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Acute and chronic toxicity assessments of 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) on the calanoid copepod *Acartia clausi*: Effects on survival, development, sex-ratio and reproduction

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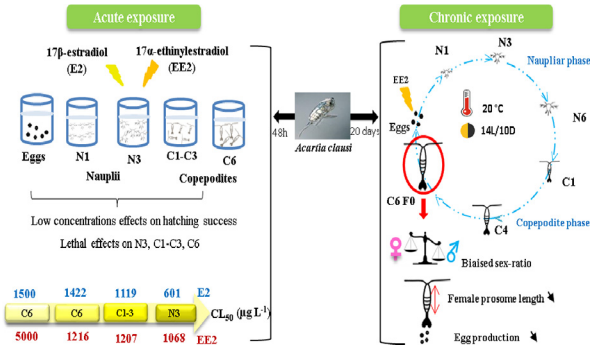
HIGHLIGHTS

- First knowledge on *Acartia clausi* response to acute and chronic estrogens exposure.
- Estrogens exhibit a U-shape curve for *A. clausi* embryos.
- Survival of N1 life stage of *A. clausi* was not sensitive to E2 and EE2 exposure.
- Sex ratio was skewed towards females at 100 μg L^{-1} of EE2.
- Reproduction and prosome length were reduced in females reared with 100 μg L^{-1} of EE2.

GRAPHICAL ABSTRACT



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ABSTRACT

Estrogens, such as the 17β-estradiol (E2) and the 17α-ethinylestradiol (EE2), have been regarded as a global threat to aquatic ecosystems due to their pseudo-persistence, their high estrogenic activity and their toxicity towards non-target species. Data regarding their ecotoxicological effects on marine calanoid copepods are very scarce. In this study, the calanoid copepod *Acartia clausi* was used as a model organism for estrogens exposure in marine pelagic ecosystems. Lethal effects of estrogens on *A. clausi* life-stages (Embryos, one day old nauplii: N1, three day old nauplii: N3, copepodites: C1-C3 and adults: C6) were investigated using 48 h acute tests. Copepods showed stage-specific responses against E2 and EE2 acute exposure. The most resistant life stage was N1 with LC_{50} values > 1500 μg L^{-1} and >5000 μg L^{-1}, respectively for E2 and EE2. For N3, C1-C3, and C6, sensitivity to estrogens decreased with age and survival was affected at concentrations above those detected in the environment reflecting low estrogens acute toxicity for these life stages. In contrast, embryonic stage revealed high vulnerability to E2 and EE2 acute effects. Embryos showed non-monotonic dose-response and hatching success was significantly reduced at low realistic concentrations of E2 (0.005, 0.5, and 5 μg L^{-1}) and EE2 (0.05 and 5 μg L^{-1}). Survival, development and sex ratio of *A. clausi* to EE2 exposure at 1 and 100 μg L^{-1} were also determined during a life cycle experiment. Fitness of the females of the generation F0 was evaluated by measuring lifespan, prosome length and egg production. The main observed effects were the decrease of females' prosome length, the feminization of the population and the reduction of the egg production for the generation F0 at 100 μg L^{-1} of EE2. This concentration is above those reported in the environment indicating the tolerance of *A. clausi* to EE2 at environmentally relevant concentrations.

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1. Introduction

Estrogens are emerging contaminants of concern and are referred to as endocrine-disrupting chemicals (EDCs) which can interact and disrupt the endocrine system functions in living organisms (Sonnenschein and Soto, 1998; Adeel et al., 2017). Based on their origin, estrogens can be divided into two groups. Natural estrogens, such as the 17β-estradiol (E2) secreted by vertebrates, play a key role in the regulation of sexual development and reproduction. Synthetic estrogens, such as the 17α-ethinylestradiol (EE2) derived from E2, are mostly used in oral contraceptive pills for hormone replacement therapy (Adeel et al., 2017). The excessive use of estrogens can lead to their possible release into terrestrial and aquatic ecosystems through numerous sources like human and animal excretions, agricultural runoff, as well as the discharge of industrial, hospital, and domestic sewage wastes into natural receptors (Pal et al., 2010).

Estrogen concentrations vary among regions and ecosystems mainly due to differences of sewage plant treatment efficiency and to the fate of estrogens in the environment (*e.g.*, biotransformation, sorption, dispersion, photolysis) (Pal et al., 2010; Adeel et al., 2017). The occurrence of both estrogens in aquatic marine environments was reported at concentrations ranging from 0.7 to 62 ng L^{-1} for E2 and from 0.4 to 6.86 ng L^{-1} for EE2 (Pal et al., 2010; Nazari and Suja, 2016). The highest reported values of E2 and EE2 were reported in the Venice lagoon and were equal to 175 ng L^{-1} and 43 ng L^{-1}, respectively (Pojana et al., 2004). Both estrogens are known by their low solubility (EE2 being the least soluble), their lipophilicity and their high absorption rate to organic matter which can explain their low concentrations in the water column in comparison with those detected in soil and sediment (Adeel et al., 2017).

The presence of steroid estrogens in aquatic ecosystems, even at low concentrations, can result in serious ecological risks due to their high estrogenic activity, their toxicity as well as their pseudo-persistence since they are continuously released into the environment (Laurenson et al., 2014). A wide range of negative effects associated with estrogen exposure has been reported previously in several aquatic organisms (vertebrates and invertebrates) such as a perturbation of endocrine and reproductive systems, alteration of the detoxification and apoptotic responses, and impairment of physiological processes (mineral homeostasis and immune function) (Tarrant et al., 2004; Souza et al., 2013). In addition to their endocrine-disrupting effects, these estrogens are also known to have genotoxic and cytotoxic effects in fish *Cyprinus carpio* at environmental concentration of 1 μg L^{-1} after 24 h (Orozco-Hernández et al., 2018).

Copepods are good bio-indicators of ecosystem health status due to their high sensitivity to environmental disturbances and climate change (Hussain et al., 2020). They play a key role in aquatic food webs as main secondary producers and as a vital link between phytoplankton and higher trophic levels (Hussain et al., 2020). They can accumulate in their tissues a considerable amount of contaminants such as estrogens and transfer them to higher trophic levels (Cailleaud et al., 2011). Despite their key role in ecosystems, available data regarding estrogens toxic effects in copepods remain scarce and lead to contradictory conclusions. Some studies did not report any negative effects on copepods development and reproduction under chronic exposure to E2 and EE2 at concentrations ≤100 μg L^{-1} (Hutchinson et al., 1999; Breitholtz and Bengtsson, 2001), while others did (Marcial et al., 2003; Forget-Leray et al., 2005). Moreover, copepods' life history and community structures can be strongly impacted by EE2 at environmental concentrations (10 ng L^{-1}) as it can alter the molting process and adult recruitment in calanoïd copepods, but also calanoïd to cyclopoïd copepod ratio (Souza et al., 2013). Bang et al. (2010) observed morphological deformities (shrinking and swelling of the urosome, loss of swimming legs, abnormal segmentation of antennules, *etc.*) in the harpacticoid copepod *Tigriopus japonicus* after E2 exposure ranging from 1 to 100 μg L^{-1}. The ability of EE2 to generate oxidative stress and to elicit apoptosis in calanoïd copepods has also been noted at 10-1000 ng L^{-1} (Souza et al., 2013). Both estrogens were reported to negatively affect the survival of adult copepods under acute exposure at concentrations in the range of mg L^{-1}, higher than those detected in the aquatic environment (Andersen et al., 2001; Breitholtz and Bengtsson, 2001).

