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Effects of Ethinylestradiol (EE2) and an Organophosphorus Flame Retardant (TCPP) on Gonadal Maturation in the Sea Urchin, *Paracentrotus lividus*

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Abstract: The sea urchin (*Paracentrotus lividus*) was used to test the effects of one of the most abundant flame retardant additives for plastics, tris (1-chloro-2-propyl) phosphate (TCPP), and the synthetic hormone ethinylestradiol (EE2) on gametogenesis and gonad development of adults. With this aim, 403 individuals of both sexes were exposed to TCPP concentrations ranging from 0.2 to 10 µg/L, EE2 (0.01 µg/L), seawater and solvent controls for 7 and 28 days. EE2 and TCPP exposure did not cause histological damage in the gonads. Some evidence of estrogenic effects of TCPP within the µg/L range and EE2 within the ng/L range is reported. Females exposed to 1 µg/L TCPP for 7 days showed a significant increase in gonad development assessed as gametogenic stage, females exposed to 10 µg/L TCPP showed increased gonad development both in terms of weight (Gonad Index, GI, at both 7 and 28 days) and maturation (Pixelar Index, PI), and females exposed to 10 ng/L EE2 showed increased PI after 28 days exposure. Male sea urchins exposed to both TCPP and EE2 for 7 days showed increased frequencies of low development gonad stage. However, the patterns of response are affected by the high inter-individual variability, the differing initial stage of the gonad, as well as the dosage administered.

Keywords: endocrine disruptors; organophosphorus flame retardant; xenoestrogens; *Paracentrotus lividus*; echinoderms

1. Introduction

The mounting presence of synthetic chemicals in natural waters, and the understanding that some of them can interfere with the action of natural hormones has led to concern about the effects of the so-called endocrine disrupting chemicals (EDCs) in aquatic ecosystems. In Europe, the European Commission has recently developed a Community Strategy for endocrine disruptors [1], within the frame of the Regulation on Registration, Evaluation, Authorization and Restriction of Chemical (REACH) [2].

The organophosphorus flame retardants (OPFR) are synthetic chemicals used by industry as additives to plastic materials and electronic devices in order to enhance their resistance to heat and avoid flammability. Their global production has recently increased as a result of their use replacing the restricted polybrominated diphenyl ethers (PBDE) [3]. The most common OPFR, tris-(2-chloro-, 1-methyl-ethyl)-phosphate (TCPP), mainly used in polyurethane foams, is the most detected synthetic chemical in many aquatic systems [4], such as rivers of Asia, America and Europe (reviewed in Iqbal,

et al. [5]). In the rivers of North-West Spain, TCPP has been typically found at levels in the 0.1–10 µg/L range [6], and in Asian coastal waters in concentrations ranging from 0.09 to 1.4 µg/L [7]. Some OPFRs pose environmental risks due to their androgenic or estrogenic effects, mimicking male or female hormones, respectively [8–10]. Possible endocrine-disrupting effects of TCPP on marine invertebrates have not been evaluated in depth yet.

Ethinyl-estradiol (EE2), a synthetic derivative of estradiol used in oral contraceptive pills, is more resistant to metabolic biotransformation and environmental degradation than the natural hormone [11,12]. In addition, EE2 showed higher estrogenic potency compared to the other estrogens (estradiol and estrone) [13]. Concentration of EE2 in surface waters within the range of 1.1 to 191 ng/L have been reported [14–16]. Previous use of steroids in ecotoxicological assays with echinoderms resorted to peristomal injection on *Paracentrotus lividus* [17,18], and dietary administration in other sea urchin species, such as *Lytechinus variegatus* [19] and juveniles of *Pseudocentrotus depressus* [20,21]. To the best of our knowledge, no investigation has previously assessed the effect of water-borne steroids using environmentally realistic exposure conditions.

