Environmental Pollution 178 (2013) 237–243

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Low concentrations, potential ecological consequences: Synthetic estrogens alter life-history and demographic structures of aquatic invertebrates

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ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form
12 March 2013
Accepted 18 March 2013

Keywords:
Aquatic systems
Caspase-3
Copepods
Estrogens
Glutathione *S*-transferase

ABSTRACT

Contraceptive drugs are nowadays found in aquatic environments around the globe. Particularly, 17α-ethinylestradiol (EE_2) may act even at low concentrations, such as those recorded in natural ecosystems. We evaluated the physiological effects of EE_2 on cyclopoids and calanoids, common copepods in both marine and freshwater communities. We used three EE_2 concentrations and assessed its impact on activity of different physiological endpoints: Acetylcholinesterase (neurotransmission), Glutathione *S*-transferase (detoxifying system), and Caspase-3 (apoptosis). While EE_2 exerts, distinctive effect on detoxifying and apoptotic systems, no effect on AChE was observed at environmental doses. Our results show that EE_2 exposure affects differently copepod physiology endpoints, altering moulting process, adult recruitment in calanoids and calanoid to cyclopoid ratio. The ecological consequences of this underlying physiological process may affect since life history to population and community structures, and this represent a new aspects of this xenobiotic in natural systems.

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1. Introduction

Over the past decades an overwhelming amount of studies have evaluated the impact of man-made chemicals, such as pesticides and plasticizers on different environment compartments (Forget et al., 2003; Minutoli et al., 2002; Sarkar et al., 2006). Recently, however, an increasing concern has arisen regarding pharmaceutical products consumed by humans and thereafter indirectly spread into the environment (Clubbs and Brooks, 2007; Forgetleray et al., 2005; Kaptaner et al., 2009; Marfil-Vega et al., 2012; Skillman, 2006; Ying et al., 2002). Due to the enormous amounts consumed, compounds belonging to analgesics, antibiotics and contraceptives drugs are nowadays found in almost all aquatic environments (Gilbert, 2012; Pereira et al., 2007). Contraceptive drugs are now in focus especially because they are potent endrocrine disruptors (EDs) that can mimic endogenous hormones and alter hormonal metabolism in a broad spectrum of organisms (Casey et al., 2003; Gilbert, 2012; Segner et al., 2003). EDs include natural compounds such as phytoestrogens and also a wide range of synthetic chemicals (Cailleaud et al., 2011; Labadie and Budzinski, 2006), and one of the most potent compounds with estrogenic activity is the synthetic hormone 17α ethinylestradiol (EE_2) (Desbrow et al., 1998) (Desbrow et al., 1998). This compound is present in many contraceptives pills and hormone replacement therapy (Teede, 2002), and is characterized by being more resistant to degradation than natural estrogens and therefore exhibits a long persistence in the environment (Jürgens et al., 2002). Although EE_2 is excreted from the body in a conjugated form, it can be reverted to active form as a result of microbial action increasing its environmental relevance (Panter et al., 1999).

Many endocrine disruptors are reactive chemicals that may trigger multiple mechanisms of toxicity by acting at different sites within the organism, and causing biological responses not only linked to endocrine effects (Gilbert, 2012; Greco et al., 2007; Hutchinson, 2002). Recent studies in fish physiology, e.g. in *Salmo salar* (Greco et al., 2007; Mortensen and Arukwe, 2007), have demonstrated that EE_2 can modulate activity of different physiological endpoints, such as Acetylcholinesterase (AChE, an enzyme crucial for neurotransmitter control) and Glutathione *S*-transferase (GST, a detoxifying enzyme linked with the metabolism of several toxic compounds). Moreover, EE_2 also causes imbalance between apoptosis (programmed cell death) and cell proliferation in the fish species (Nadzialek and Kestemont, 2010). Apoptosis plays a crucial

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http://dx.doi.org/10.1016/j.envpol.2013.03.038

role in several physiological processes of multicellular organisms and, for example, controls the subtle balance between cell division and cell death (Lockshin and Zakeri, 2001), with a particular importance in the development and moulting of aquatic organisms. However, most of the available information is derived from studies of vertebrates and can generally not be directly applied to lower trophic levels, such as invertebrates. Hence, our knowledge is limited regarding the effects of these chemicals on lower trophic levels, such as invertebrates, and even less information is available regarding the physiological and molecular key mechanisms. Invertebrates, such as insects and crustaceans, are widespread in nature; in both terrestrial and aquatic environments, and constitute important links in natural ecosystems and are crucial for many ecosystem services, e.g., mineralization and pollination (Garibaldi et al., 2011; Hutchinson, 2002; Wilson, 1999). Therefore it is important to evaluate the potential effects of pharmaceuticals on such non-target organisms. In aquatic environments, copepods are very widespread crustacean taxa which constitute a major group in planktonic communities (Adrian, 1997), and also function as an important link between primary producers and higher trophic levels, such as fish, in both marine and freshwater food webs (Stibor et al., 2004). They are also characterized by a complex life cycle with several stages, as well as a highly integrated neuroendocrine system (Hutchinson et al., 1999; Lenz et al., 2000). If compounds such as endocrine disruptors can reach the aquatic ecosystems and affect organisms at a cellular level, then this effect will affect organism fitness, and consequently affect the whole community structure and function.

