatrienoic acid (12-HHTrE) formed by COX enzymes (Gabbs et al., 2015) and HETEs derived from the LOX pathways (e.g., 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE and 15-HETE). From the LA, α-LA and EPA pathways measured oxylipins included derivatives of the LOX pathways: HODEs, oxo-octadecadienoic acids (KODEs), HOTrEs -octadecatrienoic acids (KOTrEs) and HEPEs (e.g., 5-HEPE, 8-HEPE, 12-HEPE and 15-HEPE). In addition, epoxide products from the CYP epoxygenase-dependent metabolism of AA (e.g., 14,15-DiHETrE), LA (e.g., 12(13)-EpOME to 12,13-DiHOME, and 9(10)-EpOME to 9,10-DiHOME) and α-LA (e.g., 12(13)-EpODE) were detected (Table 1). A epoxy-keto-octadecenoic acid derivative (EKODE) belonging to the La pathway was also detected. The abundance for LA-derived oxylipins was found to be higher than AA-derived oxylipins.

Table 1
Concentrations of quantified oxylipins in *D. magna*. Control (*i*) refers to the fluoxetine and carbamazepine control samples, and control (*ii*) refers to diazepam control samples. An oxylipin nomenclature list is provided in Table SI1.

PUFA	Oxylipins	Control (*i*) (ng/*daphnia* ± SD)	Fluoxetine (ng/*daphnia* ± SD)	Carbamazepine (ng/*daphnia* ± SD)	Control (*ii*) (ng/*daphnia* ± SD)	Diazepam (ng/*daphnia* ± SD)
AA	5-KETE	2.94 ± 0.17	3.03 ± 0.18	3.88 ± 0.32	2.03 ± 0.05	3.89 ± 0.13
	12-HHTrE	3.24 ± 0.23	7.27 ± 0.15	4.08 ± 0.33	6.83 ± 0.35	5.59 ± 0.26
	14,15-DiHETrE	0.55 ± 0.03	0.74 ± 0.03	0.58 ± 0.04	0.35 ± 0.02	0.70 ± 0.04
	9-HETE	2.54 ± 0.25	2.80 ± 0.07	3.01 ± 0.13	2.52 ± 0.06	3.10 ± 0.26
	8-HETE	2.95 ± 0.05	3.27 ± 0.18	4.08 ± 0.15	1.82 ± 0.05	2.62 ± 0.12
	5-HETE	5.35 ± 0.11	5.91 ± 0.10	5.88 ± 0.33	3.21 ± 0.11	6.97 ± 0.15
	11-HETE	2.08 ± 0.04	2.45 ± 0.08	2.38 ± 0.18	1.42 ± 0.01	2.28 ± 0.09
	15-HETE	2.50 ± 0.02	4.06 ± 0.22	3.85 ± 0.25	1.99 ± 0.12	3.30 ± 0.05
	12-HETE	1.34 ± 0.05	1.57 ± 0.05	1.87 ± 0.10	0.54 ± 0.02	1.82 ± 0.07
	EKODE	10.83 ± 0.59	33.19 ± 2.85	18.75 ± 0.80	15.84 ± 0.59	33.93 ± 2.88
LA	9-KODE	83.32 ± 1.63	116.64 ± 5.39	93.69 ± 3.19	84.39 ± 1.82	139.36 ± 4.22
	13-KODE	40.58 ± 1.87	51.23 ± 1.37	47.07 ± 2.04	37.52 ± 1.13	51.40 ± 1.54
	9(10)-EpOME	13.99 ± 0.50	25.69 ± 3.12	17.56 ± 0.83	20.84 ± 0.47	31.87 ± 0.42
	12(13)-EpOME	11.60 ± 0.54	17.61 ± 2.15	13.27 ± 0.53	14.05 ± 0.15	19.73 ± 0.14
	9,12,13-TriHOME	5.27 ± 0.33	7.39 ± 0.27	5.02 ± 0.30	4.54 ± 0.46	7.28 ± 0.73
	9,10-DiHOME	15.27 ± 0.30	18.68 ± 0.64	16.34 ± 0.14	16.48 ± 0.57	17.14 ± 0.14
	9,10,13-TriHOME	12.94 ± 1.25	16.42 ± 0.19	12.90 ± 0.57	12.87 ± 1.19	16.53 ± 0.56
	12,13-DiHOME	13.19 ± 0.34	14.88 ± 0.77	13.78 ± 0.21	14.14 ± 0.37	13.91 ± 0.19
	9-HODE	89.03 ± 4.85	121.73 ± 6.05	102.48 ± 2.49	84.67 ± 0.85	133.19 ± 2.54
	13-HODE	72.02 ± 1.58	106.28 ± 1.87	92.11 ± 1.77	72.70 ± 0.94	78.79 ± 1.44
	13-HOTrE	27.67 ± 1.76	39.14 ± 1.58	34.88 ± 0.91	24.62 ± 0.46	45.20 ± 1.03
α-LA	9-KOTrE	14.25 ± 1.82	19.39 ± 1.80	14.08 ± 1.10	13.77 ± 0.40	28.61 ± 0.42
	9-HOTrE	47.12 ± 6.21	62.72 ± 5.48	49.34 ± 3.13	42.44 ± 1.95	83.02 ± 0.73
	12(13)-EpODE	2.17 ± 0.08	3.98 ± 0.23	2.75 ± 0.12	3.11 ± 0.10	4.27 ± 0.10
	8-HEPE	1.37 ± 0.05	1.44 ± 0.08	2.04 ± 0.12	0.83 ± 0.05	0.96 ± 0.05
EPA	15-HEPE	1.19 ± 0.01	1.97 ± 0.08	1.70 ± 0.10	1.31 ± 0.10	1.23 ± 0.09
	12-HEPE	0.93 ± 0.05	1.09 ± 0.09	1.37 ± 0.16	0.88 ± 0.06	0.97 ± 0.03
	5-HEPE	2.55 ± 0.07	2.50 ± 0.11	2.94 ± 0.25	1.64 ± 0.03	2.90 ± 0.06

