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Developing an *in Vitro* Thicklip Grey Mullet (*Chelon labrosus*) Gonad Explant Culture for Assessing Effects of Reproductive Endocrine Disrupting Chemicals

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ABSTRACT

Sex steroid hormones necessary for regulation of gametogensis are produced through the coordinate actions occurring along the hypothalamic-pituitary-gonadal (HPG) axis. The process is complex with various potential steps liable to disruption due to exposure to external chemical stimuli such as the presence of reproductive endocrine disrupting chemicals (EDCs), eventually leading to reproductive failure. The toxic effects of reproductive EDCs in aquatic organisms can be elucidated effectively using in vitro culture techniques. The goal of this study was to develop a simple cost effective in vitro protocol to culture *C. labrosus* gonad explants to investigate the effects of EDCs. Mullets are useful sentinel organisms of environmental pollution in the Southern Bay of Biscay, where they have been shown to suffer an intersex condition as a result of exposure to xenoestrogens. Fish were sampled in Plentzia port and in the Bilbao river during the spring months (April–June 2015). The ovaries were sliced and explants cultured in L-15 supplemented media for 5 days, to study the effects of incubation with different concentrations of 17β-estradiol and testosterone at 18°C. The morphology of explants was evaluated histologically and the cell viability was analysed measuring the amount of lactate dehydrogenase released into the culture medium. Finally, 5S/18S rRNA ratio was determined in the ovaries and the transcriptional levels of steroidogenesis (*cyp19a1a*), and germ cell differentiation genes (*tfiiia* and *piwil1*) were quantified by qPCR. Histologically, explant culture conditions did not alter the morphology of oocytes, either previtellogenic or vitellogenic, but disruption of the stromal connective tissue and follicular cells around the oocytes was observed in some ovary explants after 5 days of culture. Cell viability analysis revealed no significant differences after 2 and 5 days in culture, but the levels of cyto-toxicity were very high both in control and in 17β-estradiol incubated explants. Culture conditions did not affect the production of 5S rRNA which was high both in previtellogenic (PV) and vitellogenic (V) ovarian explants at time 0, 5S rRNA transcript levels being higher in ovaries with PV oocytes than in those with V oocytes. A significant down-regulation of *cyp19a1a* was observed in the cultures, both under control and hormone incubation conditions. The same happened with *tfiiia* after 5 days of in vitro culture, but incubation with E2 this time resulted in the maintenance of the time 0 transcription levels. *piwil1* transcript levels were not affected by the culture conditions. These results demonstrate that the developed mullet ovary explant in vitro assay was effective in preserving oocyte morphology and function, but not in somatic cell maintenance. In the present culturing circumstances, no effective analysis of the steroidogenic process can be undertaken, but the analysis of exogenous supply of steroid hormones on oocytes could be effectively analysed. Improving gonad explants culturing conditions will be a prerequisite in order to apply such explants as cost-effective, in vivo test substituting and high-throughput technology for the analysis of basic thicklip grey mullet reproductive endocrinology and of the effects of reproductive endocrine disrupting chemicals.

Key Words**:** Gene Transcription; Gametogenesis; cyp19a1a; tfiiia; piwil1; 17β-estradiol; 5S/18S rRNA Index

INTRODUCTION

The occurrence of endocrine disrupting compounds (EDCs) in the environment has become widespread (Jobling et al. 1998, Vajda and Norris 2011). Most relevant in environmental toxicology are reproductive EDCs that affect both human and animals through impacting the reproductive system interfering with the hypothalamic-pituitary-gonadal (HPG) hormonal signalling axis. The normal development, growth and function of vertebrate reproductive systems are regulated by this HPG-axis. Reproductive behaviour in fish in response to environmental and physiological cues is also regulated at the level of this axis (Kah and Dufour 2011). The hypothalamus instructs pituitary glands through neuroendocrine neurons by secreting GTRHs (Weltzien et al. 2004). In response, the pituitary gland produces two GTHs); luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones reach the gonads via the blood circulation and regulate the processes of gametogenesis and steroidogenesis. During steroido-genesis the ovaries and testes of the fish release estradiol E2 and 11-KT, respectively (Norris and Lopez 2011, Weltzien et al. 2004). These sex steroid hormones in turn are transported to the hypothalamus and pituitary through the blood circulation where they bind to their corresponding estrogen and androgen receptors (Figure 1). In doing so they are able to regulate the release of GTRHs and GTHs that can bring a negative or positive feedback depending upon the reproductive stage of the fish (Weltzien et al. 2004, Zohar et al. 2010). Reproductive EDCs can alter this essential and evolutionary conserved system by mimicking, altering, or inhibiting the action of endogenous hormones responsible for endocrine homeostasis in any of the steps described above. Furthermore, they are normally active at very low concentrations and are increasingly a big concern with the environment, both due to the fact that they are ubiquitous and present in complex mixtures in the environment and because reproduction is a key factor for the resilience of a population to environmental alterations (Damstra et al. 2002, Dietrich and Krieger 2009).