The aim of this study was to assess the physiological sublethal effects of EE_2 (including concentrations reported in natural environments) on two groups of copepods, cyclopoids and calanoids. We designed a laboratory experiment with three different concentrations of estrogen and assessed the effects of this treatment on the activity of three different enzymatic systems: Acetylcholinesterase (AChE), Glutathione *S*-transferase (GST), and Caspase-3 (CASP-3). We selected these endpoints in order to include responses from different physiological mechanisms not directly linked with hormonal function.

2. Material and methods

2.1. Test organisms

The laboratory experiments were performed in Lund, Southern Sweden (55.7 °N, 13.5 °E), using the copepod species *Eudiaptomus gracilis* (calanoid) and *Mesocyclops luckarti* (cyclopoid). The animals were collected with a plankton net (125 μm mesh size) from surface waters (0–0.5 m depth) in Dalby Quarry. This lake is situated within a natural reserve close to Lund and receives no input of estrogens from effluent or sewage. Zooplankton were transported to the laboratory and maintained overnight with containers full of natural lake water at 18 °C. Prior to the experiment they were rinsed in filtered (10 μm) lake water. The age structure of the studied populations showed that *E. gracilis* was composed of 50% of adults and 50% of copepodites, while *M. leuckarti* had 40% of adults and 60% of copepodites. The experiments were initiated with these proportions.

2.2. Chemicals

Estrogen stock solution, was prepared by dissolving 2 g L^{-1} of 17α ethinylestradiol (EE_2) (≥98% Sigma–Aldrich, code: E4876) in Dimethyl Sulfoxide (DMSO Sigma–Aldrich, code: 41639) as vehicle. The stock solution was then diluted in DMSO to obtain 50 mL EE_2 solutions with concentrations of 0.16 mg L^{-1}, 1.6 mg L^{-1} and 16 mg L^{-1}. DMSO for the vehicle-control was treated in the same way as the EE_2 solutions. The dilution series was stored in darkness when not in use.

2.3. Experimental setup

We performed five different treatments, Control (C), Control + DMSO (C+ Vehicle), 10 ng L^{-1}, 100 ng L^{-1} and 1000 ng L^{-1} of EE_2 and each treatment was replicated 4 times. Each replicate consisted of a four-litre aquarium with dechlorinated tap water and the addition of 250 μl of EE_2 solutions to obtain nominal concentrations of 10 ng L^{-1}, 100 ng L^{-1} and 1000 ng L^{-1}, and the same volume of DMSO was added to the vehicle-control (C+ Vehicle), while the control (C) treatment lacked DMSO and EE_2 addition. Due to the EE_2 half-life (Hallgren et al., 2012), new estrogen solution (150 μL) was added to the experimental units every 72 h to recover original concentration. Previous assays under the same experimental system (in the same greenhouse where the experiment took place, using the same containers and with the same temperature and light regime) indicate that the difference between nominal and in-water concentration (actual exposure concentration) is below to 5% (Hallgren et al., 2012), so accumulation of EE_2 over time can be excluded. These EE_2 concentrations were chosen since levels around 10 ng L^{-1} have been reported from aquatic ecosystems (Casey et al., 2003; Gilbert, 2012; Rudder, 2004). Copepods were exposed for 14 days, and fed with *Chlamydomonas* sp. cultured every third day. The experiment was run maintaining constant light and temperature regimes (20 °C and a 14/10 h light/dark).

At the end of the experiment, animals from each replicate were concentrated to 400 mL with a plankton net (80 μm mesh size) and a subsample of 40 mL was taken out for counting and determination of group (cyclopoid, calanoid) and ontogenetic stage (adult, juvenile). All animals of each treatment were frozen at −80 °C until enzymatic determination or counting, was carried out. The identification and counting of copepod species and stage were done using a 32–40× magnification (Olympus SZ 40).

2.4. Enzyme activity determinations

Since both calanoid and cyclopoid copepodites (juveniles) were scarce at the end of the experiment enzymatic analyses were performed only on adult copepods. For GST and AChE activities, animals were homogenized using a glass-Teflon homogenizer with ice-cold 50 mM potassium phosphate buffer (pH = 7.7) containing 1 mM EDTA and 0.1% Triton X-100 (Souza et al., 2010a). Homogenates were centrifuged at 10 000 × *g* and 4 °C for 10 min and supernatants used as GST and AChE sources.