To our knowledge, there is no study that compares the sensitivity of copepod life-stages under estrogens acute exposure. Moreover, laboratory studies on copepod life-cycle evaluating reproduction, development, and sex-differentiation sensitivity to EDCs have been focused mainly on harpacticoid copepods which present different biological (physiology, metabolism, morphology) and ecological (life strategies) characteristics in comparison to calanoïd copepods (Hutchinson et al., 1999; Andersen et al., 2001; Breitholtz and Bengtsson, 2001). The calanoïd copepod *Acartia clausi* is a suitable biological model for ecotoxicology tests due to its ubiquitous occurrence, short life-cycle, small size and high sensitivity to environmental disturbances and contaminants (Carotenuto et al., 2020; Tato et al., 2018).

The present study aims to (1) investigate the lethal toxic effects of E2 and EE2 under acute exposure on various developmental stages of *A. clausi* in order to compare their apparent sensitivity, (2) evaluate the sublethal effects of chronic exposure to EE2 on *A. clausi* by investigating different life cycle endpoints: adult survival rate, development from embryo to adult and sex-ratio. Fitness of the females of the F0 generation was also estimated by measuring egg production rate (EPR), female's lifespan and prosome length (PL). The present study aims to expand our knowledge on the effects of estrogens on copepods as well as to provide, for the first time, information on *A. clausi* life stages sensitivity to estrogens under acute and chronic exposures.

Defining water quality guidelines is essential for the regulation of emerging pollutant discharge in the aquatic environment and the protection of aquatic life against their severe acute and long term effects on survival, growth and reproduction. Acute and chronic data derived from this study could be used for the elaboration of the aquatic life criteria of estrogens (Criterion Maximum Concentration (CMC) and Criterion Continuous Concentration) which requires laboratory ecotoxicological information on the most sensitive life stages of aquatic species (namely median lethal concentrations (LC_{50}) or median effect concentrations (EC_{50}) (Stephan et al., 1985).

2. Materials and methods

2.1. Copepods sampling

Sampling was carried out during the autumns 2018 and 2019. Zooplankton was collected with a WP2 plankton net (200 μm mesh size) by horizontal hauls at a fixed station (43°23′59.10″N; 3°36′37.15″E) in Thau lagoon. Temperature varied between 16 and 20 °C and salinity was of 37 psu during the samplings. The zooplankton was maintained in cool boxes and immediately transported to the laboratory within 1 h after collection.

2.2. Copepods cultures

About 300 ovigerous females of *A. clausi* were isolated and incubated for 48 h in a 5 L beaker filled with 0.2 μm filtered natural seawater with a gentle permanent aeration. To avoid egg predation by adults, females were separated from their eggs using a perpex chamber with a 180 μm mesh false bottom. The temperature was maintained at 20 °C since egg production and hatching success are optimum at this temperature for *A. clausi* (Castro-Longoria, 2003). A light: dark regime of 14:10 h was used to prevent the production of diapause eggs (Chinnery and Williams, 2004). Copepods were fed with a mixture of the haptophyte *Isochrysis galbana* and the chlorophyte *Tetraselmis* sp. at a carbon ratio of 1:1 provided *ad libitum* (*>300* μg *C* L^{-1}) (Klein Breteler and Schogt, 1994). Algal cell concentration was estimated

daily and adjusted from counts on a Malassez cell. The females were removed after 48 h. Consequently, all individuals were roughly of same age within the 5 L beaker. After egg hatching, about 450 nauplii with different ages (≤24 h old and >72 h old) were used to perform acute toxicity tests. Others were reared in the laboratory until copepodite stages were reached.

2.3. Estrogens stock solutions

The natural estrogen 17 β-estradiol (98% purity) and the synthetic estrogen 17 α-ethinylestradiol (98% purity) were supplied from Sigma Aldrich chemicals. Dimethylsulfoxide (DMSO, Sigma Aldrich, and purity ≥ 99.8%) was used as a carrier solvent. Stock solutions of 100 mg mL^{-1} were prepared by dissolving 100 g of E2 or EE2 in 10 mL of DMSO. The solutions were stirred to facilitate solubilization and were stored in darkness at 4 °C until use. Each stock solution was then used to make a further ten-fold dilution series in 100% DMSO. Nominal concentrations were prepared by the addition of 10 μL of the dilution series in 100 mL seawater. The final concentration of DMSO in the solvent control and in E2 and EE2 treatments was 0.0001% (v/v).

2.4. Toxicity test procedures

2.4.1. Lethal effects of short-term estrogens exposure on A. clausi life-stages

48 h acute toxicity tests were performed on five different life stages: i) *A. clausi* embryos, ii) nauplii 1 (N1: ≤24 h old), iii) nauplii 3 (N3: >72 h old), iv) copepodites (C1-C3) reared in the laboratory and on v) wild females (copepodite 6: C6). Individuals of the targeted stage were identified under a dissecting microscope and captured by a mouth pipette of proper size. The number of replicates varied according to the number of individuals available for each life-stage (Fig. 1A). For each stage, two control treatments were conducted: a seawater control (SW) and a solvent control (DMSO) (final DMSO concentration of 0.0001%). Experiments were performed using natural seawater, sampled from the Thau lagoon and filtered through a 0.2 μm filter. Experiments were run in glass vials of different volumes depending on the size and the number of individuals used in tests in order to keep a comparable density of individuals among the different tests (Fig. 1A). During acute tests, no food was provided to *A. clausi* life stages. The test solution was prepared 24 h prior to the beginning of the test. Tests were performed using the same conditions described above for the cultures.