The aquatic models most often used to assess endocrine disruption effects of OPFRs are freshwater fishes such as zebrafish (*Danio rerio*) [9,22–28], goldfish (*Carassius auratus*) [29], killifish (*Oryzias latipes*) [29], and freshwater gobies (*Gobiocypris rarus*) [30] or the common carp (*Cyprinus carpio*) [31]. Very few studies addressed the issue of endocrine disruption of OPFRs in aquatic invertebrates [32], even though endocrine disruption effects of other xenobiotics have been reported in adult echinoderms [33,34] and the sea urchin early life stages are a common model in marine ecotoxicology [35–40]. Although the function of estradiol and other steroid hormones in fish is very well known, their role in controlling the female gametogenesis in invertebrates is under discussion [19,41–43]. This study aims to explore the possible effect of TCPP and EE2 on gametogenesis and gonad growth in adult males and females of the *P. lividus* sea urchin.

2. Materials and Methods

2.1. Sea Urchins

Adult sea urchins (*Paracentrotus lividus*) ranging from 4 to 6 cm in diameter were collected from the intertidal zone of Toralla Island (42°12′07.1′′ N, 8°48′05.0′′ W), at the Ria de Vigo (Galicia, NW Iberian Peninsula) on two occasions (November 2016 and September 2017). For the acclimation, the sea urchins were maintained in an open-flow system at the Toralla Marine Station (ECIMAT, CIM, University of Vigo).

2.2. Experimental Design

The experiments were performed using 184 individuals in 2016, and 219 individuals in 2017. The sea urchins were distributed in groups of 12 individuals in 2016 and 10 individuals in 2017, using 25-L glass aquariums, and 5 days (d) acclimation was allowed before exposure to the different treatments. These treatments consisted of control filtered seawater (FSW) of oceanic characteristics (35 ± 1 psu), solvent control (acetone), EE2 (0.01 µg/L) and TCPP (0.2, 1, 5 and 10 µg/L). The aquariums were kept in an isothermal room at 16 ± 1 °C, at intervals of 12 h light: 12 h dark photo-period, and gentle aeration was provided allowing dissolved oxygen > 90% saturation. Water was renewed three times per week and the sea urchins were fed with *Laminaria* sp., one 4 cm^2 piece per individual. Four aquariums were used per treatment. Two of the aquariums were sampled after 7-d exposure to assess acute effects, and the other two after 28-d exposure to assess subchronic effects.

2.3. Chemicals and Stock Solutions

EE2 (ref. 46263, purity ≥ 98%, Sigma-Aldrich, Switzerland) and TCPP (ref. 32952, analytical standard, mixture of isomers, Sigma-Aldrich, Germany) were used in this study. The structures of these compounds are shown in Figure S1 (Suppl. Material). Stocks of EE2 and TCPP were made

up by dissolving 0.010 g/L of EE2, and 10 g/L (in 2016) and 5 g/L (in 2017) of TCPP, respectively, in acetone (pure, pharma grade, PanReac AppliChem, ITW Reagents, Spain). For TCPP, secondary stocks were made up with serial dilutions of the TCPP primary stock in acetone, in order to add exactly the same amount (25 µL) of solvent to each aquarium. Fresh stocks were made up weekly. Nominal concentrations were analytically checked in 2016 to determine the stability of the compound in the test media, showing a reduction in the concentration of around 30–40% between 2–3 days, suggesting that there is no water renewal due to the physicochemical characteristics of the substance. After water renewal, concentrations ranged between 0.9 and 10.7 µg/L and before water renewal, they ranged between 0.6 and 6.9 µg/L, for concentrations of 1 and 10 µg/L, respectively [44]. Stability of EE2 in seawater using the present experimental design was confirmed by Fernández-González [45].

For each individual, diameter of the test (D) was measured using a caliper (0.05 cm accuracy) and gonads were removed and weighed fresh (gonad wet weight, GWW ± 0.01 g). In 2017, two gonads per individual were then dried in an oven at 60 °C until constant weight and then weighed (gonad dry weight, GDW ± 0.0001 g).

The gonad index (GI) of each individual was calculated, following the expression modified from Ouréns, et al. [46]:

$$GI = \frac{GDW\ (g)}{D\ (cm)} \times\ 100 \quad (1)$$

In 2016, GI was estimated from the GDW/GFW ratio obtained from the 2017 data (33.1 ± 17.2%).