Total glutathione *S*-transferase (GST) activity was determined at 25 °C by using 100 mM phosphate buffer (pH = 6.5) with 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) in acetonitrile (1% v/v) and 1.2 mM glutathione GSH as substrates (Habig et al., 1974). The GST activity was then assessed as absorbance at 340 nm after two minutes using a Beckman Coulter DU800 Spectrophotometer. Specific activity of GST was expressed in nanomoles of product developed per minute per mg of protein (nmol prod. min^{-1} [mg prot]$^{-1}$) at saturating substrate concentrations. Linearity of the reaction with protein quantity per assay was determined ($r^2 = 0.975$).

Total Acetylcholinesterase (AChE) activity was determined at 25 °C following the colorimetric method of Ellman according to (Forget et al., 2003), at saturating concentrations of acetylthiocholine iodide as substrate and dithiobisnitrobenzoate (DTNB) as reagent. The AChE activity was then assessed as absorbance at 412 nm after two minutes. Buthyrilcholinesterase (BChE) was specifically inhibited by adding 1 mM iso-OMPA (Kristoff et al., 2006). AChE activity was expressed as μmol product developed min^{-1} (g protein)$^{-1}$. Substrate linearity of the reaction with protein quantity per assay was determined ($r^2 = 0.962$).

The caspase-3 (CASP-3) activity was determined by using the specific peptide acetyl-Asp-Glu-Val-Asp p nitroanilide as substrate (Ac-DEVD-pNA; Sigma–Aldrich) (Souza et al., 2012). The fact that the core components of the cell death machinery are conserved through evolution (Richardson and Kumar, 2002), allowed us to determine CASP-3 activity in both groups of copepods with this kit. Briefly, animals of each treatment replicate were homogenized using a glass-Teflon homogenizer with ice-cold lyses buffer containing 250 mM HEPES (pH = 7.4), 25 mM 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 25 mM dithiotreitol (DTT), and leupeptin as protease inhibitor. Then the samples were held on ice for 20 min. Supernatants of homogenates, centrifuged at 20,000 × *g* and 4 °C for 15 min, were used as enzyme source. Extracts were added to the Caspase-3 reaction buffer (200 mM HEPES, pH 7.4, 1% CHAPS, 50 mM DTT, 20 mM EDTA) with Ac-DEVD-pNA substrate and then incubated at 37 °C for 2 h. Upon proteolytic cleavage of the substrate by endogenous caspase-3 enzyme, unbound pNA concentration was determined as absorbance at 405 nm using a Beckman Coulter DU800 Spectrophotometer. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm based on a calibration curve prepared with pNA standard solutions ($r^2 = 0.9997$). The specific activity of CASP-3 was expressed in μmol of product developed per minute per mg of protein (μmol prod. min^{-1} [mg prot]$^{-1}$). From each treatment replicate of each measured enzyme, three sub-samples were determined (method replication for accuracy determinations).

2.5. Protein determination

The enzymatic activities were normalized to protein concentration. Protein determination was performed according to (Lowry et al., 1951) with bovine serum albumin as standard. The protein quantity per assay was 7.32 ± 0.08 μg proteins per reaction for GST or AChE activities, and was 45.68 ± 6.03 μg proteins per reaction for CASP-3 determination.

2.6. Data analysis

Results are expressed as mean ± standard error. For independent samples we used a Student's *t*-test and One Way ANOVAs. When significant differences were obtained in the ANOVA, we applied a posthoc multiple comparison Tukey test. All analyses were performed in Sigma Stat 3.5 software for Windows. To compare the percentage of copepodites among treatment arcsin-squared root transformed was performed.

3. Results

The results obtained revealed physiological effects of EE_2 exposure for both copepod groups evaluated. We obtained distinctive effects of EE_2 exposure on each enzyme activity (GST, AChE and CASP-3) in both calanoid and cyclpoid copepods.

3.1. Glutathione S transferase activity

GST activity differed between copepod groups, cyclopoids showed higher base-level GST activity under control conditions (C = 1041 ± 134 nmol prod. min^{-1} [mg $prot]^{-1}$) than calanoids (C = 620 ± 81 nmol prod. min^{-1} [mg $prot]^{-1}$) (*t*-test, $p < 0.001$). In calanoids GST activity decreased under all EE_2 tested concentration (Fig. 1A) (One Way ANOVA $p = 0.027$, posthoc Tukey test C vs. 10 ng L^{-1}, $p = 0.049$, C vs. 100 ng L^{-1}, $p = 0.021$, C vs. 1000 ng L^{-1}, $p = 0.0006$), while in cyclopoids GST activity decreased only at the two highest concentrations of this xenoestrogen (100–1000 ng L^{-1}) (Fig. 1B) (One Way ANOVA $p = 0.004$ posthoc Tukey test C vs. 100 ng L^{-1}, $p = 0.023$; Tukey test C vs. 1000 ng L^{-1}, $p = 0.022$).