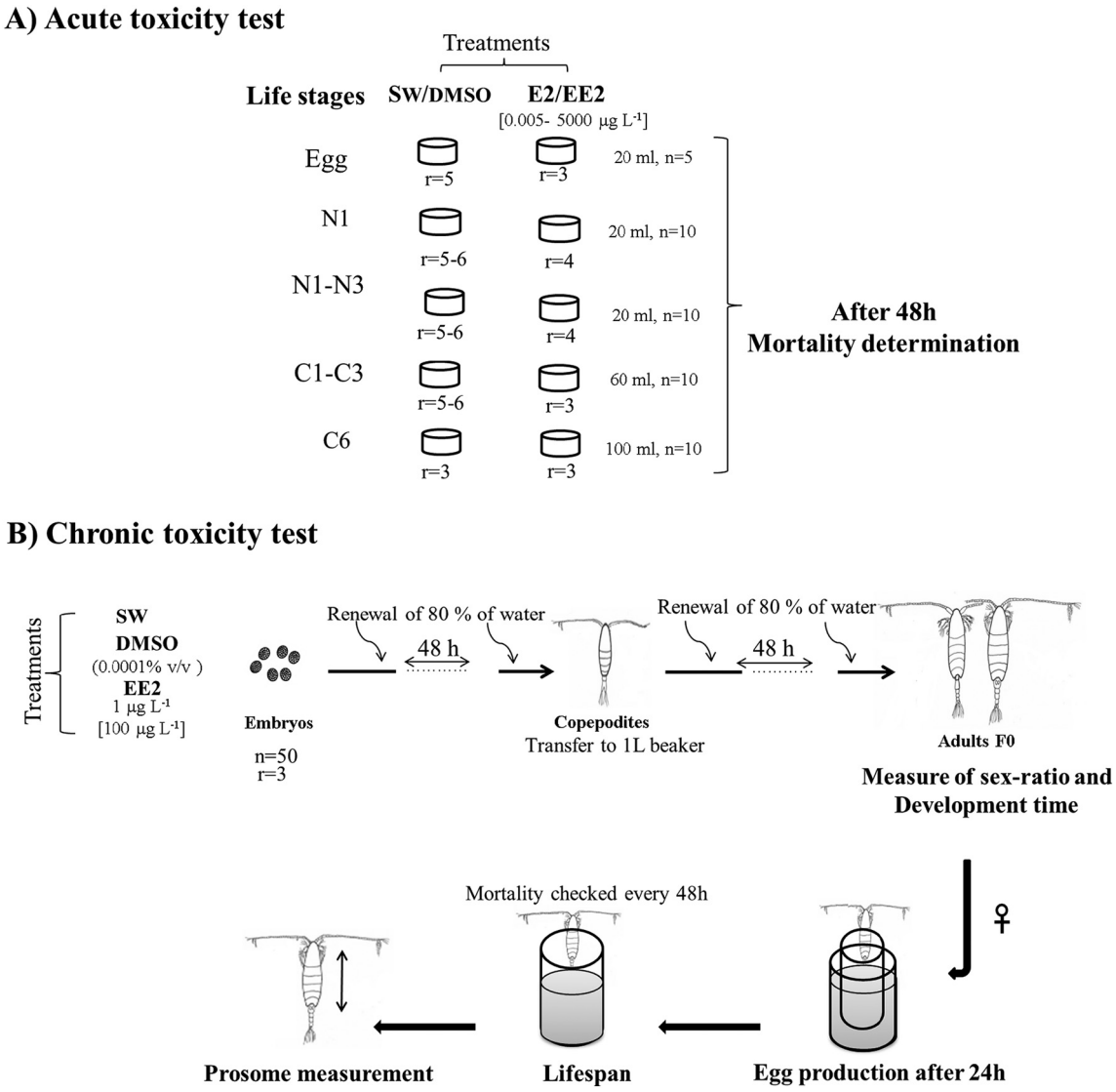

Fig. 1. Experimental setup of (**A**) acute (48 h) toxicity test using the copepod *A. clausi* exposed to concentrations of E2 and of EE2 ranging from 0.005 to 5000 μg L^{-1} and (**B**) chronic toxicity (20 days) test using the copepod *A. clausi* exposed to concentrations of EE2 of 1 and 100 μg L^{-1}. r = number of replicates; n = number of individuals per replicate; F0: generation 0 of *A. clausi*; SW: Sea Water control; DMSO: solvent control containing 0.0001% DMSO (v/v).

2.4.1.1. Embryos. Test with embryos was performed according to Gorbi et al. (2012) and the ISO 14669 protocol (ISO, 1999 revised in 2015) with some modifications. For each estrogen, about 240 embryos were used to test 7 nominal concentrations (0.005, 0.05, 0.5, 5, 50, 500, 5000 μg L^{-1}) ranging from concentration below or close to environmental concentrations (E2 = 0.83 ng L^{-1}; EE2 = 4.6 ng L^{-1}) (Pal et al., 2010) to environmentally unrealistic high levels of hormone. For each concentration, triplicates of 5 embryos groups were incubated in 20 mL petri dish filled with the tested solutions. For each hormone, controls in triplicates with SW and with the tested solvent DMSO were prepared.

2.4.1.2. Nauplii (N1 and N3). Tests with naupliar stages were run according to ISO 14669 (1999 revised in 2015) and Forget-Leray et al. (2005) with some modifications. Preliminary exposure tests were performed with a broader concentration range than those tested on embryos, before setting up the final test in order to better determine the concentration inducing 0% and 100% mortality. The final tests were conducted using six concentrations of E2 (5, 15, 50, 150, 550, 1300 μg L^{-1}) and EE2 (15, 50, 150, 550, 1500, 3300 μg L^{-1}). For each concentration, 4 groups of 10 nauplii were added to 60 mL vials containing 20 mL of tested solution. For the controls (SW and DMSO), 5 replicates were set up.

2.4.1.3. Copepodites (C1-C3). Acute tests on copepodites were carried out following the ISO 14669: 1999 revised in 2015 with slight modifications. As larger and older life-stage is expected to be more resistant, E2 and EE2 were tested respectively at concentrations of 50, 150, 550, 1300 μg $L^{-1}$and 150, 550, 1500, 3300 μg L^{-1}. For each concentration, triplicates of 10 copepodites groups were incubated in 70 mL vials. Controls were also carried out in triplicates.

2.4.1.4. Adult females (C5-C6). Acute tests on copepodites were carried out following the ISO 14669: 1999 revised in 2015 with little modifications. E2 and EE2 concentrations were tested at concentrations of 200, 400, 750, 1500 μg L^{-1} and 200, 550, 1700, 5000 μg L^{-1}, respectively. For each treatment and controls, 3 replicates were done. Groups of 10 females were incubated in 250 mL beakers.

During the test, none of the solutions was renewed or aerated. To assess the median lethal concentration (LC_{50}), dead individuals were counted at the end of the incubation. Animals were considered dead when they exhibited no swimming or appendage movements within 10s. Tests were considered valid if there was less than 20% of mortality in controls (SW and DMSO controls) for nauplius stage and less than 10% in controls for copepodite and adult stages. Embryos were considered dead when they did not hatch and changed color. The hatching success was calculated from the number of nauplii observed compared with the initial number of embryos incubated.

2.4.2. Sublethal effects of long-term EE2 exposure on A. clausi life cycle

Based on the results of EE2 lethal toxicity tests on females and published data for EE2 that have caused sublethal effects on copepods species, two nominal EE2 concentrations were chosen to study sublethal effects on *A. clausi* developmental cycle (Fig. 1B). Copepod life cycle experiments initiated with embryos were run at 1 μg L^{-1} and 100 μg L^{-1}, corresponding respectively to 1/1000 and 1/10 of LC_{50} of the most sensitive stage of *A. clausi* (N3), for 20 days under controlled laboratory conditions. Embryos were obtained from non-exposed *A. clausi* females sampled in the Thau lagoon, according to the above-described procedure. Three replicates for each EE2 concentration and controls (SW and DMSO) were set up using groups of 50 embryos in 250 mL beakers. Cultures were maintained in a laboratory incubator at 37 psu, 20 °C, under a fixed light:dark regime of 14:10 h. Each beaker was continuously aerated. Cohorts were fed *ad libitum* (>300 g C L^{-1}) with *Dunaliella salina* (20.000 cells mL^{-1}), *Nannochloris* sp. (10.000 cells mL^{-1}), and *I. galbana* (100.000 cells mL^{-1}) in the ratio of 1:1:1 from N2 to adult, as the first naupliar stage does not have a mouth (Baud et al., 2002). Algal cell concentration was estimated daily with a Malassez cell and adjusted from counts. Beakers were visually checked daily and when 50% of the cohorts had reached the copepodite stage, individuals were transferred to 1 L beakers. Due to EE2 half-life of 72 h (Ying et al., 2003), 80% of the incubation volume was renewed every 48 h and food was supplied at the same time. Prior to discarding old solution, three replicates of 20 mL were sampled in 30 mL tinted glass bottle from each condition to determine the concentration of EE2 at 2, 14 and 20 days.

At the end of the experiment, copepodites survival rates were determined for each treatment, and development time was calculated. C5 and C6 stage frequency distributions, from counts of males and females in each cohort, were used to calculate the cohort development time defined as the time needed for 50% of the individuals to reach the adult stage. For each treatment, males and females from triplicates were pooled to determine sex-ratios.

Adult females of the F0 generation were then selected and transferred to seawater to further explore the chronic exposure effect of EE2 by measuring several endpoints such as EPR, Female individual lifespan and PL.

For each cohort, ovigerous females were individually incubated in 250 mL glass beakers filled with 100 mL filtered seawater for 24 h with the same photoperiod and temperature used during the life cycle experiment. Due to the low abundance of F0 females, the number of replicates varied in each cohort (n = 6 to 17). Eggs were counted after 24 h under a Leica stereomicroscope and EPR was expressed as the number of eggs produced per female per day. The females' individual lifespan was then followed each 48 h until they died. Females were fed each 48 h *ad libitum*. When dead, the female PL (mm) was measured using a stereomicroscope.

2.5. EE2 analysis in during the life cycle experiment

In order to determine the EE2 concentrations after 2, 14 and 20 days of exposure, a competitive direct enzyme-linked immunosorbent assay (ELISA) was used to measure EE2 in the exposure media (Ecologiena, Japan), according to manufacturer's instructions. The protocol is described in details in Wernicke von Siebenthal et al. (2018). The absorbance was measured at 450 nm using a TECAN infinite microplate reader (Tecan Group Ltd., Switzerland) and the detection range was 0.05-3 μg L^{-1}.