Many chemicals that have been developed for agricultural, industrial and personal care purposes contain endocrine disrupting capacity. EDCs arrive to the aquatic ecosystems via sewage flow from waste treatment plants, runoff from agricultural lands and animal feeding operation lots. Gonochoristic teleost fish

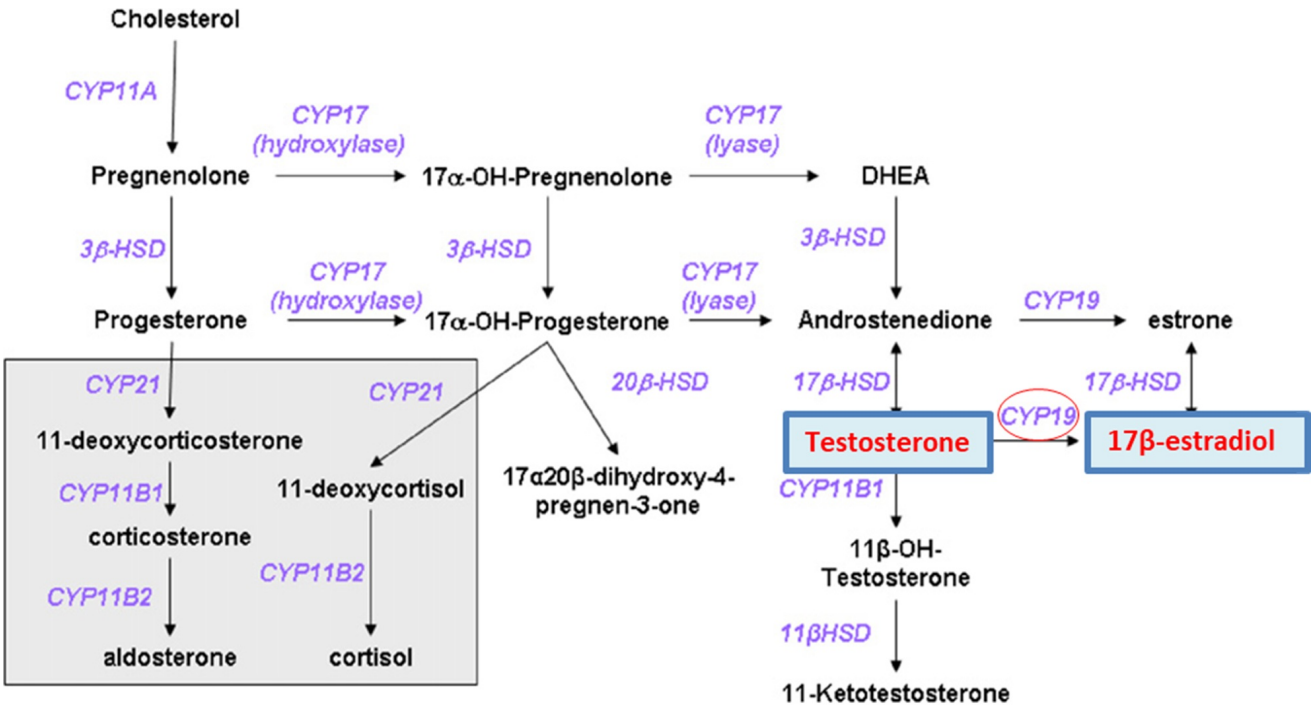

Figure 1. Major steps involved in steroid hormone biosynthesis in fish (Villeneuve et al. 2007). Abbreviations for enzyme names are shown in italics. CYP=cytochrome P450; HSD=hydroxysteroid dehydrogenase. Normal background indicates major pathways/products occurring in fish gonad tissue. Gray background indicates predominantly adrenal pathways/products. Hormones analyzed in the present are boxed in blue boxes and the only gene in the pathways analyzed is circled in red.

species are very sensitive to these reproductive EDCs, particularly in their early stages of sexual determination and differentiation before gametogenesis first begins. The fates of the gonadsand differentiation into either testes or ovaries are largely determined by sex steroid hormones in teleosts (Dietrich and Krieger, 2009). Impossibility to supply the correct amount of sex steroids has major consequences on the development of gonads. Aquatic species exposed to EDCs have been shown to display impaired reproductive endocrine status (Leblanc et al. 1997, MacLatchy et al. 2003) and secondary sex characteristics (Parrott et al. 2004), skewed sex ratios and abnormal feminization of testis or masculinisation of ovary (Jobling et al.1998, Mathiessen et al. 1998, Harris et al. 2011, Ortiz-Zarragoitia et al. 2014), abnormal production of female specific proteins such as vitellogenins in males (Mathiessen et al. 1998), diminished gonad size and low egg production (Parrott et al. 2004), as well as abnormal early life stage development (Maack and Segner 2004, Boudreau et al. 2005). The prevalence of intersex condition in river and marine fish living in diverse polluted aquatic environments is becoming a common and worldwide observed event. Reproductive EDCs potentially minimize the ability of the individual to synthesize and produce viable gametes at the right moment in response to environmental and nutritional cues. As a result, this could lead to the collapse of the fish populations affected as it has been proved in studies performed under laboratory and real field conditions (Harris et al. 2011).