One of the five gonads of each animal used in the experiments was fixed in Davidson's solution [47] for 24 h at 4 °C, and then processed using an automatic tissue processor (Leica TP1020, Leica Microsystems) in the histology service of ECIMAT. Samples were embedded in paraffin, the blocks were sectioned at 5 µm, and the tissue sections were stained with hematoxylin and eosin. The slides were observed under the microscope (Nikon Eclipse 90i) with image analysis software (NIS-Elements BR v4.0) and a gametogenic stage was assigned to each individual using the scale by Byrne [48]: Stage I: recovery; Stage II: growing; Stage III: premature; Stage IV: mature; Stage V: partly spawned.

The stained gonad sections were photographed at high resolution (total magnification100x) using TIFF format, and analyzed using the Pixelar Index (PI) developed by Mantilla-Aldana, et al. [49], following the expression:

$$PI\ (\%) = \left(\frac{v}{v+p}\right) \times\ 100$$

where v computed for pixels coming from the violet layer (v), which represent only germinal or gametogenic cells, P the pixels coming from pink layer representing other cellular populations such as the nutritive phagocytes and non-germinal accessory cells (see Figure S2). The histological analysis shows these two cell types with a different pattern of color, because the germinal cells are stained in violet given their basophilic nature, whereas the somatic cells are shown in pink by their eosinophilic nature.

2.4. Statistical Analysis

Analysis of variance (ANOVA) was conducted for GI and PI and normality and homogeneity of variances were verified using the Shapiro–Wilk test and the Levene test, respectively. Dunnet's post-hoc test was used for multiple comparisons between control/solvent control and experimental treatment. Contingency tables and Pearson's chi square (χ^2) test were used for sex ratio and gametogenic stage analyses. All statistical tests were performed by using SPSS Inc. v.24 (IBM, Chicago, EEUU).

3. Results and Discussion

3.1. Mortality

Some apparently random mortality was recorded, characterized by animals losing spikes. Dying individuals were discarded as soon as identified; in 2016, two animals died in acetone 7-d group, three

animals in the TCPP (1 µg/L) 7-d group, 2 animals in the TCPP (10 µg/L) and 1 animal in the acetone 28-d group; in 2017, one animal dies from the 0.2 µg/L 28-d group.

3.2. Sex and Gametogenic Stage

The relative frequencies of ovarian and testicular maturity stages for the two sets of experiments are illustrated in Figures 1 and 2. For two years consecutively, microscopical analyses of the histological samples showed that individuals were in five of the six stages described by Byrne (1990) [48]; Figure S3 (Suppl. Material) depicts the histological appearance of the maturation stages. In 2016, 101 individuals were females and 82 males, while in 2017, 120 were females and 99 males. Therefore, the overall proportions were 55% females and 45% males, resulting in a sex ratio of 1.24:1, not significantly different from 1:1 (χ^2, $P > 0.05$). In contrast with that reported by Byrne (1990) [48], for the Irish coast, no hermaphroditic individuals were observed in the samples obtained from this experiment. Acetone (solvent control), TCPP and EE2 did not induce tissue damage in any processed sample compared to individuals of seawater control (Figure S4).