2.6. Statistical analysis

The 48 h lethal concentration values (LC_{50}) for EE2 and E2 were calculated for each life stage using REGTOX, a curve fitting macro for Microsoft Excel (version 7.0.3. available at https://www.normalesup.org/~vindimian/en_index.html). The Hill model was used to calculate the 48 h LC_{50} of E2 and EE2 for each replicate. The LC_{50} are given as mean ± standard deviation.

Data from lethal and sublethal tests are presented as means associated with their standard deviation (SD). Prior to parametric analysis, data were tested for normal distribution and homogeneity of variance and were log-transformed, if necessary. When the assumption was met, one-way ANOVA with a Tukey post-hoc test was performed to determine significant difference between DMS and exposure conditions. If the assumption was not met, data were analyzed using a non-parametric Kruskal-Wallis test by ranks, followed by Mann/Whitney tests to highlight which treatment was different from DMS in acute and chronic tests. Data analysis was performed using SPSS (version 18). The level of significance was set at $p < 0.05$.

3. Results

No statistically significant differences were determined between SW and DMSO controls in terms of studied endpoints in acute and chronic tests ($p > 0.05$). Therefore, data for SW are not shown and DMSO was considered as the control (*i.e.* solvent control) in subsequent analyses.

3.1. Lethal effects of short-term estrogens exposure on A. clausi life-stages

The results obtained by exposing various life-stages of *A. clausi* to E2 and EE2 for 48 h are shown in Figs. 2 (Embryos) and 3 (N1, N3, C1-C3, C6).

3.1.1. Embryos

Lethal effects of E2 and EE2 on *A. clausi* embryos were assessed by measuring hatching success (HS, Fig. 2). Eggs hatching success showed a U-shaped curve in response to 48 h estrogens exposure. E2 induced a significant decrease in HS at low concentrations of 0.005 μg L^{-1}, 0.5 μg L^{-1}, and 5 μg L^{-1}, under which HS was equal to 76.7% ± 5.8 compared to 96.7% ± 5.8 recorded in DMSO ($p < 0.05$). Similarly to E2, HS was significantly reduced at low concentrations of EE2 of 0.05 and 5 μg L^{-1}, for which it was equal to 83.3% ± 5.8 in comparison with DMSO showing a 96.7% ± 5.8 HS ($p < 0.05$). For both estrogens, the higher concentrations, ranging from 50 to 5000 μg L^{-1}, exhibited no negative effect on HS ($p > 0.05$).

3.1.2. Nauplii (N1 and N3)

After 48 h, N1 survival was not affected by E2 or EE2 exposure (Fig. 3). For both estrogens, survival rates were more than 70% for all tested concentrations, with no significant difference between DMSO and estrogens' treatments ($p > 0.05$). LC_{50} was not calculated for N1 due to their tolerance to estrogens acute exposure.

In contrast to N1, the life stage N3 exhibited a significant survival reduction following estrogen exposure (Fig. 3) ($p < 0.05$). This pattern was significant at concentrations ranging from 150 μg L^{-1} to 1300 μg L^{-1} of E2, for which survival varied between 85% and 0%, respectively and from a concentration of 150 of EE2 for which survival was 72.5%. No nauplii survived after exposure to the highest concentration of 3300 μg L^{-1} of EE2 ($p < 0.05$). LC_{50} values for N3 are 601 ± 9 μg L^{-1} and 1068 ± 376 μg L^{-1} for E2 and EE2, respectively (see Figs. S1 and S2, Supplementary data and Table 1).

3.1.3. Copepodites (C1-C3)

Exposure to E2, up to a concentration of 150 μg L^{-1}, had no significant effect on C1-C3 survival compared with control (Fig. 3). A significant effect ($p < 0.05$) was observed at the two E2 highest concentrations of 550 μg L^{-1} and 1300 μg L^{-1} for which 77.5% ± 5 and less than 30% of the exposed copepodites were still alive, respectively. For EE2, the copepodites survival was significantly reduced at concentrations ranging from 550 μg L^{-1} to 3300 μg L^{-1} at which survival varied between 76.7% ± 5.7 and 0% after 48 h ($p < 0.05$). LC_{50} values for copepodites C1-C3 were 1119 ± 76 μg L^{-1} and 1207 ± 195 μg L^{-1} for E2 and EE2, respectively (see Figs. S3 and S4, Supplementary data and Table 1).

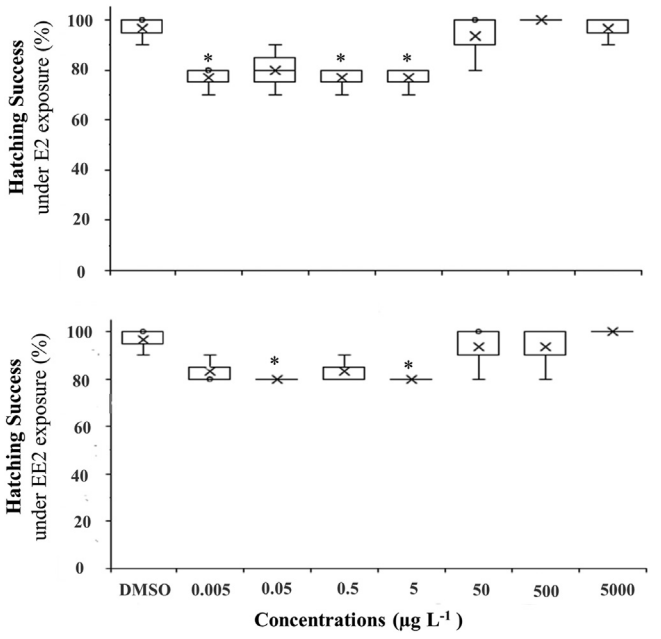

Fig. 2. Hatching success of *A. clausi* eggs after 48 h of exposure to solvent (DMSO 0.0001% v/v) controls, E2, and EE2. Boxes extend from the lower (25) to the upper (75) quartile with an internal segment for the median. Crosses "×" indicate mean values. Asterisks (*) indicate a significant difference from the DMSO (solvent control) (Kruskal-Wallis, $p < 0.05$). (5 replicates for control and 3 for treatments).

3.1.4. Adult females

Females' survival rates decreased significantly, in comparison to control, at E2 concentrations of 750 μg L^{-1} and 1500 μg L^{-1} for which survival rates varied between 50% and 63% ($p < 0.05$). Similarly, EE2 has a significant negative effect on survival rates, starting at concentrations of 550 μg L^{-1}, reaching 0% at the highest concentration 5000 μg L^{-1} of EE2 ($p < 0.05$). Adult females LC_{50} values are 1422 ± 265 μg L^{-1} and 1216 ± 176 μg L^{-1} for E2 and EE2, respectively (see Figs. S5 and S6, Supplementary data and Table 1).

3.2. Sublethal effects of long-term EE2 exposure on A. clausi life cycle

3.2.1. EE2 analysis

The measured concentrations of EE2 in the chronic test are shown in Table 2. After 48 h, EE2 measured concentrations were half the nominal concentrations. Results showed an increase of measured EE2 concentrations over time. After 14 days, measured EE2 concentrations were equal to 0.9 ± 0.4 μg L^{-1}, at 1 μg L^{-1} nominal concentration, and 48.3 ± 15.3 μg L^{-1} at 100 μg L^{-1} nominal concentration. The highest measured EE2 concentrations were observed after 20 days of exposure, where the measured EE2 concentrations were equal to 1.2 ± 0.1 and 88.0 ± 18.3 at 1 μg L^{-1} and 100 μg L^{-1} nominal concentrations, respectively.