3.2. Acetylcholinesterase activity

As for GST, the AChE activity also differed markedly between groups, but, in contrast to GST, AChE showed a higher activity in calanoids (above 70 μmol prod. min^{-1} [g $prot]^{-1}$) than in cyclopoids (generally below 10 μmol prod. min^{-1} [g $prot]^{-1}$) (Fig. 2) (*t*-test, $p < 0.001$). The AChE activity of calanoid copepods showed no response to the addition of EE_2 (Fig. 2A) (One Way ANOVA NS). However, in cyclopods we found that the exposure to the highest dose of EE_2 (1000 ng L^{-1}) caused a significant decrease in AChE activity (Fig. 2B) (One Way ANOVA $p = 0.019$, Tukey test $p = 0.009$).

3.3. Caspase-3 activity

In both copepod groups, we observed a similar pattern of CASP-3 response to EE_2 exposure, though stronger in calanoids (Fig. 3A and B). Calanoids showed a strong and significant decrease for all EE_2 doses (One Way ANOVA $p < 0.002$; posthoc Tukey test C vs.

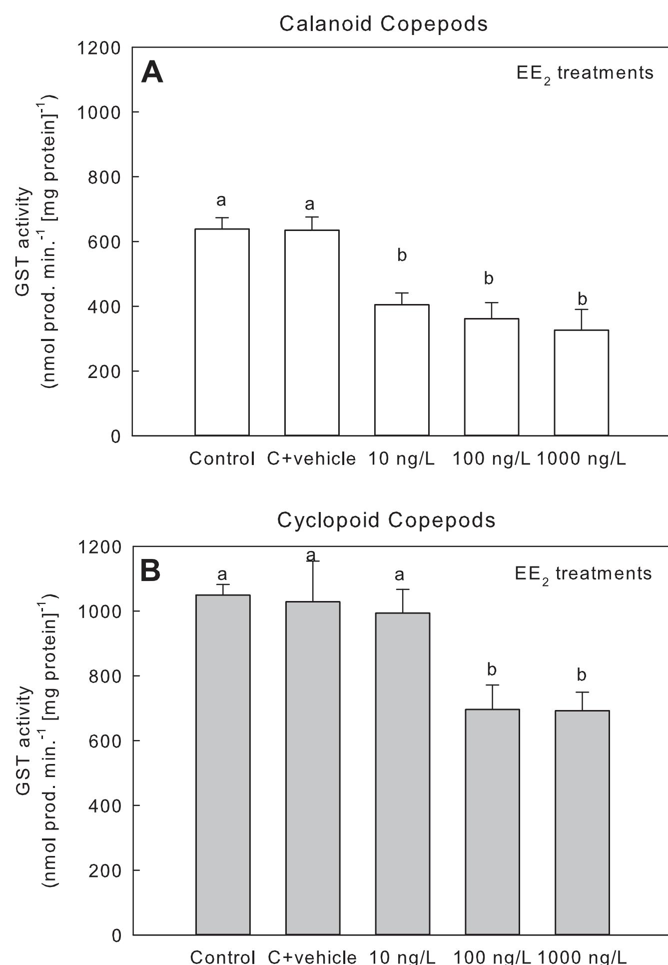

Fig. 1. Glutathione *S*-transferase (GST) activity under different EE_2 concentration for: (**A**) calanoid and (**B**) cyclopoid copepod. DMSO treatment was indicated as C+ Vehicle, whereas the control treatment lacked DMSO and EE_2 addition. Letters inside the graphs indicate homogeneous groups (treatments with non-significant differences) of enzyme activity as shown by the posthoc analysis.