Environmental risk assessment is typically performed to evaluate the effect of a chemical through in vivo animal exposure to a test compound under laboratory conditions. The advantages of in vivo studies are mainly in regard to the fact that the full array of physiological responses and toxicokinetic processes are integrated when the whole animal is exposed to the contaminant/chemical. The effects of the contaminant observed at the individual level can in this way be extrapolated to the possible effects at the population and ecosystem levels (Gray et al. 1997). Hence, in vitro testing is becoming an important methodological approach in predictive Ecotoxicology to forecast ecological risk from the use of certain chemicals (Villeneuve and Garcia-Reyero 2011). As a result, several in vitro testing methods have been developed to screen the effects of panels of chemicals (Celander et al. 2011). Different fish organ explant cultures have been developed in different laboratories. For instance, ovarian and brain tissue cultures have been developed from Atlantic salmon in order to assess the effects of chemicals that inhibit the activity of aromatase (Lee et al. 2006). The effects of reproductive xenobiotics on steroidogenesis has been effectively demonstrated using ovary explants cultures from fathead minnows (Villeneuve et al. 2007), or brown trout (testis and ovary) (Pereira et al. 2011). These studies enabled the detection of chemical inhibition of enzymes involved in the production of T and E2. Testes explants from rainbow trout have been also used for reproductive studies (Bouma et al. 2005). Thus, gonad explants from many different fish species can be maintained under in vitro conditions up to 36 days, tissues remaining functional and showing effects comparable to those observed in vivo (Miura et al. 1991, Bouma et al. 2005, Keyaba et al. 2008).

In the present study, *C. labrosus* was selected to establish a protocol for the first time utilizing the culture of gonade explants for future application in ecotoxicological studies. Currently, the assessment of environmental risks of EDCs in aquatic environments are founded on the responses of model laboratory fish species such as zebrafish or medaka which may not be representative of the responses of the animals present in our local environments (Beitel et al. 2014). These model species do have very specific reproductive strategies that may not be relevant in the case of many other species, such as the thicklip grey mullet which for instance need longer time to mature and spawn and spawning once or twice per spawning season (Pereira et al. 2011). The levels of hormones are also inconsistent between such species, particularly during gonad development and maturation (Hutchinson et al. 2006) and consequently affect their sensitivity to the exposure of reproductive xenobiotics. In order to extrapolate correctly the finding from these studies into population level effects, and in different teleost fish species as well, we need to develop better and high-throughput chemical screening systems through rigorous studies incorporating more sentinel species. Therefore, developing an in vitro culture of a key organ system of the pollution sentinel species *C. labrosus* would be desirable. This gonad assay could be employed in the identification of substances that disrupt the activity of enzymes involved in the sex steroid biosynthesis, or the sexual differentiation process itself, and thus could be applied to evaluate the health status of the aquatic environment. It will also apply to the screening of the normal functioning of the reproductive endocrine systems of fish.

MATERIALS AND METHODS

Chemicals

L-15 medium, Bovine Serum Albumin (BSA), Streptomycin and Fungizone were obtained from Life Technologies (Carlsbad, CA, USA); HEPES, 5-bromo-2-deoxyuridine (BrdU), 17β-estradiol (E2) and testosterone (T) from Sigma-Aldrich (St. Louis, MI, USA). Growth media (200 mL) were prepared by adding 200 mL of L-15 medium, 0.48 g HEPES, 1.0 g BSA, 4 mL penicillin-streptomycin, 0.2 mL fungizone antimycotic and the pH was adjusted to 7.4 by using 1 M NaOH. The solution was stored at 4°C until use. For longer use it was stored at -40°C. 20 μg mL^{-1} stock solution of E2 was prepared by mixing 1 mg of E2, 1 mL of absolute alcohol and 49 mL of basal L-15 medium and stored at 4 °C until immediate use. Similarly, 20 μg mL^{-1} stock solution of T prepared from 1 mg testosterone, 2 mL of absolute alcohol and 48 mL of L-15 medium and stored at 4°C until immediate use. For longer use both hormone stock solutions were stored in -40°C.

Animals

The experimental animals (*Chelon labrosus*) were captured by fishing rode both from Plentzia port and in Bilbao river. Fish captured were transferred to plastic tanks with aeration and transported to the Plentzia Marine Station (PiE-UPV/EHU) as soon as possible, where fish were transferred to a 2000 L plastic aquarium until used. The aquarium was held at 18ºC with a 12:12 h light:dark photoperiod under a continuous seawater flow and aeration. The fish were fed once daily with commercial pellets.

Gonad explants culture preparation and incubation
The culture of *C. labrosus* gonad explants was carried out following the methodology developed by McMaster et al. (1995) and Miura et al. (1991), which has been modified slightly. The fish were sacrificed and the gonads removed immediately. Ovaries from seven female *C. labrosus* were used for the in vitro experiments, while only one male was studied. Portions of the left gonad were used as a control group while the right gonad was used to produce the explants that were incubated with the test hormones. The gonads were cut into three sections longitudinally and sliced into portions of approximately 3 mm^3, that were placed in a Petri dish containing ice-cold PBS buffer, pH 7.6. Gonad fragments from each fish were transferred to 24-well plates. In advance each well in the plates was filled with 1 mL L-15 medium supplemented with HEPES, penicillin-streptomycin solution, fungizone and BSA, pH 7.4. After 2 hours incubation, test hormones with graded concentrations (E2; 10, 100 and 1000 pg mL^{-1} and T; 10, 100 and 1000 ng mL^{-1}) were added to different wells. The doses were chosen based on previous studies performed for other species. The control gonad explants were incubated in growth media alone. Gonad explants were incubated for 5 days at 18°C with growth medium renewal every 2 days including the test hormones. At the end of the incubation period, the gonad explants were harvested. Some samples were frozen in liquid nitrogen and stored at -80ºC for RNA extraction, and others fixed in 10% buffed formalin containing 1% glutaraldehyde for either histological or immunohistochemical evaluation. The same was done with tissue explants at time 0 directly upon animal dissection.