3.2.2. Survival, development time and sex-ratio in the F0 generation

Results of *A. clausi* survival rates after 14 days and 20 days of exposure to EE2 at 1 μg L^{-1} and 100 μg L^{-1} are shown in Table 3. Chronic exposure to EE2 at 1 μg L^{-1} did not significantly affect survival rates (54.7% ± 21.6) of *A. clausi* after 14 days ($p > 0.05$). At the highest concentration of 100 μg L^{-1} of EE2, survival rate was significantly lower (14% ± 3.5) than the one observed for DMSO (28% ± 3.4) ($p < 0.05$). Survival rates were not constant over time and decreased with increasing time in different cohorts (control and EE2 concentrations). After 20 days, survival rates in DMSO, at 1 μg L^{-1} and at 100 μg L^{-1} were equal to 8.7% ± 6.4, 24% ± 6.9 and 10% ± 5.3, respectively. No significant difference was observed in survival rates between DMSO and EE2 treatments (1 μg L^{-1} and 100 μg L^{-1}) after 20 days ($p > 0.05$).

Exposure to EE2 at sublethal concentrations of 1 and 100 μg L^{-1} did affect the development of *A. clausi*. The duration of copepod development from embryos to C5-C6 in DMSO was 16.3 ± 0.6 days and were 16.4 ± 0.8 days and 16.9 ± 0.6 days for 1 μg L^{-1} and 100 μg L^{-1} of EE2, respectively (Table 3). No significant difference was detected among groups (estrogens and DMSO) ($p > 0.05$).

Results of sex ratio are shown in Table 3. The sex ratio (F/M) was higher for copepods exposed to 100 μg L^{-1} of EE2 (2.75) than for DMSO (1.25) and 1 μg L^{-1} of EE2 (1.25).

3.2.3. Prosome length, lifespan and reproductive traits in females of the F0 generation

Adult females' lifespan is presented in Table 3. There was no significant difference between groups and adult females' lifespan was equal to 12.1, 14.2 and 10.4 days for females reared in DMSO, 1 μg L^{-1} and 100 μg L^{-1}, respectively ($p > 0.05$).

Values of PL of the F0 females are indicated in Table 3 and varied between 0.58 ± 0.02 mm and 0.63 ± 0.01 mm. There was no significant

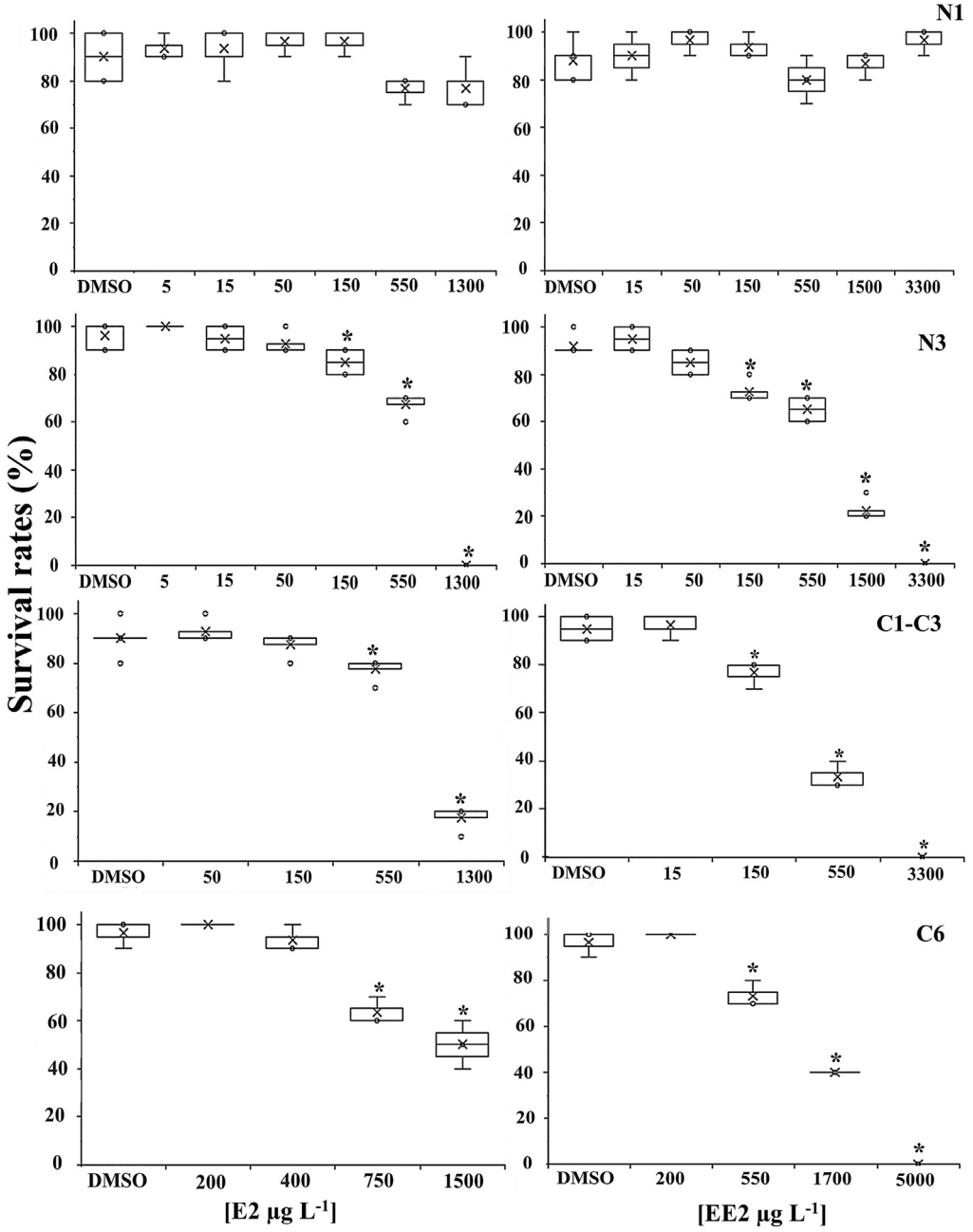

Fig. 3. Survival rates (%) of one day old nauplii: N1, three days old nauplii: N3, copepodites: C1-C3, and adult females: C6 *A. clausi* life stages after 48 h of exposure to E2 and EE2 at concentrations ranging from 0 to 5000 µg L^{-1}. Boxes extend from the lower (25) to the upper (75) quartile with an internal segment for the median. Crosses "×" indicate mean values. Asterisks (*) indicate a significant difference from the DMSO (solvent control) (Kruskal-Wallis, $p < 0.05$).

difference between individuals cultured in DMSO and 1 µg L^{-1} of EE2 ($p > 0.05$). However, a significant reduction in PL was observed for females reared at the highest concentration of 100 µg L^{-1} of EE2 and DMSO ($p < 0.05$).

In order to investigate whether EE2 exposure during development impacts reproductive capacities, EPR was measured in F0 females incubated in seawater for 24 h (Fig. 4). A statistical difference in the number of eggs produced by females was noticed between the different treatments ($p < 0.05$). EPR of females raised with 1 µg L^{-1} of EE2 did not differ from EPR in control ($p > 0.05$). In contrast, EPR of females reared at 100 µg L^{-1} of EE2 was significantly low 1.16 ± 0.75 eggs $female^{-1}$ day^{-1} compared to 12.6 ± 9.08 eggs $female^{-1}$ day^{-1} in control ($p < 0.05$).

Table 1
Lethal concentrations (LC_{50}) in *Acartia clausi* life stages (N1, N3, C1-C3, C6) exposed to estrogens E2 and EE2 for 48 h, with associated 95% confidence intervals (CI). LC_{50} values are expressed as µg L^{-1} and as means ± Standard Deviation.

Life stages	N1	N3	C1-C3	Female C6
E2 (µg L^{-1})	>1500	601 ± 9 (501-1488)	1119 ± 76 (567-1199)	1422 ± 265 (906-1812)
EE2 (µg L^{-1})	>5000	1068 ± 376 (544-699)	1207 ± 195 (815-1402)	1216 ± 176 (936-1697)

Table 2
Nominal and measured concentrations of EE2 (μg L^{-1}) at 2, 14 and 20 days of the test. 80% of the test medium were changed every 48 h. Data are expressed as means ± Standard Deviation.