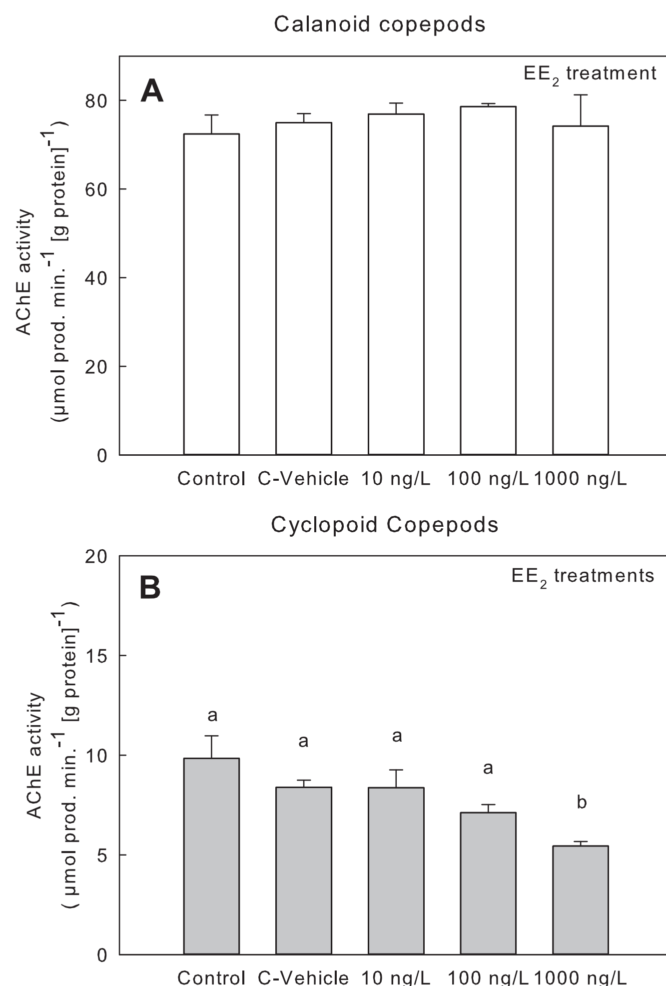

Fig. 2. Acetylcholinesterase (AChE) activity under different EE_2 concentration for: (**A**) calanoid and (**B**) cyclopoid copepod. DMSO treatment was indicated as C+ Vehicle, whereas the control treatment lacked DMSO and EE_2 addition. Letters inside the graphs indicate homogeneous groups (treatments with non-significant differences) of enzyme activity as shown by the posthoc analysis.

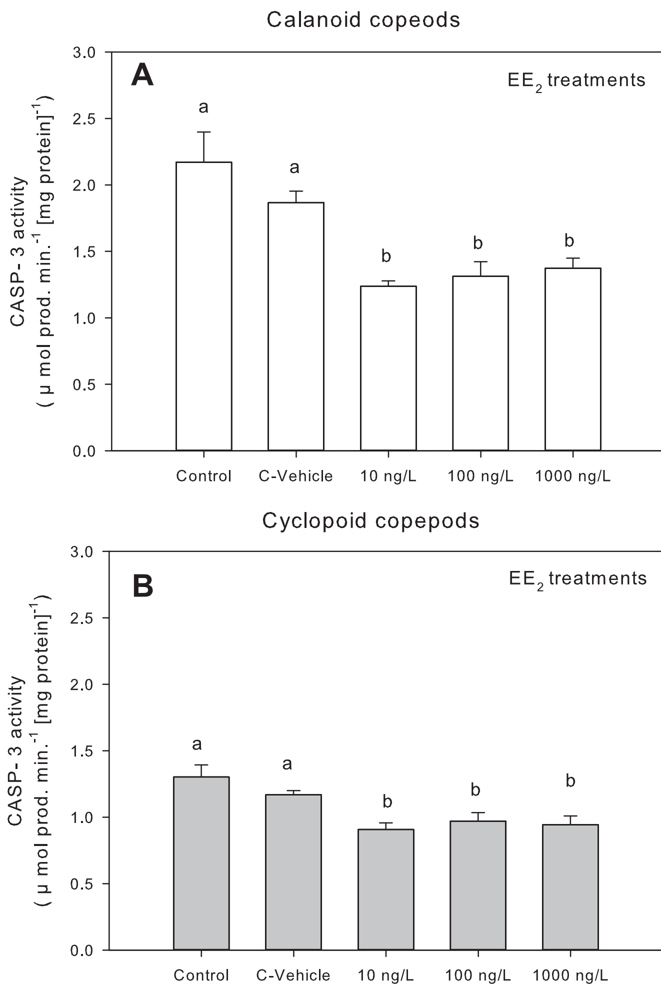

Fig. 3. Caspase-3 (CASP-3) activity under different EE_2 concentration for: (**A**) calanoid and (**B**) cyclopoid copepod. DMSO treatment was indicated as C+ Vehicle, whereas the control treatment lacked DMSO and EE_2 addition. Letters inside the graphs indicate homogeneous groups (treatments with non-significant differences) of enzyme activity as shown by the posthoc analysis.

10 ng L^{-1}, $p = 0.005$; Tukey test C vs. 100 ng L^{-1}, $p = 0.006$; Tukey test C vs. 1000 ng L^{-1}, $p = 0.006$), whereas cyclopoids showed also a significant, decrease in CASP-3 activity (One Way ANOVA $p < 0.002$; posthoc Tukey test C vs. 10 ng L^{-1}, $p = 0.004$; Tukey test C vs. 100 ng L^{-1}, $p = 0.009$; Tukey test C vs. 1000 ng L^{-1}, $p = 0.015$).