Histological Analysis

Gonad explants were fixed for 24 hours and then rinsed twice with 70% ethanol. They were dehydrated in a series of ethanol dilutions and embedded in paraffin at 60ºC. Five micrometer sections of each sample were cut in a rotary microtome and stained with hematoxylin and eosin. Stained sections were examined using a light microscope to compare the morphology of the hormone-incubated and non-incubated tissue explants with the time 0 gonad tissue removed directly after dissection and to confirm the sex and the stage of gonad development using the description in McDonough et al (2005) and in Bizarro et al. (2012).

Tissue Viability Analysis: Lactate Dehydrogenase (LDH) Test

The enzyme released in the media has been evaluated using the Thermo Scientific™ Pierce™ LDH Cytotoxicity Assay Kit protocol.

Gene Transcription Levels and 5S/18S rRNA Ratio

The expression levels of three genes (Table 1) involved in oocyte differentiation and in esteroid hormone synthesis (*cpy19a1a, tfiia, and piwi1*) was carried by qPCR in thicklip grey mullet gonad explants and in time 0 samples.

Table 1. Primer sequences used to amplify each specific gene. Concentration of primers in all cases was 12.5 pMol.

Gene	Forward	Reverse	Tm (ºC)	NCBI Accesion	Reference
cyp19a1a	5′-TGCAGCGCAGCAAACG -3′	5′-ACGTGAGCCAAACTGT-3	60	KC684586	Bizzaroet al. 2012
tfiiia	5′-CCCTACAAGTGTCTGGCTCAAG-3	5′-GTTCTTCAAGCGGCATTGGT-3′	58	AY648780	Diaz de Cerio et al. 2012
piwil1	5′-AAGCCACAGATGGAGTCTCG-3′	5′-ACCTTTTCTCCGTTCCTGGT-3	59	KF663555	Diaz de Cerio et al. 2012

Total RNA Extraction

Total RNA was extracted from both frozen gonad explants and gonad tissues at time zero using Trizol (Invitrogen, Carlsbad, CA, USA) method; following manufacturer's protocol.

RNA Quantification

RNA quantification was carried out using 2 µl of the sample in a BioTeks Epoch spectrophotometer (Biotek, Winoosky, USA) and the absorbance was read at 260 and 280 nm. When the reading was out of the range value, the samples were diluted and the concentration measured again.

Total RNA Electrophoresis and Calculation of 5S/18S rRNA Ratios

Electrophoresis of 200 ng total RNA were run using Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) with the RNA 6000 Nano Lab Chip kit. All steps necessary for sample and reactive preparations have been performed following the manufacturer´s protocol. The ratio 5S to 18S rRNA was determined from the electropherograms obtained after running the electrophoresis (Rojo-Bartolomé et al. in press). The Time Corrected Area of each peak was used to calculate the 5S/18S rRNA ratio. The logarithm of the ratio was calculated in order to develop an index that allowed clear visualization of the differences between samples.

cDNA synthesis

Aliquots of 2 µg total RNA were retrotranscribed using the Affinity Script Multiple Temperature cDNA Synthesis Kit and random hexamer primers (Agilent Technologies) following the manufacturer´s instructions in the 7300 thermocyclers (Life Technologies, Foster City, CA, USA) and stored at -40ºC until used in subsequent analyses.

Quantification of cDNA concentration: Quant-iT

cDNA samples were diluted by a factor of 0.01x taking into account the middle points of the standard curve. Secondly, oligonucleotide standard M13 stock solution is diluted (1 µl of M13 + 49 µl of RNAase free H_2O) and a standard curve was generated (0, 0.02, 0.2, 1 and 2 ng μL^{-1}). Then, 50 µL of each sample in replicates were (49.75 µl of H_2O + 0.25 µl of R) transferred to a 96-well flat-bottom plate in triplicates covered with aluminium foil to minimize photo-bleaching, and 50 µL of Quant-iTreactives were added to each sample well and incubated for 5 minutes at room temperature. Finally, the absorbance was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm using a BioTek Synergy fluorimeter (Biotek, Winoosky, USA) connected to a Gen5 software. The cDNA concentration was determined from the standard curve.

SYBR Green and relative qPCR quantification

2 µL of the diluted sample mixed with 18 µl of PCR reaction mix in 96 wells, sealed with plastic adhesives, were laid in a 7300 Applied Biosystems Thermocycler to quantify the expression levels of genes in question (Table 2). The qPCR reaction master mix comprised 10 µl of SYBER Green (FastStart Universal SYBR Green Master, Roche, Indianapolis, USA), 0.06 µL of forward primers, 0.06 µL of reverse primers, and 7.88 µL rNAase free water per sample. The reactions were run at a total volume of 20 µl and the conditions were 1 cycle at 95°C for 10 min, followed by 50 cycles at 95°C for 10 seconds, at appropriate annealing temperature (see Table 2) for 15 sec and at 72°C for 15 sec. Relative gene transcription levels were calculated using the values from the standard curve quantification normalized by the

ngs of sample cDNA quantified from Quant-iT with kinetic PCR efficiency correction (equation below).

$$\text{Relative quantification} = \frac{(1+E)\ ^{\wedge}\text{-}\Delta C_T\ (C_T \text{ sample-} C_T \text{ control})}{\text{ng cDNA}}$$

where E is the real time PCR efficiency, ΔC_T is the crossing point difference between the unknown sample and the control sample and ng cDNA is the value obtained from the Quant-iT quantification. Negative controls were measured in all experiments and data was omitted where they showed any gene expression. SYBR®Green reaction efficiencies were also calculated per gene using the equation E=10^ (-1/slope)-1.