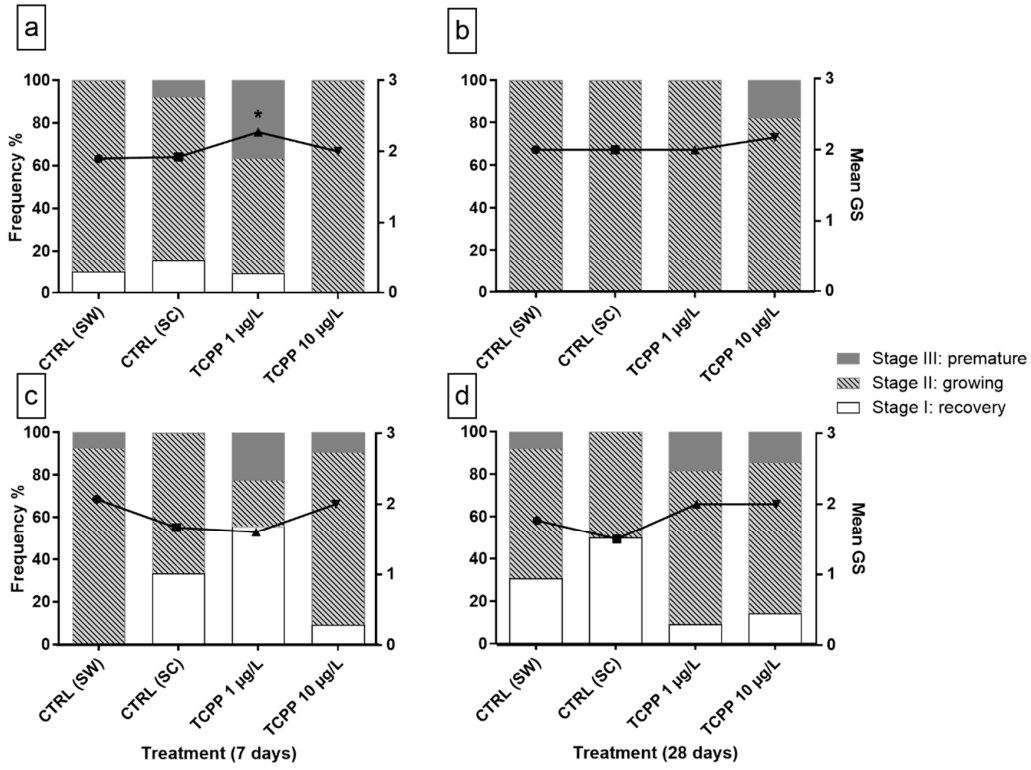

Figure 1. Relative frequencies of the gametogenic stages in histological sections of female (**a**,**b**) and male (**c**,**d**) *P. lividus* sea urchins exposed for 7-d (**a**,**c**) and 28-d (**b**,**d**) in 2016 (n = 7 to 17). The black line is the weighted average of the gametogenic stage (GS) for each treatment (Table S1). Asterisk indicates significant ($P < 0.05$) differences with respect to solvent control (CTRL (SC)).

In November 2016, disregarding sex, all individuals were in early stages of the gametogenic cycle (stages I to III) (see Figure 1), whereas in September 2017, many individuals showed signs of more advanced stages (mature and partially spawned; stages IV and V, respectively) (see Figure 2).

In 2016, 15.1% of control individuals (both seawater and solvent controls) were at stage I, 81.7% at stage II, and 3.2% at stage III, whereas in 2017, 10% of individuals were at stage I, 25% at stage II, 42.5% at stage III, 20% at stage IV, and 2.5% at stage V. Sex significantly affects the proportion of individuals at each gametogenic stage ($P = 0.027$ in 2016 and $P = 0.0001$ in 2017), with females showing a more advanced stage than males (see Table S1), likely due to the greater energy investment in the maturation process of oocytes compared to spermatocytes. In 2017, although sampling was conducted

two months earlier, the population showed a consistently more advanced gonad development for both males and females.