	Measured concentrations		
Nominal concentrations	2 days	14 days	20 days
1 μg L^{-1}	0.4 ± 0.1	0.9 ± 0.4	1.2 ± 0.1
100 μg L^{-1}	51.9 ± 3.7	48.3 ± 15.3	88.0 ± 18.3

4. Discussion

4.1. Lethal effects of short-term estrogens exposure on A. clausi life-stages

Results from acute tests performed on various life-stages of *A. clausi* (Embryos, N1, N3, C1-C3, and C6) showed high LC_{50} values of E2 and EE2, which reflect the relatively low toxicity of these estrogens for the copepod *A. clausi*. Estrogens mortality effects on various crustacean species, essentially copepods, are reported in Table 4. Our results are in line with findings recorded previously indicating that estrogens concentrations affecting crustacean species survival, under short-term exposure, are well above those occurring in the marine aquatic environment (Pal et al., 2010). For example, the recorded 48 h LC_{50} values for N1 *A. clausi* in the present study were >1500 μg L^{-1} and >5000 μg L^{-1}, respectively for E2 and EE2, in the same range as those reported by Goto and Hiromi (2003) and Brennan et al. (2006) for the neonates of *D. magna* (48 h LC_{50} = 2870 μg L^{-1} and >5000 μg L^{-1}, respectively for E2 and EE2). Data reported in the literature on copepods refer mainly to the adult stage and exposure durations are very diverse, making the comparison with our 48 h LC_{50} values delicate. The sensitivity of *A. clausi* adult stage to E2 (48 h LC_{50} = 1422 μg L^{-1}) is similar to that of *Acartia tonsa* (48 h LC_{50} > 1000 μg L^{-1}) but lower than for the harpacticoid copepod *T. japonicus* (48 h LC_{50} = 3350 μg L^{-1}), suggesting an apparent species-specific sensitivity to estrogens acute exposure, as observed by Andersen et al. (2001) and Marcial et al. (2003).

Furthermore, our results reveal a life-stage-specific sensitivity to E2 and EE2 acute exposure. There is a general opinion that earlier life stages of crustacean species are more vulnerable to environmental stress than older stages (Kulkarni et al., 2013). However, early life-stage vulnerability is not a constant rule for all copepods and as reported by Jager et al. (2016) who stated that there is no general pattern in copepods life stages sensitivity. Based on the estimated 48 h LC_{50}, N1 of *A. clausi* evidenced the highest resistance to both estrogens lethal effects in comparison to the other life stages. In contrast, N3, C1-C3 and C6 showed different responses to both estrogens which might be linked to estrogens' mode of toxic action. N3 (LC_{50} = 601 μg L^{-1}) displayed a higher sensitivity to E2 than C1-C3 (LC_{50} = 1119 μg L^{-1}) which were slightly more sensitive than C6 (LC_{50} = 1422 μg L^{-1}). Whereas, under EE2 exposure, no sensitivity difference was observed among C1-C3 (LC_{50} = 1207 μg L^{-1}) and C6 (LC_{50} = 1216 μg L^{-1}) which were relatively more resistant to EE2 than N3 (LC_{50} = 1068 μg L^{-1}).

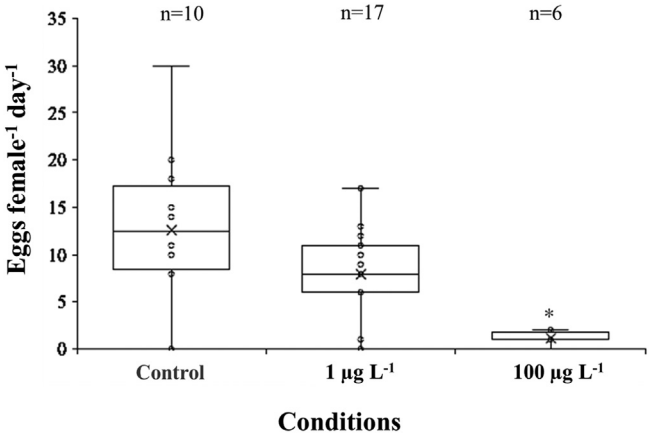

Fig. 4. Number of eggs produced by F0 females, raised in different conditions, incubated in seawater for 24 h expressed as eggs $female^{-1}$ day^{-1}. Boxes extend from the lower (25) to the upper (75) quartile with an internal segment for the median. Crosses "×" indicate mean values. Control corresponds to the DMSO and SW. The asterisk (*) indicates a significant difference from the control (Kruskal-Wallis, $p < 0.05$). n values denote the number of females incubated in seawater.

Embryos were the most sensitive life-stage to both estrogens and presented negative effects in HS at low concentrations (E2: 0.005 μg L^{-1}, 0.5 μg L^{-1}, and 5 μg L^{-1} and EE2: 0.05 and 5 μg L^{-1}). Results from the present study corroborate previous findings observed for the marine copepod *Calanus finmarchicus* exposed to organic chemicals water-soluble fractions of fresh and weathered oil, which are lipophilic contaminants like estrogens, where older, larger, and more lipid-rich life stages turn out to be more sensitive than nauplii (Hansen et al., 2011). Similar results have been asserted in the harpacticoid copepod *Nitocra lacustris* exposed to phenanthrene where no sensitivity difference has been observed between nauplii and copepodites, whereas adult females evidenced more vulnerability to this pollutant than all other stages (Lotufo and Fleeger, 1997). Moreover, Calbet et al. (2007) did not observe any difference in mortality between naupliar and adult stages of the marine copepod *Paracartia grani* to polycyclic aromatic hydrocarbon.

The difference in sensitivity among copepod life-stages may either be explained by the difference in intrinsic sensitivity as well as the toxicokinetics processes difference (uptake and elimination processes of pollutant), or may either result from the way physiological processes interact to produce the observed effect as stated by Jager et al. (2016). Differences in lipid content among life stages might also be an additional factor causing sensitivity difference especially under exposure to hydrophobic pollutants such as estrogens. High lipid content in advanced life stages can protect them against short-term acute toxicity of pollutants as it can be immobilized in the lipids reservoirs (Hansen et al., 2016; Jager et al., 2016). Anatomy and physiology differences can also explain in part the result observed in *A. clausi*, since the digestive apparatus is not differentiated in the N1 stage in opposition to the next life stages for which mouth and anus are both present and functional (Baud et al., 2002). Copepods may take up pollutants *via* their

Table 3
A. clausi life-history parameters under EE2 chronic exposure. Adults survival rates (%), development time from embryos to adult (DT, days), sex ratio (F/M), adult female lifespan (days), and female PL (mm) measured in *A. clausi* adults of the generation F0. Values are mean ± Standard Deviation (SD), except for sex-ratio.

Treatments	*A. clausi* life-history parameters					
	Survival rates (%)		DT (days)	Sex- ratio (F/M)	Lifespan (days)	PL (mm)
	14 days	20 days				
DMSO	28 ± 3.4	8.7 ± 6.4	16.3 ± 0.6	1.25	12.1 ± 7.5	0.63 ± 0.01
1 μg L^{-1} EE2	54.7 ± 21.5	24 ± 6.9	16.4 ± 0.8	1.25	14.2 ± 4.7	0.62 ± 0.01
100 μg L^{-1} EE2	14 ± 3.5*	10 ± 5.3	16.9 ± 0.6	2.75	10.4 ± 4.8	0.58 ± 0.02*

The asterisk (*) indicates a significant difference from the DMSO (solvent control) (Kruskal-Wallis, $p < 0.05$ or one-way ANOVA, $p < 0.05$).

Table 4
Lethal concentration LC_{50} (μg L^{-1}) of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) for different life-stages of *A. clausi* and other invertebrate aquatic species.