The quantified oxylipins are mapped in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa et al., 2014) for AA, LA and α-LA in *Daphnia pulex* (see Supplementary Figures SI1, SI2 and SI3, respectively), in order to contextualize them in the biology of *D. magna*. As it can be observed, most of the oxylipins are end products of the pathways. However, not all the oxylipins appear in the KEGG database and therefore could not be represented, such as 12-HHTrE for AA pathway, 9-KOTrE for the LA pathways and 12(13)-EpODE for the α-LA pathway.

The abundance of some *S* and *R* stereoisomers of monohydroxy lipid mediators was determined using chiral chromatography. Due to the low abundance of most of these compounds, chirality could only be determined for 9- and 13-HODE. For both compounds, the *S* was the more predominant form, with an enantiomeric excess between 44% and 66% for 9-HODE and between 14% and 30% for 13-HODE. Results for the specific treatments are detailed in Table 2.

3.2. Effects of anti-depressant pharmaceuticals

Fold changes of quantified oxylipins are given in Fig. 2, where more significant ones ($p < 0.05$) have been marked. Of the three tested drugs, diazepam produced the most pronounced changes in oxylipin levels (Fig. 2). Increased levels ($p < 0.05$, Student *t*-tests) were found for the AA-derivatives, 5-KETE, 5-HETE and 12-HETE, LA-derivatives, 9-KODE, 9(10)-EpOME, 12(13)-EpOME and 9-HODE, α-LA derivatives, 9-HOTrE, 13-HOTrE, 9-KOTrE and 12(13)-EpODE and the EPA-derivative 5-HEPE. Exposure of *D. magna* with fluoxetine led only to increases ($p < 0.05$, ANOVA) in 12-HHTrE. Only the LA-derivative 13-HODE increased after carbamazepine exposure ($p < 0.05$, ANOVA) (Fig. 2).