Statistical Analyses

Statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics version 10) Microsoft excel. The data were checked for normality using the Shapiro-Wilk test and significant differences between groups were determined by one-way analysis of variance (ANOVA) followed by post hoc Tukey test after checking the homogeneity of variance. Data which failed to meet the normality assumption and homogeneity of variance were analysed by non-parametric test Kruskal Wallis one-way ANOVA followed by Mann-Whitney pairwise multiple comparison. In both cases the significance level was set at $p<0.05$.

RESULTS

Histological Analysis: Maturity Stage of Studied Gonads

All the studied gonads were histologicallyanalysed and their maturity assigned to any of the 5 stages of maturity described by McDonough et al (2005) for *Mugil cephalus*. The only males analysed in the present study and captured in Aprilwere in the ripe stage. The only 2 female mullets captured in April were also in mature stage showing big vitellogenic oocytes filled with yolk content and with thickened oocyte extracellular layer or vitelline envelope (Figures 2b and 2c). Some clustered perinucleolar oocytes could still be observed between the big vitellogenic oocytes (Figures 2b, 2d and 4). All the 5 females captured in May and June, showed a developing ovary in just started oogenesis and with previtelo-genicperinucleolar oocytes (Figures 2a and 3). The somatic cells were observed in close association with the oocytes with well-preserved stromal connective tissue morphology.

Ovarian Tissue Explants in Vitro

Histological analysis revealed that the morphology of the ovary was preserved in ovary explants maintained under culture conditions for 5 days (Figures 3 and 4). Histologically, no necrosis was observed in the ovarian tissue explants during the course of culturing period, but disruption of connective tissue surrounding the oocyte follicles was noted under culture (Figures 3f and 4b to 4f). In general, the structure of the oocytes remained unaltered, especially in the case of perinucleolar oocytes, with some disruption of the cytosolic cortex in the case of the big vitellogenic oocytes in the animals captured in April (Figure 4b to 4f). Although paraffin embedding of the ovarian samples does not allow for the better morphological preservation, explants showed detachment of the follicular cell layer from the oocyte envelope (Figures 4b to 4f), both in control and in hormone incubated explants after 5 days in culture. In most vitellogenic oocytes, a significant thickening of the follicular cell layer was observed (Figure 4d to 4f). No morphological evidence of any change in oocyte type composition was found in regard to the effect of subjecting the ovarian explants to the steroid hormones for 5 days in comparison to either the control explants or the ovary processed for histology directly after dissection. This was so in the ovaries in the two different developmental stages studied (Figures 3 and 4). Sever disturbance in testis explants morphology were also observed after 7 days of culture (Figure 6b).

Cell Viability Assay in Ovarian Explants

LDH activity was measured in the supernatant of the ovarian tissue explants at 48 hours and at the end of 5 days of culture. This was done only in the case of the explants of ovaries from May-June containing only previtellogenic oocytes. Results showed that there was no significant increase in LDH release from the first measurement to the last one at the end of the culturing period, although the release of LDH on the average was higher at 120 hours (Figure 5). Incubation with 17β-estradiol did not elicit any significant increase in LDH