Taxonomic groups	Species	Life stages	Incubation time	Lethal Concentrations (LC50) (μg L^{-1})		References
				E_2	EE_2	
Copepods	*Acartia clausi*	Nauplii N1	48 h	>1500	>5000	Present study
		Nauplii N3		601	1068	
		Copepodite C3		1119	1207	
		Adults		1422	1216	
	Acartia omorii	Adults	96 h	–	>100	Hossain, 2004
	Acartia tonsa	Adults	48 h	>1000	1100	Andersen et al., 2001
	Eurytemora affinis	Nauplii	96 h	45	–	Forget-Leray et al., 2005
	Nitocra spinipes	Adults		1600	510	Breitholtz & Bengtsson, 2001
	Tisbe battagliai	Nauplii	21 days	≥100	≥100	Hutchinson et al., 1999
	Tisbe japonicus	Adults	48 h	3350	–	Marcial et al., 2003
Cladocerans	*Daphnia magna*	Adult	24 h	10,000	–	Brennan et al., 2006
		neonates (<24 h)	48 h	2870	–	
				–	>5000	Goto and Hiromi, 2003
	Diaphanosoma celebensis	Adults	24 h	10,370	–	Marcial and Hagiwara, 2007
Amphipods	*Gammarus pulex*	Juveniles	10 days	–	840	Watts et al., 2001

digestive tract when they feed (phytoplankton, detritus, and bacteria) or *via* their body surface (exoskeleton) (Wang and Fisher, 1998). Consequently, the most possible estrogen absorption pathway for N1 could be the exoskeleton. Nevertheless, the exoskeleton can constitute a barrier against the effect of various contaminants, which can allow them to cope with the toxicity of some pollutants (Yoon et al., 2019). The reasons for N1 *A. clausi* estrogens resistance remain unclear, but we suggest that this tolerance might be explained by a moderate uptake of estrogens by earlier life stages relative to the older ones.

EDCs such as estrogens and Bisphenol A are frequently associated with non-monotonic dose-response (Lagarde et al., 2015). Both estrogens exhibited a U-shape curve for *A. clausi* embryos (Fig. 2) which suggests a non-monotonic dose-response. This response is consistent with previously reported results in the crustacean species *Daphnia pulex* and *Americamysis bahia* under acute exposure (96 h), where EE2 displayed high toxicity at low concentrations (Daigle, 2010). Alteration of HS at environmentally realistic concentrations indicates that the presence of these chemicals in the aquatic environments may alter the dynamics and functioning of ecosystems by impairing eggs hatching. The high vulnerability of *A. clausi* embryos suggests that the embryonic stages of crustacean species can be good bio-indicators of acute toxic effects of estrogens. The present study reveals that the evaluation of estrogens toxicity at high concentrations, like those tested in the present work (50 μg L^{-1}, 500 μg L^{-1} and 5000 μg L^{-1}), can underestimate the toxicity of pollutants as they cannot predict their toxic effects, observed at low concentrations, in some aquatic species. Additional works are needed to assess the molecular mechanism behind such non-linear relationships.

4.2. Sublethal effects of long-term EE2 exposure on A. clausi life cycle

4.2.1. Survival rate, development time, and sex-ratio in the F0 generation

Full life cycle studies can highlight the toxic effects of contaminants that only manifest after long-term exposure. The life cycle experiment showed low survival rates of *A. clausi* raised in the laboratory which is not in agreement with the commonly accepted criteria for chronic tests conducted on crustacean species such as harpacticoid copepods (control survival ≥ 70%) and cladoceran species like *Daphnia magna* (control survival > 80%). *Acartia* species show low densities when cultured in the laboratory in comparison to harpacticoid copepods (Abolghasem et al., 2011), thus the validity criterion of survival ≥70% in controls seems difficult to reach for calanoid copepods especially *Acartia* species.

To our knowledge, there are no standardized methods for acute and chronic toxicity tests with the copepod *A. clausi*. Previous ecotoxicological studies with *A. clausi* have focused mainly on sublethal effects of pollutants (eggs viability, respiration, uptake, and accumulation) under acute exposure and no study has been dedicated to evaluate chronic effects of contaminants on *A. clausi* using full life cycle (Carotenuto et al., 2020). *A. clausi* has been proposed in ecotoxicology bioassays as an alternative model species to *A. tonsa,* for which an internationally harmonized test OECD method is available for reproductive and developmental effects of endocrine disrupters (ICRAM-APAT, 2007; Kusk and Wollenberger, 2007). For this reason, our results on survival rates were compared to those recorded in *A. tonsa*. Results of survival rates in both controls (27% for SW and 28% for DMSO) after 14 days are close to those reported previously in *A. tonsa* after 14 days of laboratory culture where the validity criterion for survival in mass culture control was not fulfilled because only 30 to 40% of survival rates were observed in the controls (Barata et al., 2002; Kusk and Wollenberger, 2005). In the present study, adult survival rates of *A. clausi* in controls decreased after 14 days as observed previously by Abolghasem et al. (2011). Moreover, Medina and Barata (2004) indicated that survival of *A. tonsa* was less than 43% after 10 days in all cultures and decreased to less than 12% after 26 days. *A. clausi* population density in the culture can be influenced by numerous intrinsic and extrinsic factors. Among the intrinsic factors, the important natural mortality of naupliar stages induces a decline of the population size (Uye, 1982; Kusk and Wollenberger, 2005). Mortality in early-stages can be linked to the shifts from yolk-related endogenous foods to exogenous sources and to the morphological and physiological changes related to the metamorphosis of nauplii into copepods (Almeda et al., 2010). Water physicochemical parameters may also act as limiting factors for the development, growth and survival of copepods. Temperature (20 °C), salinity (37 psu) and photoperiod (light: dark regime of 14:10 h) of our experiments are well inside the optimal environmental range of *A. clausi* in Thau lagoon and are suitable for its development in laboratory conditions (Person-Le Ruyet, 1975; Klein Breteler and Schogt, 1994; Abolghasem et al., 2011). The exchange of 80% of water each 48 h should have avoided the degradation of water quality parameters.

Food plays also an important and major role in copepods culture as it controls their growth and their population density (Zhang et al., 2013; El-Tohamy et al., 2021). In this study, a mixture of three algae (*I. galbana*, *D. salina*, N. sp.) was used to feed *A. clausi* as monoalgal diets do not meet the nutritional requirement of copepods namely *Acartia sp* (Zhang et al., 2013; Dayras et al., 2021). The suitability of these algae in terms of nutritive value for crustacean species has been previously confirmed (Støttrup and Jensen, 1990; Alvarez-González et al., 2001; Payne and Rippingale, 2001). Our experiment was conducted at satiating food levels where a sufficient carbon concentration (>300 μg C L^{-1}) was provided in order to meet the carbon requirement of this copepod (Klein Breteler and Schogt, 1994; Zhou et al., 2016).

Other factors could have partially enhanced *A. clausi* mortality in the present study such as the handling and the filtration of organisms during water renewal. The high natural mortality of Acartia sp. observed in control conditions in the present study and previous works (Barata et al., 2002; Kusk and Wollenberger, 2005) clearly suggest revising the validity criterion during chronic tests (Kusk and Wollenberger, 2005).

The development time of *A. clausi*, from embryos to adulthood, was around 16 days, which is faster than the duration of 20 days reported by Person-Le Ruyet (1975) at the same temperature of 20 °C. This indicates that the controlled conditions fixed for the culture of this copepod in this study were convenient for development of *A. clausi* culture chronic exposure to EE2 did not affect the duration to reach the adult stage even at the highest tested concentration of 100 μg L^{-1}. Interestingly, this concentration is much higher than the maximum recommended concentration of EE2 to protect biota in surface water (0.35 ng L^{-1}) (Caldwell et al., 2008), suggesting that the development of crustacean species is not a sensitive endpoint to EE2 chronic effect. Data supporting our results have been observed in the harpacticoid copepods *Nitocra spinipes* and *Tisbe battagliai* where EE2 concentrations in the range of 50-100 μg L^{-1} did not impact nauplius to adult development in these species (Hutchinson et al., 1999; Breitholtz and Bengtsson, 2001). Copepods might be able to eliminate EE2, as observed in the estuarine copepod *E. affinis* (Cailleaud et al., 2011), since crustacean species can biodegrade vertebrate steroids to glucose and sulfate conjugate (Hutchinson et al., 1999; Cailleaud et al., 2011). Copepods could therefore potentially cope with the toxic effects of EE2 by biodegradation into innocuous compounds.