3.4. Changes in life cycle

At the start of the experiment (To), the adult: copepodite ratio was 50:50 in *E. gracilis* population while in *M. leuckarti* this ratio was 40:60. However, during the 15 days of experimentation, most of the copepodites of the controls molted into the adult stage (Fig. 4, see increase in adults of control at the end compared with To), resulting in an increase of the proportion of adults (about 95%) with only 5% of copepodites at the end of the experiment (Fig. 4 A & C). While a similar copepodite decrease was observed in the EE_2 treatments (for calanoid One Way ANOVA, $p = 0.1$ posthoc Tukey test C vs. 10 ng L^{-1}, $p = 0.038$; Tukey test C vs. 100 ng L^{-1}, $p = 0.011$; Tukey test C vs. 1000 ng L^{-1}, $p = 0.052$ and cyclopoid One Way ANOVA, $p = 0.77$) the number of adults in these treatments did not increase as was observed in the controls (Calanoids One Way ANOVA $p < 0.001$, posthoc Tukey test C vs. 10 ng L^{-1}, $p = 0.0004$; Tukey test C vs. 100 ng L^{-1}, $p = 0.011$; Tukey test C vs. 1000 ng L^{-1}, $p = 0.090$ and for cyclopoids $p = 0.012$ posthoc Tukey test C vs. 10 ng L^{-1}, $p = 0.031$; Tukey test C vs. 100 ng L^{-1}, $p = 0.028$; Tukey test C vs. 1000 ng L^{-1}, $p = 0.090$) (Fig. 4 A & C). The lack of increase in adults resulted in an increase in the proportion of juveniles under EE_2 exposure in both groups (Fig. 4B & D). This increase was statistically significant only for calanoid copepods under 100 ng L^{-1} EE_2 treatment (Fig. 4 B) (One Way ANOVA, $p = 0.034$ posthoc Tukey test, $p = 0.022$).

4. Discussion

When having passed the human body, natural and synthetic estrogens end up in aquatic environments, such as streams, lakes and coastal marine ecosystems, via sewage treatment plants (Casey et al., 2003; Clubbs and Brooks, 2007; Desbrow et al., 1998; Gilbert, 2012). Hence, though aquatic organisms are not target organisms they may be exposed and affected by these substances. Our results show that EE_2 released in natural aquatic systems will affect physiological mechanisms that reduce organism fitness. The novelty of physiological approach is based on the concept that impact of a contaminant, in this case EE_2, will act at different levels, from cellular to ecological ones. The studies of these enzymes responses allow as to obtain a fast diagnosis before ecological consequences are evident. However, the effect of EE_2 depends on the physiological system analysed. While EE_2 exerts, at almost all concentrations, distinctive effect on detoxifying and apoptotic systems, no effect on the AChE neurotransmission system was observed at doses reported for natural systems. It has been suggested that in vertebrates, estrogens can alter neurotransmission by altering the synapses and stimulate membrane receptors in the central nervous system (Kohtz and Frye, 2012; Küppers et al., 2000). However, the potential effects of estrogens on neurotransmission in invertebrates are poorly understood and in this study we found no direct effect of environmental doses of EE_2 on AChE activity in copepods.

The enzymatic activities analysed in our study showed different susceptibility to EE_2 exposure in calanoids and cyclopoids. The basal level of GST activity was significantly higher in cyclopoid than in calanoid copepods. In addition, calanoid GST activity decreased with all EE_2 concentrations, while in cyclopoid copepods it only decreased at the highest concentration. GST induction is an adaptive response acting as part of the primary detoxifying mechanism protecting cells from chemical insults (O'Brien et al., 2000). GSTs constitute a very ancient and highly versatile superfamily that carry out a wide range of functions, such as the removal of reactive oxygen species and regeneration of S-thiolated proteins (both of which are consequences of oxidative stress), catalysis of conjugations with endogenous ligands, and catalysis of reactions in metabolic pathways not associated with detoxification (Sheehan et al., 2001). A significant decrease in this crucial enzyme activity will decrease protection when organisms face other environmental substances, including several antropogenetic pollutants as naphthalenes, pesticides, heavy metals and a wide range of other prooxidant compounds (Hansen et al., 2008; Kim et al., 2009; Lee et al., 2007). Particularly in copepods, this enzyme activity is also linked with and increased tolerance to UVR, one of the most important pro-apoptotic stimuli for crustaceans (Souza et al., 2007, 2010b, 2012).

According to our results, naturally occurring doses of EE_2 (around 10 ng L^{-1}) (Clubbs and Brooks, 2007) decreased significantly the GST activity in calanoids, but not in cyclopoids. The different sensitivity to EE_2 exposure will be linked to a dissimilar defence capability between these two copepod groups when they face environment with several stressors acting at the same time. Thus, this physiological advantage in cyclopoids may result in shifts