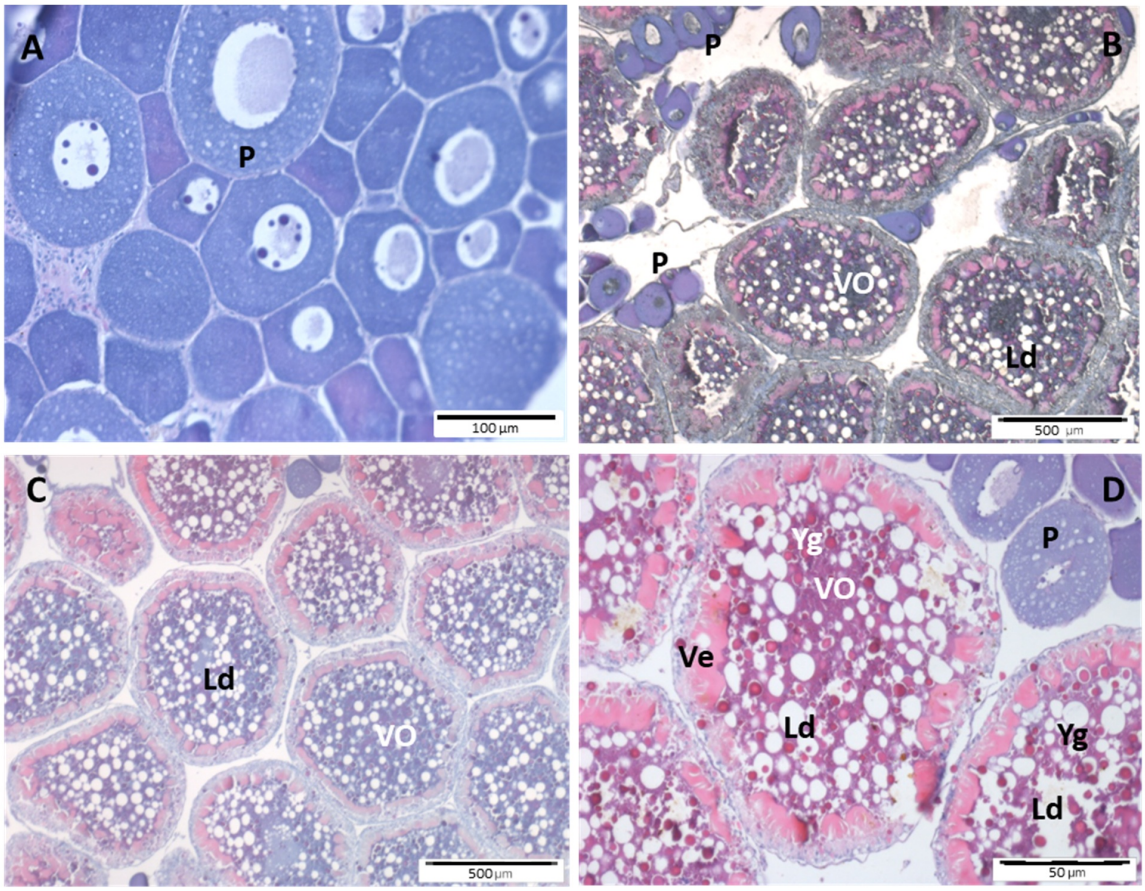

Figure 2. Morphology of representative ovary samples analyzed at time 0 during the present study. A. Ovary of a mullet captured in June showing previtellogenic perinucleolar oocytes (P); B-D. Mature ovary displaying vitellogenic oocytes (VO) characterized by the accumulation of yolk (Yg) and lipid droplets (Ld) within the cytoplasm with a thick vitelline envelope (Ve). Previtellogenic perinuclear oocytes are found scattered in clusters in between the vitellogenic follicles.

activity either in comparison to the control at 48 or at 120 hours. Anyway, when we analysed the percentage of cytotoxicity at 120 hours in comparison with the control group at 48 hours we observed that all the explant groups, control of the highest tested hormone concentration (E3), showed quite significant cytotoxicity, in the range 32% to 40%, while at 48 hours the most severe cytotoxicity in comparison to its control was shown by the highest 17β-estradiol concentration group (15%, Figure 5).

Ovaries with Vitellogenic Oocytes in Early Spring

The 5S to 18S rRNA ratio of in the ovarian tissue explants of *C. labrosus* captured in February are presented in Figures 8b and 8c. The ratio varies from 4.6 to 9.2 for tissue explants exposed to 17β-estradiol, and 6.3 to 8.3 for testosterone. The control explants displayed a lower index value, in comparison to the steroid treated explants and also in comparison to the samples in pre-vitellogenesis. Despite no statistical evidence, the two smallest doses of E2 displayed the highest 5S/18S rRNA index values in the ovaries with vitellogenic oocytes. On the other hand, no differences across the treatment groups were observed in ovarian tissue explants incubated with the androgen hormone T (Figure 6b). The index value at the time 0 was higher (Figure 6) in the ovaries containing only previtellogenic oocytes than in the ovaries containing vitellogenic stage oocytes together with scattered clusters of previtellogenic oocytes (8 vs 6).

Gene Transcription Analyses: *Previtellogenic oocytes*

The expression of tfiiia in thicklip grey mullet (captured in late spring) ovaries with previtellogenic oocytes was