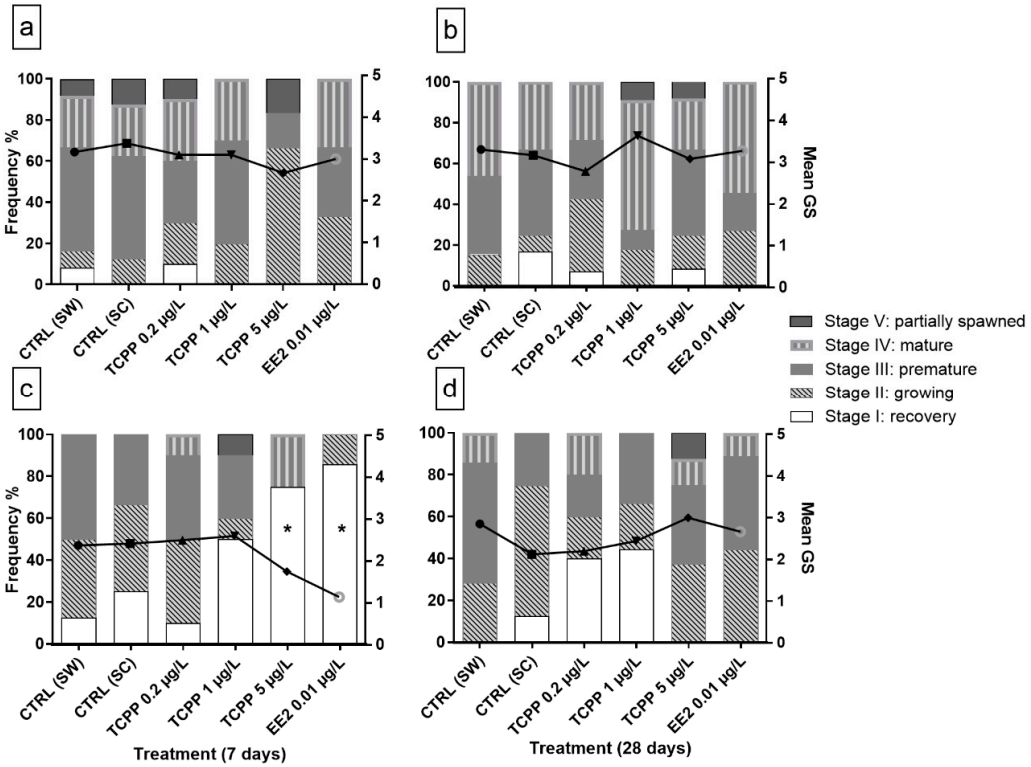

Figure 2. Relative frequencies of the gametogenic stages in histological sections of female (**a**,**b**) and male (**c**,**d**) *P. lividus* sea urchins exposed for 7 (**a**,**c**) and 28 (**b**,**d**) days in 2017 (n = 4 to 14). The black line is the weighted average of the gametogenic stage (GS) for each treatment (Table S1). Asterisks indicate significant ($P < 0.05$) differences with respect to solvent control (CTRL (SC)).

Concerning individuals exposed to the experimental treatments, the most advanced gonad development, indicated by higher mean gonad stage (Figure 1a), was achieved in females at 1 µg/L TCPP after 7-d, and at 10 µg/L TCPP after 28-d in 2016, and at 1 µg/L TCPP after 28-d in 2017. This trend towards more advanced gonad stages in females exposed to TCPP did not reach statistical significance except for the 1 µg/L TCPP treatment at day 7 ($P = 0.013$), due to the strong inter-individual variability.

In males, significantly less advanced gametogenic stages were found for the 5 µg/L TCPP and the 10 ng/L EE2 treatments after 7-d exposure compared to individuals of solvent control ($P = 0.014$, $P = 0.035$, respectively), but these effects disappeared after 28-d exposure.

3.3. Gonad Growth and Pixelar Index

In 2016, an increase in GI with increasing TCPP concentrations was observed for female sea urchins (Figure 3a), and despite high inter-individual variability those exposed to 10 µg/L TCPP showed significant increased GI values after both 7 and 28 days compared to the solvent control ($P = 0.022$). In males (Figure 3b), the opposite trend was observed with TCPP-exposed males showing GI values lower than solvent control, but the high variability prevented statistically significant differences. The patterns observed in the 2016 experiments were not confirmed in the 2017 experiments (Figure 4) conducted with sea urchins in a more advanced gametogenic stage (see Figure 2), nor were clear trends in GI observed that year (Figure 4a,b).