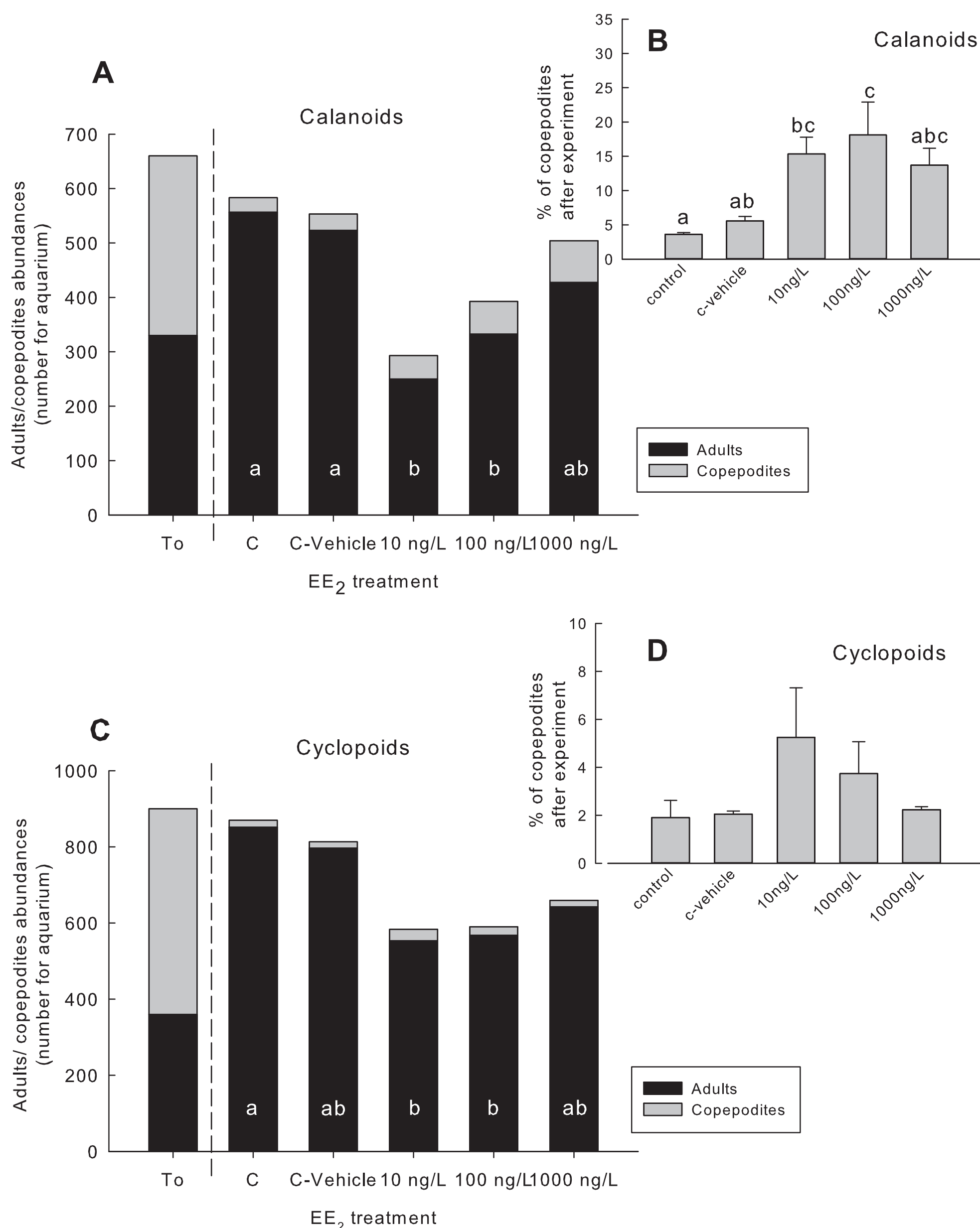

Fig. 4. Adults/copepodites abundances under different EE_2 concentrations for: (**A**) calanoid and (**C**) cyclopoid copepod. DMSO treatment was indicated as C+ Vehicle, whereas the control (C) treatment lacked DMSO and EE_2 addition. The initial Adult/copepodite ratio at the beginning of experiment is indicated as To. Little boxes indicate the proportion of copepodites after EE_2 treatment in calanoids (**B**) and cyclopoids (**D**). Letters inside the graphs indicate homogeneous groups (treatments with non-significant differences) as shown by the posthoc analysis.

in calanoid to cyclopoid ratio. At long term exposures, these low concentrations effect would result in changes in the zooplankton community structure. Since most calanoid copepods are grazers, whereas cyclopoids generally are predators or omnivores, a change in the calanoid/cyclopod ratio would imply an important shift in the matter-energy transfer in the food web.

Regarding to molecular events implied in vertebrate estrogen GST modulation, several studies on vertebrates indicate that some endocrine disruptors, including EE_2, can alter gene expression patterns of enzymes involved in drug biotransformation, such as GST (Mdegela et al., 2006; Mortensen et al., 2007). Although copepods apparently lack the orthologue receptors of EE_2 (Breitholtz and Bengtsson, 2001), the effect of this disruptor may still affect gene expressions in invertebrates, and thus affect the activity of GST as we observed in our results.