Available data regarding the effects of EE2 on development and molting processes in copepod species are variable. For example, Andersen et al. (1999) indicated a stimulation of female gonads maturation in the copepod *A. tonsa* at 23 μg L^{-1} of E2. In contrast, Andersen et al. (2001) reported an inhibition of naupliar development in *A. tonsa* by EE2 at concentrations of 88 μg L^{-1}, concentration measured in our experiment after 20 days, which may suggest that *A. tonsa* was more sensitive to EE2 chronic effect than our target species *A. clausi*. Moreover, Forget-Leray et al. (2005) and Marcial et al. (2003) reported a delay in naupliar development of the calanoid copepods *Eurytemora affinis* and *T. japonicus* at concentrations of 6 and 1 μg L^{-1} of E2, respectively. It is worth noting that the physiological and molecular key mechanisms behind estrogens' negative effects on reproduction and development of crustacean species remain unclear. The development of calanoid copepods includes 12 different postembryonic stages with 6 naupliar and 6 copepodite stages, the last being the adult stage. Molting processes and metamorphosis in crustacean species are regulated by the ecdysteroids system and metamorphosis is presumably controlled by compounds similar to juvenile hormones controlling insect development (Lafont and Mathieu, 2007). Previous studies have suggested that the ability of estrogens to disrupt copepods might be due to the structural features of estrogens allowing them to bind ecdysteroid receptors in the same way that they might bind estrogenic receptors preventing endogenous ecdysteroids from binding and activating their receptor (Forget-Leray et al., 2005).

Sex ratio in DMSO showed the dominance of females in the population with in a higher proportion of females (79%) in comparison to solvent control 53%, indicating a feminization of *A. clausi* population through EE2 exposure. Unlike previous studies that did not report a significant effect of EE2 (50–100 μg L^{-1}) on the sex ratio of harpacticoid copepods *N. spinipes* and *T. battagliai* (Hutchinson et al., 1999; Breitholtz and Bengtsson, 2001).Our results are consistent with previous findings observed in the amphipod *Gammarus pulex*, for which EE2 skewed the sex ratio in favor of females at a concentration < 10 μg L^{-1} (Watts et al., 2001). The variability in results reported in the literature might be attributed to species specificity. Sexual differentiation in crustacean species is regulated by the androgenic glands (AGs). In males, the primordial AGs develop and synthesize the androgenic gland hormone (AGH), which induces testicles development and male sexual differentiation. In females, sexual differentiation is induced spontaneously because AGs are absent (Hasegawa et al., 1993). The feminization of copepods population under EE2 exposure can suggest the interference of EE2 with *A. clausi* sexual differentiation. Vandenbergh et al. (2003) stated three EE2 possible mechanisms of action behind the impairment of sexual differentiation in the freshwater amphipod *Hyallela azteca*. EE2 can disturb the function of AGs, or can interact with the AGH or the hormone metabolizing enzymes and subsequent changes in AGH (Vandenbergh et al., 2003).

4.2.2. Prosome length, lifespan and reproductive traits in females of the F0 generation

To further explore the chronic exposure effects of EE2, PL, lifespan and EPR were investigated in adult females having been exposed to EE2 through their whole life, from embryos to adult.

Results showed that lifespan in *A. clausi* F0 females was not affected by EE2 chronic exposure. As a general rule, the lifespan of *A. clausi* decreases with time (Hirst et al., 2010). Hirst et al. (2010) investigated *A. clausi* females' lifespan in laboratory and stated that less than 20% of incubated females remain alive after 20 days. To our knowledge, there are no studies in the literature focusing on copepods lifespan as a sensitive endpoint to estrogens. In contrast to lifespan and development time, females reared at 100 μg L^{-1} of EE2 showed a significant decrease in their PL in comparison to DMSO. This result is consistent with the findings of Bang et al. (2010) which stated a significant reduction of the body length of the harpacticoid copepod *T. japonicus* (F1) exposed to E2 at a concentration of 0.1 to 30 μg L^{-1}.

The reproductive capacity of F0 females was sensitive to EE2 chronic exposure as EPR decreased at 100 μg L^{-1} of EE2. Previous results from laboratory studies are contradictory and varied between species. Our results are in lines with those reported in the cladoceran freshwater species *D. magna*, EE2, at 100 μg L^{-1}, was associated with impairment of reproductive traits (Goto and Hiromi, 2003). In contrast, our results are not consistent with those recorded for the harpacticoïd copepods *N. spinipes* and *T. battagliai* (Hutchinson et al., 1999; Breitholtz and Bengtsson, 2001), which were tolerant to estrogens, suggesting that our target copepod can be a good bio-indicator of EE2 chronic toxic effect. Alteration of reproduction in *A. clausi* can impact the fitness of the offspring or subsequent generations resulting in the alteration of marine community structure and the modification of the pelagic trophic food web. Exposure to EE2 did not disturb the development of *A. clausi* from embryo to adulthood, but has impacted reproduction in females F0, indicating that exposure to EDCs during development can provoke negative and permanent effects which can manifest even in later adulthood.

In the life-cycle experiment, EPR, sex ratio, and PL were the most sensitive endpoints measured in *A. clausi* exposed to EE2. These results may suggest that EE2 is physiologically active in *A. clausi*, but the precise mechanism underlying these processes is unclear (Lafont and Mathieu, 2007). We cannot draw a conclusion on the interference of EE2 in reproductive impairment and sexual differentiation in copepods due to the conflicting results in the literature and the lack of molecular information about copepods endocrine system.

It is worth noting that the EE2 measured concentration under which the negative chronic effects were observed in *A. clausi* is above those reported in the field. Thus, EE2 measured concentration in the present study may reflect the chronic effect of EE2 on copepods upon accidental exposure. Moreover, the extensive production and continuous release of estrogens compounds will lead to the increase of estrogens levels in the future which results in potential risk in the aquatic environment.

5. Conclusion

This work is the first report of the sensitivity of *A. clausi* to E2 and EE2 acute exposure. Results revealed that N1 of *A. clausi* were not acutely sensitive to E2 and EE2 exposure even at concentrations in the mg L^{-1}

range. Both estrogens exhibited low concentrations negative effects on HS with presumably a non-monotonicity response. Nevertheless, under chronic exposure during the whole *A. clausi* life cycle, EE2 appears to affect the sexual differentiation and fecundity of the F0 population. The present findings encourage future research focusing on early life stages to better understand the mechanisms of estrogen uptake by the earlier life stages of *A. clausi*, their functional role and their possible mode of action in copepods. Furthermore, the multigenerational and population effects of estrogens should be studied to highlight the carry-over effects of E2 and EE2 on offspring.

CRediT authorship contribution statement

Emna Djebbi: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, writing - original draft. **Mohamed Néjib Daly Yahia:** Writing - review & editing. **Emilie Farcy:** Conceptualization, Methodology, Estrogen analysis, Validation, Writing - review & editing. **Olivier Pringault:** Validation, Writing - review & editing. **Delphine Bonnet:** Conceptualization, Funding acquisition, Methodology, Formal analysis, Project administration, Resources, Validation, Writing - review & editing.

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Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Declaration of competing interest

The authors declare that they have no financial interests or personal relationships that could influence the work reported in this article.

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Appendix A. Supplementary data

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