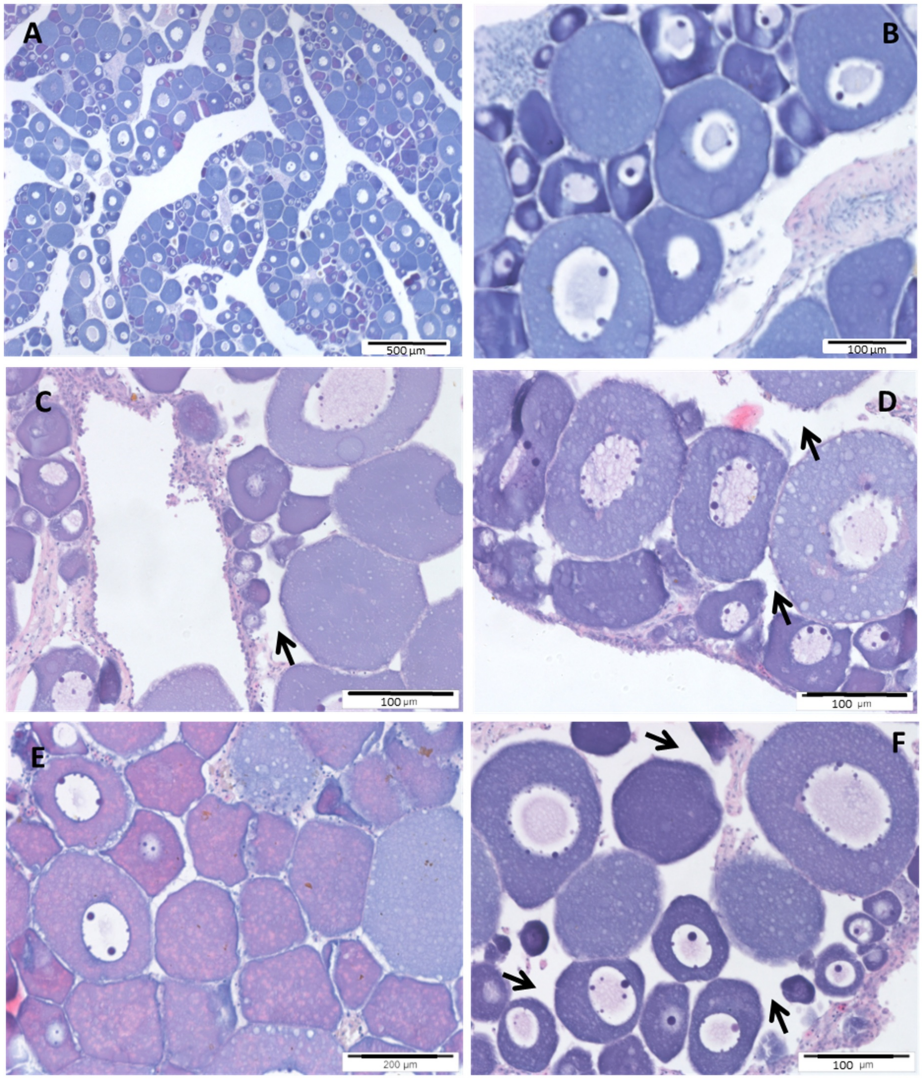

Figure 3. Morphology of representative previtelogenic ovary samples analyzed in mullets captured in May and June. A. Time 0 ovary histology where previtellogenic perinucleolar oocytes and well preserved interfollicular connective tissue preservation can be observed; B. Control explant sample after 5 days in culture. C, D and E represent the histology of explants after incubation with 10, 100 and 1000 pg mL^{-1} of 17β-estradiol, respectively for 5 days; F. The micrograph shows a detail of an explant after 5 days in culture showing severe disruption of the connective tissue and follicular cells surrounding the oocytes (arrows).

significantly regulated (ANOVA, p=0.041) after exposure to different concentrations of 17β-estradiol (Figure 7). Culture conditions resulted in a significant down-regulation of tfiiia transcription levels in comparison to the time 0 ovaries. 17β-estradiolon the other hand, up-regulated tfiiia transcript levels in a concentration-dependent way, showing a relative to the controlsignificant 3-fold, 2.2-fold and 1.6-fold increase after incubation with 10, 100 and 1000 pg mL^{-1} E2, respectively. There were no significant differences among groups in piwil1 transcription levels, control values after 5 days of culture, showing a high degree of variability (Figure 8). cyp19a1a expression levels showed significance differences between treatment groups (Figure 8). Culture conditions resulted in a significant down-regulation of cyp19a1a that was observed both under control conditions and after incubation with the 3 17β-estradiol concentrations tested.

Vitellogenic oocytes

The mRNA expression levels *of tfiiia, piwil1* and *cyp19a1a* genes in ovaries with vitellogenic oocytes incubated with various concentrations of 17β-estradiol