As a result of structural similarities between vertebrate estrogens and the invertebrate moulting hormone ecdysone (Segner et al., 2003), it has been suggested that endocrine-active pharmaceuticals may disturb ecdysteroid system by binding to ecdysteroid nuclear receptor or atering ecdysteroid metabolism and, as a consequence, altering with the invertebrate endocrine system (Clubbs and Brooks, 2007) (Breitholtz and Bengtsson, 2001). However there are contrasting results regarding how synthetic estrogens can interfere with or disrupt endocrine processes that regulate crustacean development and reproduction (Breitholtz and Bengtsson, 2001). While Hutchinson et al. (1999) did not observe any significant effects on development, reproduction or sex ratio in the marine copepod *Tisbe battagliai*, other studies report that exo- estrogens may alter maturation of reproductive organs in crustaceans (LeBlanc, 2007; Oehlmann

et al., 2000) or, particularly in copepods, quite low concentrations of 17b-estradiol or bisphenol A (20–35 mg L^{-1}) stimulate ovarian maturation, and increasing in the rate of egg production (Forgetleray et al., 2005) or even produced a delay in the moulting process (Andersen et al., 2001). Moulting in invertebrates is a complex process in which apoptosis plays a crucial role (Heyland and Moroz, 2006). Apoptosis involves a series of steps providing a selective advantage to the best adapted offspring by preventing flawed offspring from competing for resources (Kiess and Gallaher, 1998). At cellular level, apoptotic events induce morphological and biochemical alterations including cell shrinkage, disintegration and activation of specific caspases that lead to enzymatic breakdown of DNA (Lockshin and Zakeri, 2001). This signalling mechanism plays an essential role in physiological processes such as differentiation, tissue remodelling and immune system regulation, so a dysfunction or alteration of those mechanisms would affect moulting and hence normal population age structure (Porter and Jänicke, 1999; Romano, 2003). Here we used the CASP-3 activity as a proxy of apoptosis, since this protease is a key component in the apoptotic signalling network (Gewies, 2003). In our experiment, we observed a distinct decrease of CASP-3 enzymatic activity under all EE_2 treatments. Nadzialek and Kestemont (2010) demonstrated that EE_2 produces an imbalance between the apoptosis process and cell proliferation in testicle culture of fish. However, few studies have focused on the effective doses of these chemicals on invertebrates and how it can modulate apoptosis. All concentration tested in this study (10 ng L^{-1}, 100 ng L^{-1} and 1000 ng L^{-1}) provoked similar decreases (about 20–30%) in copepod CASP-3 activity. Alterations in apoptosis (as decrease in CASP-3 activity observed) could induce a delay in the moulting process and this in turn will be reflected in changes in the adult/juvenile ratio as we observed for calanoid copepods. In the control treatment of our experiment we observed that the adult number and adult/copepodite ratio increased at the end of the experiment. This means that the increase in adults resulted from the moulting of copepodites to adulthood. If EE_2 alters moulting then the consequence of the exposure can result in two alternative possibilities. Firstly, copepodites that became unable to moult because of the EE_2 effect will stay as copepodites and so the number of adults, copepodites and the ratio between them would remain unchanged along the experiment, this would be a “Dorian Gray” effect, that is the permanence in a juvenile stage. On the contrary, if EE_2 uncouples the moulting-apoptosis mechanisms, then most of the copepodites that became unable to moult can die as a consequence of the physiological bottleneck of this desynchronization. The consequence is that although copepodites would decrease in number, no increase in adults would be observed, as no recruitment through moulting is taking place. Our results show that in both copepod groups, the adult recruitment dropped drastically (no increase in adult numbers as observed in controls), but at the same time the copepodites were also reduced, indicating that there was no Dorian Gray effect, but more likely an increase in the mortality of copepodites as suggested by our second hypothesis. Hence, we show here that EE_2 may also alter growth and differentiation processes and, as a consequence, affect population age structure through the apoptosis/moulting process (CASP-3 activity).

CASP-3 also plays a key role in adults because the apoptosis cascade will be trigged as a response to perturbations of intracellular homeostasis by various exogenous stresses (xenobiotics, UV radiation, others prooxidants compounds) to ensure that unwanted and potentially dangerous damaged cells are efficiently removed (Souza et al., 2012). Thus, in multicellular organisms, triggering of apoptosis process may be considered part of the defence strategy against environmental stressors (Souza et al., 2012). On the contrary to GST, EE_2 reduced CASP-3 activity similarly for both taxa and at all doses tested, suggesting a similar negative effect on fitness of both copepods groups.

It has been suggested that in the last decades the cyclopod/calanoid ratio is increasing, especially in anthropogenically affected (eutrophic) waters (Adrian, 1997), although no underlying mechanisms has been suggested. Our results suggest that although several other factors may be involved in this shift, the pharmaceutical-active compounds, as EE_2, that are being released to aquatic ecosystems are altering some copepod population features. Thus, EE_2 is acting not only as endocrine disruptors, but also altering other physiological mechanisms related to invertebrate defence capabilities (such as GST and CASP-3 activities) and thereby affecting life histories and age structure. At the physiological scale we recorded these effects even at ecologically relevant doses and therefore linked with changes in fitness of different copepod groups. In a broader perspective this implies that estrogens released through the sewage system are likely to affect species abundances, dominance patterns and as a consequence the community composition in natural aquatic ecosystems, altering the energy matter transfer in the food web.

Acknowledgements

This work was carried out during a stay at Lund University (Sweden) of MSS supported by CONICET (Argentina). The authors acknowledge the support of the Swedish Research Council (VR) to LAH.

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