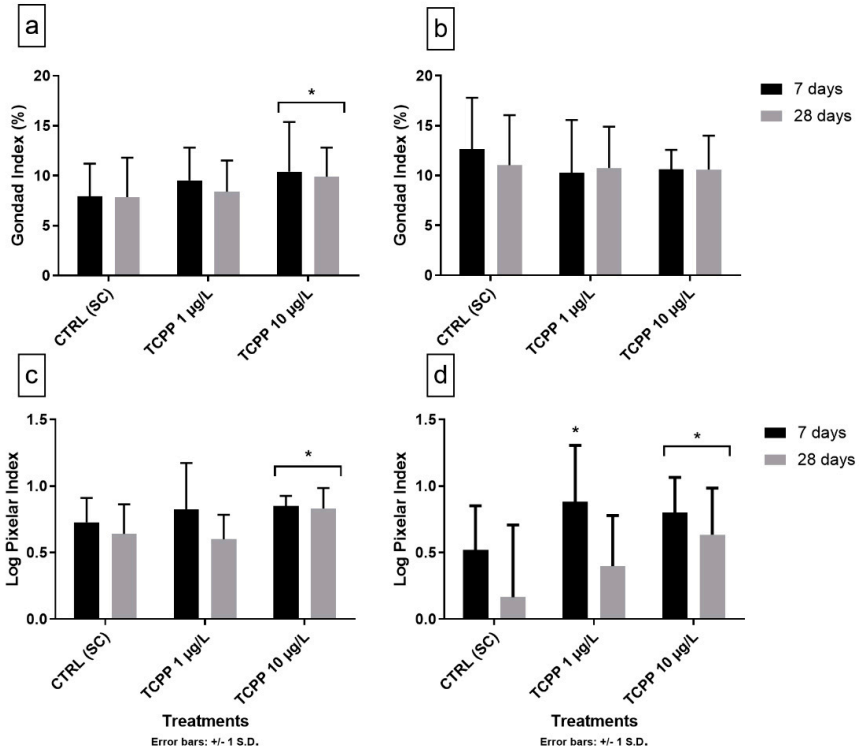

Figure 3. Gonad Index (**a**,**b**) and Pixelar Index (**c**,**d**) (mean ± SD) of female (**a**,**c**) and male (**b**,**d**) *P. lividus* sea urchins exposed for 7-d (black bars) and 28-d (grey bars) to tris (1-chloro-2-propyl) phosphate (TCPP) in 2016 (n = 7–17). Asterisks indicate significant ($P < 0.05$) differences with respect to solvent control (CTRL (SC)).