Heat map representation of fold changes of oxylipins across treatments is shown in Fig. 3. As illustrated in Fig. 3, hierarchical clustering separated exposure treatments fluoxetine and carbamazepine from diazepam. The oxylipins EKODE and 12-HETE were represented in red, with fold changes values higher than 3, for fluoxetine and diazepam treatments, respectively. Fluoxetine showed elevated fold changes for EKODE, 12-HHTrE, 9(10)-EpOME and 12(13)-EpODE, whereas carbamazepine did so for EKODE and 15-HETE. Diazepam treatment increased levels of some AA, LA, EPA and most of the detected α-LA-derivatives being the exception 12(13)-EpODE, whose fold change was around 1.

4. Discussion

In the present study, we used a comprehensive UPLC-MS/MS method to determine changes in eicosanoids and other oxylipins in *D. magna* exposed to three different psychiatric drugs. Free PUFAs are oxygenated by three families of enzymes (COX, LOX and epoxygenase CYP) into distinct classes of oxylipins (Massey and Nicolaou, 2013).

Enantiomeric excess of 9-(*S*) HODE and 13-(*S*) HODE over 9-(*R*) HODE and 13-(*R*) HODE, respectively (as determined by chiral chromatography), suggests that there is an enzymatic activity involved in the production of these compounds in *D. magna*. Other LOX-derived

Table 2
Contribution of the *R* or *S* enantiomers of 9-HODE and 13-HODE resolved by the chiral method in the *D. magna* samples. Also, the enantiomeric excess was calculated.

	% 9(*S*)-HODE	% 9(*R*)-HODE	Enantiomeric excess	% 13(*S*)-HODE	% 13(*R*)-HODE	Enantiomeric excess
Control (*i*)	75	25	50	60	40	20
Fluoxetine	83	17	66	65	35	30
Carbamazepine	74	26	48	62	38	24
Control (*ii*)	72	28	44	58	42	16
Diazepam	78	22	56	57	43	14