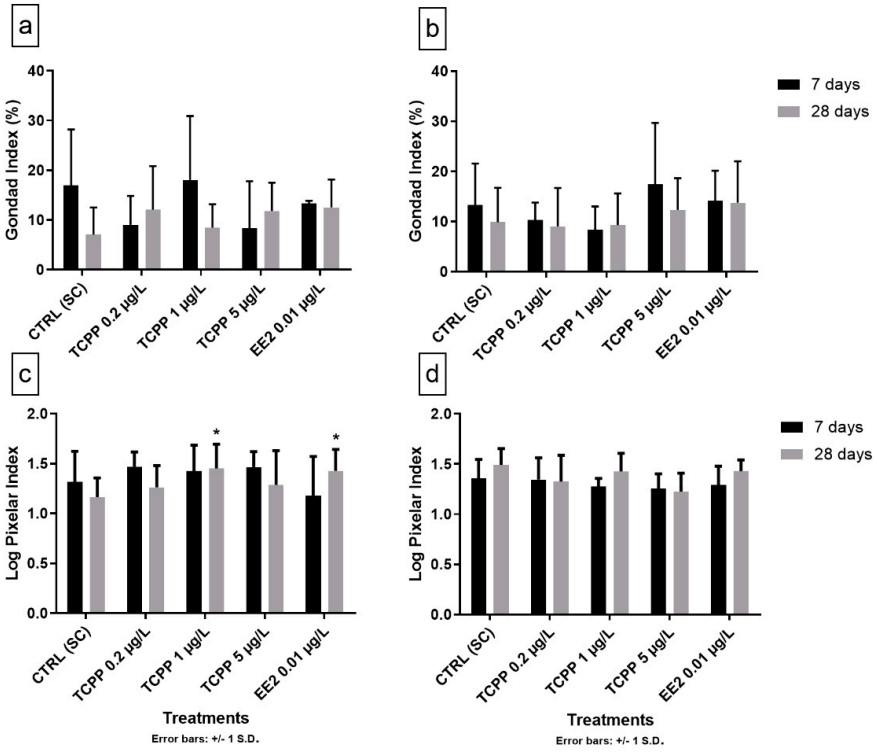

Figure 4. Gonad Index (**a**,**b**) and Pixelar Index (**c**,**d**) (mean ± SD) of female (**a**,**c**) and male (**b**,**d**). *P. lividus* sea urchins exposed for 7-d (black bars) and 28-d (grey bars) to TCPP in 2017 (n = 4–14). Asterisks indicate significant ($P < 0.05$) differences with respect to solvent control (CTRL (SC)).

The Pixelar Index is a quantitative and objective tool used for the assessment of the stage of development in male and female gonads [49]. In 2016, females exposed to 10 µg/L TCPP showed significant higher values than control regardless of exposure time ($P = 0.039$ on day 7 and $P = 0.006$ on day 28). Significant differences were also observed in males, with an increase in PI values at a concentration of 1 µg/L ($P = 0.030$) on day 7, and at a concentration of 10 µg/L ($P = 0.041$) at day 28 compared to solvent controls. However, in the case of males, significantly decreased PI values were also observed in the solvent controls compared to seawater controls ($P = 0.002$), and no significant differences were found between TCPP treatments and seawater controls.

In 2017, PI values in females were significantly higher than solvent controls for the 1 µg/L TCPP and the 10 ng/L EE2 treatments after 28-d exposure ($P = 0.015$ and $P = 0.025$, respectively), whilst no significant effects were observed in males at any treatment.

The high inter-individual variability in gonad development may hinder statistical detection of differences among treatments. Future experimental designs can be improved by increasing sample size and taking several gonad samples from the same individual.

3.4. Natural and Anthropogenic Factors Affecting the Reproductive Cycle

The gametogenic cycle of *P. lividus* populations was described in Ireland [48], France [50], and in the Mediterranean [51,52] and Atlantic [53–56] coasts of Spain. The main exogenous factors that seem to control the reproductive cycle are temperature, photo-period and food availability, but as to the endogenous mechanisms that control gametogenesis, the role played by steroid hormones and the possible estrogenic effects of synthetic xenobiotics in echinoderms are actually poorly understood. The role of estrogens in the control of gametogenesis in invertebrates is under debate, and while some findings support the induction of gametogenesis mediated by oestrogen receptors [57–60], other authors advocate for an ancient non ER-mediated mechanism of control of gonad maturation [61]. Concerning echinoderms, there is considerable evidence published suggesting that vertebrate-type sex steroids, progesterone, estrogens (17β-estradiol and estrone) and androgens (testosterone) are synthesized by sexually mature individuals [41,62,63], and that they may have a role in the control of growth and reproduction [64]. However, estradiol and testosterone levels are maturity-stage and sex independent, and no clear patterns of seasonal differences in neither testosterone nor estradiol contents were found (Lavado, et al. [33,34], for *P. lividus* and *Antedon mediterranea*; and Barbaglio, et al. [43], for *P. lividus*). Injection of estradiol to adult sea urchins for 10 weeks did not influence the maturation stage of the gonads and the development of the gametes [18]. In addition 80 to 99.8% of testosterone and estradiol in *A. mediterranea* were in esterified form [34], as a consequence of phase II activity (acyl-transferases) [33]. In vertebrates, esterified steroids are assumed to be inactive (do not bind to the estrogen receptor), but in echinoderms no ER was identified up to date and ER-independent mechanisms of activity for the esterified steroids cannot be discarded.