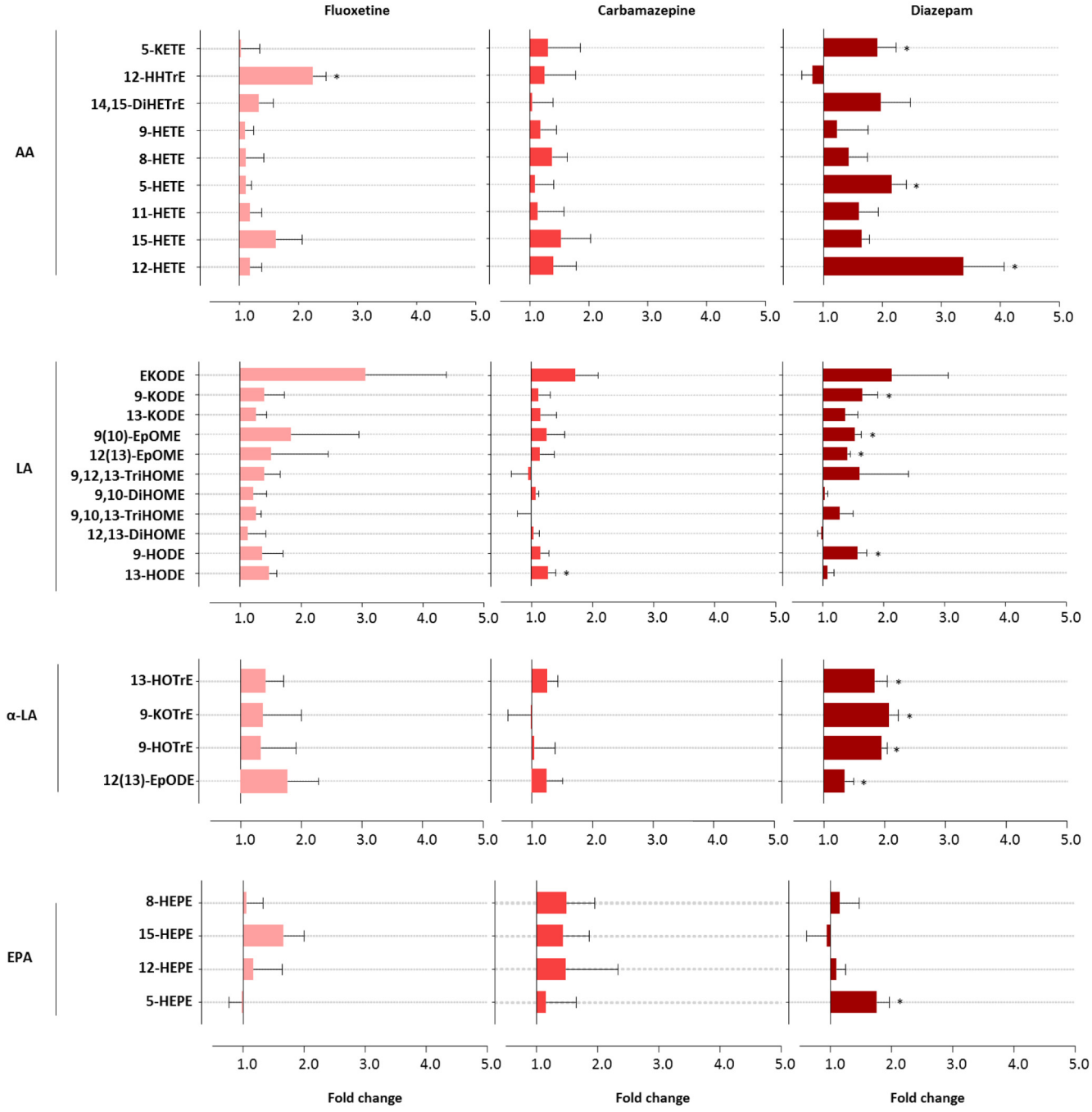

Fig. 2. Representation of the fold changes of each oxylipin in each exposure treatment respect to the control samples. Oxylipins with significant changes relative to controls ($p < 0.05$, ANOVA or Student's *t*-tests) are marked with an asterisk.

oxylipins quantified in *D. magna* (Table SI2), such as 5-HETE and 15-HETE, could not be detected using the chiral method due to their lower abundance.

The most detected oxylipins were final products of the enzymatic pathways involved, which may indicate a high rate of turnover of intermediated products (Fig. 1). Within AA-pathways related oxylipins, HETEs (e.g., 8-HETE, 9-HETE, 11-HETE, 12-HETE and 15-HETE), as well as 5-KETE and 14,15-DiHETrE are end-products for LOX and CYP pathways, respectively. In addition, KODEs (9-KODE and 13-KODE) and TriHOMEs (9,12,13-TriHOME and 9,10,13-TriHOME) are LA-derived endproducts for the LOX pathway, and DiHOMEs (12,13-DiHOME and 9,10-DiHOME) are LA-derived end-products for the CYP pathway. Besides, HOTrEs (9-HOTrE and 13-HOTrE) are also final products in the LOX pathway for α-LA.

Unfortunately, PGs could not be detected in *D. magna* samples. Previous studies indicated that prostanoids are mostly present in the ovary and are more abundant in early stages of oocyte maturation in decapods (Sumpownon et al., 2015). We sampled *D. magna* adults just after the release of eggs into the brood pouch when the ovaries have their minimum size and start a new egg provisioning cycle (Baird et al., 1991). This may have prevented our ability to detect prostanoids.

In *D. magna* the total amount of lipids is dependent upon dietary lipid content, feeding and ontogeny. Under high food ratios, within a single instar interval, the proportion of lipids can vary between 16%