Concerning the effect of the xenobiotic TCPP on the gametogenesis and gonad maturation of *P. lividus*, in the present study we found different tendencies between the two sets of experiments. When sea urchins with immature gonads were used (in 2016), TCPP showed a trend to increase the gonad index in females, statistically significant in the 10 µg/L treatment, and the decreasing trend of male GI values after TCPP is slightly evident. This was consistent with the higher mean gonad stages found in TCPP-exposed females. However, these trends disappeared in 2017, when sea urchins with more mature gonads were used. In September 2017, we found individuals still mature and in recovery stage after spawning, probably after a longer and warmer summer with seawater temperatures above 17.5 °C in August 2017, recording an anomaly of +0.9 °C with respect to 2016 and reaching 20.8 °C of maximum seawater temperature (J. González, University of Vigo, https://torallamar.info, Table S2). The gametogenic cycle depends on environmental factors varying geographically and over various years. Compounds with potential for endocrine disruption, including the well-known estrogen for vertebrates EE2, seemed not to enhance female gonad development in sea urchins on late gametogenic stages [17–19]. Finally, some evidences in vertebrates studied reported uterine weight decreased in

the offspring of female rats fed with TCPP, while two other studies reported no significant effects on reproductive parameters on females rats exposed to TCPP also in the diet [65].

4. Conclusions

This study found some evidence of estrogenic effects of TCPP on female sea urchins, especially when exposed at the beginning of their gametogenic cycle. Higher mean gonad stages were found in TCPP-exposed females compared to controls, although the differences were statistically significant for the 1 µg/L treatment only. An amount of 10 µg/L TCPP significantly increased the gonad weight and development in females ranging from recovery to premature gametogenic status, although the effects seem to disappear in more advanced stages, suggesting that individuals in early gametogenic stages are more sensitive to xenobiotic-induced endocrine disruption than mature and partly spawned individuals. These preliminary results point at estrogenic effects of TCPP that should be further investigated through more detailed dose:response experiments.

Ethinyl-estradiol does not seem to consistently accelerate gametogenesis in female sea urchins, although experiments with individuals in less advanced gonad stages would be necessary to confirm this finding.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-1312/8/8/611/s1, Figure S1: Structures of the compounds used in this study: (a) EE2 ethinylestradiol; (b) TCPP mixture of isomers: mainly tris(1-chloro-2-propyl) phosphate 66%, minor components: bis(1-chloro-2-propyl) (2-chloropropyl) phosphate and (1-chloro-2-propyl) bis(2-chloropropyl) phosphate, Figure S2: Sequence of image processing to quantify the Pixelar Index: original image in HE dye (a), bar = 100 m. Image without incomplete follicles after the cleaning process (b) with Adobe Photoshop. From b, the Colour Deconvolution plugin (v.3.0.2, ImageJ, FIJI), generates a pink layer (c), and a violet layer (d) and the respective binary images (e, f). The binary images obtained were quantified by the CellProfiler software (Carpenter et al. 2006; Lamprecht et al. 2007) creating a pipeline effect and using the Measure Image Area Occupied tool, Figure S3: Sea urchin, *Paracentrotus lividus*, developing stages of males (top) and females (bottom): I, recovery; II, growing; III, premature; IV, mature; V, partly spawned (ECIMAT histology service), Figure S4: Examples of individuals histological slides of significant data on gametogenic stages. a: Female stage III, premature, CTRL in 2016 (t = 7 d). b: Female stage III, premature, TCPP 1 µg/L in 2016 (t = 7 d). c: Male in recovery condition, stage I, EE2 0.010 µg/L in 2017 (t = 7 d). d: Male in recovery condition, stage I, TCPP 5 µg/L in 2017 (t = 7 d). Bar = 100 m, Table S1: Mean gametogenic stages1 of the sea urchins exposed to different treatments in the 2016 and 2017 experiments, Table S2: Mean of seawater and environmental temperature (± S.D.) maximum and minimum in the 2016 and 2017 experiments.

Author Contributions: R.B. contributed with the experimental design, funding acquisition and resources. Material preparation, data collection, analysis and interpretation of results were performed by P.C.-L., E.P.-P. and L.M.-A. The first draft of the manuscript was written by P.C.-L. and reviewed by R.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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