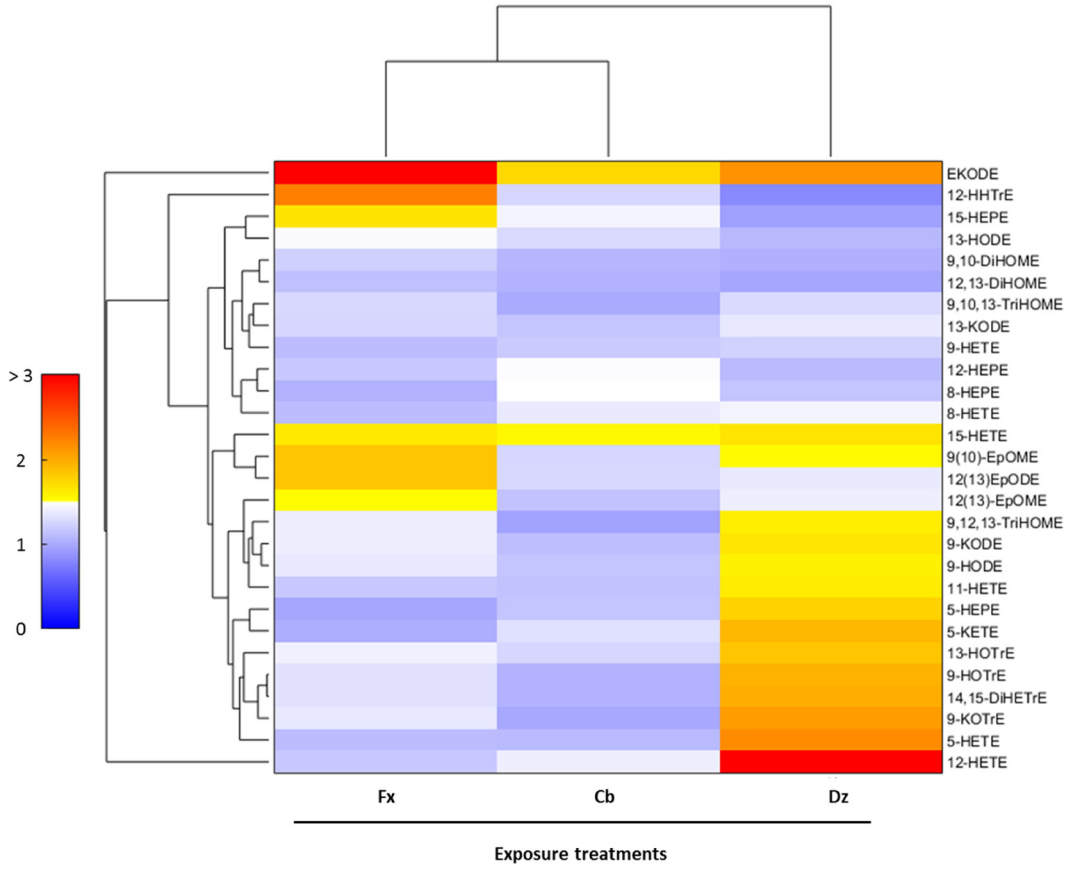

Fig. 3. Heat map representation with hierarchical clustering of fold changes of oxylipins across treatments. Data has been autoscaled relative to controls, so 0 value (blue) corresponds to no change relative to controls. Fx: fluoxetine exposure, Cb: carbamazepine exposure and Dz: diazepam exposure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and 22% of their dry mass (Barata et al., 2005; Goulden and Place, 1993). In the present study, the use of constant food conditions and a sampling scheme that consists in sampling adult females at the beginning of the intermolt interval, respectively, prevented large differences in total lipid to be found across animals. Reported fatty acid composition in *D. magna* individuals, cultured in similar conditions as those used here, indicated that among PUFAs, the C18 group is the most common including LA (18:2n-6) and α-LA (18:3n-3). PUFAs of 20 carbon chain are found only in small amounts, being AA (20:4n-6) followed by EPA (20:5n-3) the most abundant ones (Barata et al., 2005). Endogenous fatty acid synthesis contributes <5% of the accumulated total fatty acids in *D. magna* with most of them being incorporated from food (Cowgill et al., 1984; Sundbom and Vrede, 1997). The number of oxylipins quantified in whole *D. magna* tissues agrees with their relative abundance, being in decreasing order LA, AA, α-LA and EPA, respectively.

The biological role of eicosanoids in *D. magna* is still unclear. For AA, in silico and transcriptomic studies suggest that only the COX and the LOX pathways are present in *D. magna* (Heckmann et al., 2008). Other genomic studies (Baldwin et al., 2009; Kim et al., 2018), contradict the bioinformatic arguments of Heckmann et al. (2008), who reported that there is no current evidence of CYP pathway in *D. magna* and other arthropods. Detection of 14,15-DiHETrE, which is a downstream product of the CYP produced 14(15)-EpETrE, and of 12(13)-EpOME and 9(10)-EpOME provides an evidence for the existence of eicosanoid CYP epoxygenase pathway in the AA and LA pathways in *D. magna*. Our results, thus, are in line with the findings of Baldwin et al. (2009) an d Kim et al. (2018). Recently, sEH activities and epoxy fatty acids have been found in the mosquito *Culex quinquefasciatus* (Xu et al., 2015). In the present study, the detection and quantification of DiHOMEs in *D. magna*, related with the sEH activities, confirmed the presence of an eicosanoid CYP pathway. However, further research is needed to confirm that finding.

Fluoxetine enhances serotonin activity in *D. magna* (Campos et al., 2016). There is also a cross-talk between serotonin receptors and AA/PG metabolism (Tournois et al., 1998), which in insects regulates reproduction (Stanley, 2006). The increase of 12-HHTrE upon fluoxetine exposure, which was identified as a product of prostaglandin H_2 (PGH_2) (Matsunobu et al., 2013), suggests that this psychiatric drug may increase, and consequently. up-regulate PG synthesis and, hence, offspring production. Little is known about the physiological role of LOX and CYP derivate oxylipins in insects and crustaceans (Heckmann et al., 2008; Stanley, 2006). Medeiros et al. (2004) found that LOX inhibitors decreased yolk uptake in the insect *Rhodnius prolixus,* whereas a specific LOX product, leukotriene B_4 (LTB_4), stimulated its uptake. Conversely, in an early study, the same authors found that the prostaglandin E_2 (PGE_2) down-regulates the ovarian yolk uptake in the same species (Medeiros et al., 2002). Thus, the previous results indicate that LOX may be involved in reproduction. Accordingly, the observed increased levels of several LOX and CYP products by diazepam and, to a lesser extent by carbamazepine, also supports the hypothesis that the studied psychiatric drugs up-regulated oxylipins metabolism and as such reproduction in *D. magna*. In a previous work, it was reported that the three studied drugs enhanced reproduction in *D. magna* at similar low exposure levels (Rivetti et al., 2016). Our results may thus provide additional evidence for a link between observed up-regulation of COX and LOX related oxylipins products and enhanced offspring production in *D. magna,* upon exposure to psychiatric drugs.

5. Conclusions

In this study, we report for the first time, a total of 28 different oxylipins belonging to the COX, LOX and CYP pathways for the AA, LA, α-LA and EPA pathways in *D. magna* individuals. All detected oxylipins were final oxidized products of the enzymatic pathways involved. Some of the eicosanoids detected, such as 12-HHTrE, are involved in PG and leukotriene biosynthetic pathways, respectively (Hamberg and Samuelsson, 1974; Stanley, 2006), which regulate reproduction in insects (Medeiros et al., 2004; Medeiros et al., 2002; Stanley, 2006).

Exposure to environmental concentrations of psychiatric drugs enhanced the differential accumulation of specific oxylipins. Fluoxetine increased the accumulation of the PG product 12-HHTrE, whereas diazepam increased the concentration of 12 eicosanoids and other oxylipins belonging to the LOX and CYP pathways of AA, LA, α-LA and EPA. Some of them (5-HETE and 5-KEPE) are closely related to the leukotriene metabolic pathway. Carbamazepine only increased significantly one LOX metabolite from the LA pathway (13-HODE). Previous studies in insects indicated that both COX and LOX products, PGs and leukotrienes, respectively, regulate reproduction (Medeiros et al., 2004; Medeiros et al., 2002; Stanley, 2006). This means that despite having different modes of action, fluoxetine and diazepam upregulated eicosanoid pathways, closely related to known biologically active products that regulate reproduction. Results of carbamazepine are difficult to interpret, as there is no evidence in invertebrates that oxylipins from the LA-pathway affected reproduction.

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Compliance with ethical standards

The authors declare no financial conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.333.

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