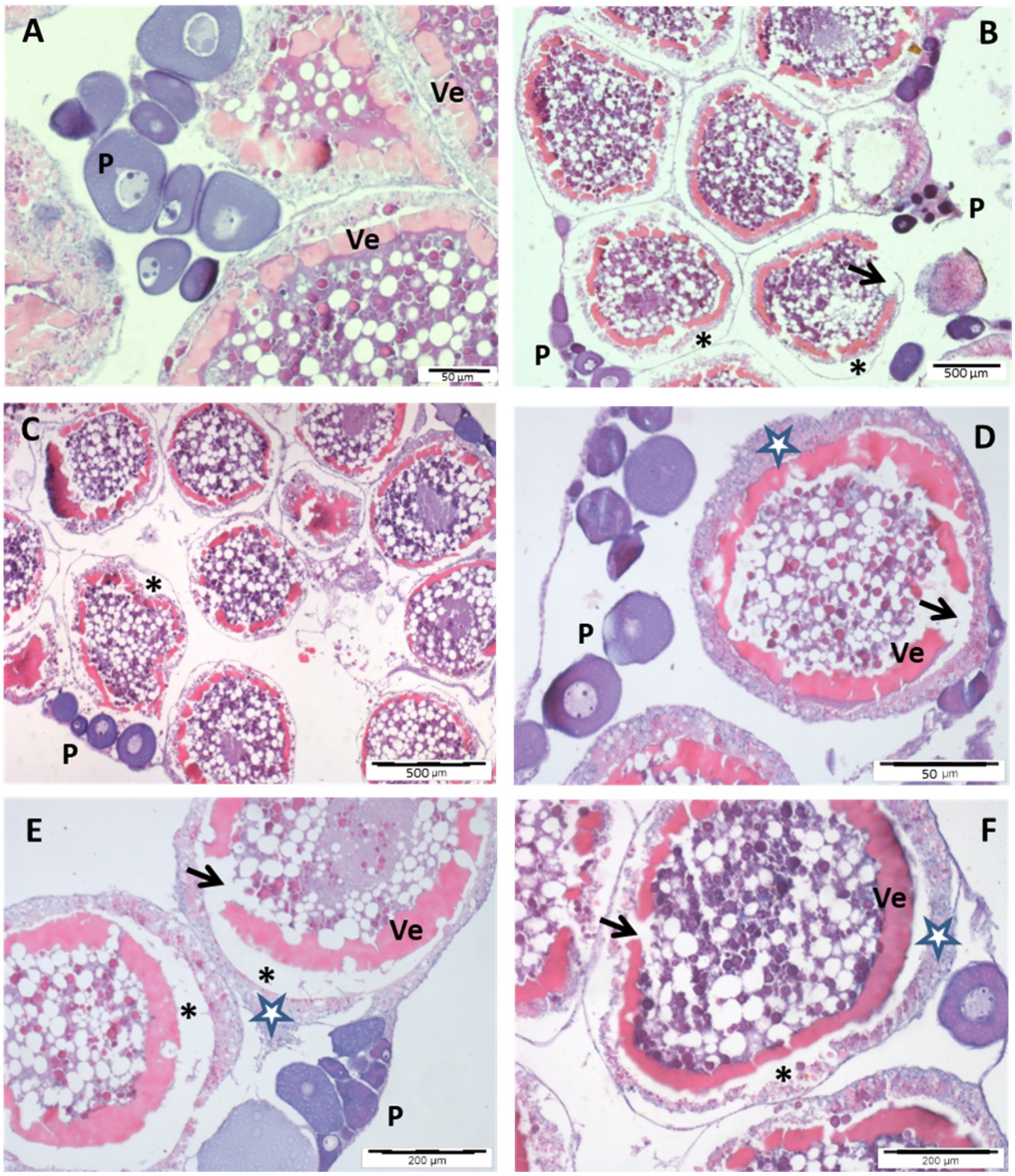

Figure 4. Micrographs showing the histology of mature gonads with vitellogenic oocytes present in the mullets captured in April. **A**. At time 0 well preserved oocytes with attached follicular cells can be observed. Clusters of perinucleolar oocytes (P) can be observed in between the vitelogenic follicles. **B**. Control ovary after 5 days in culture. **C** and **D**. Histology of explants after 5 days of incubation with 1000 ng mL^{-1} of testosterone and showing breakage (arrows) of the vitelline envelope (Ve) and detached follicular cell layers (asterisks). **E** and **F**. Detail of the vitellogenic oocytes in explants incubated with 17β-estradiol and showing thickened follicular cell layers with stromal connective tissue cells (stars).

and testosterone are shown in Figures 10 and 11. As only two female individuals were analysed, no statistical analysis could be done, but the qPCR analysis allowed to observe that the three genes studied are actively transcribed in ovaries under the explant culture conditions employed in the study. In general transcript levels were similar at time 0 and after 5 days of culture with the exception of a slight increase in *piwil1* transcript levels under culture. Both for tfiiia and for the piwil1 incubation with 17β-estradiol and testosterone in the 3 different concentrations tested resulted in transcriptional up-regulation in comparison to time 0 (Figure 8). No changes in cyp19a1a transcription levels were observed, but a slight increase with testosterone incubation.

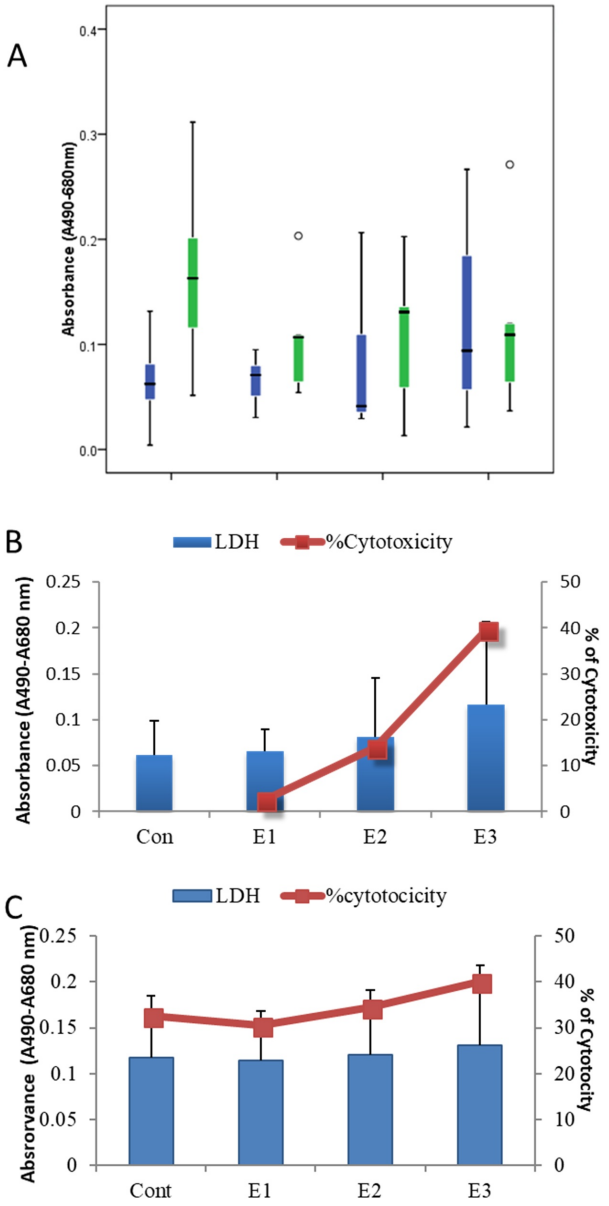

Figure 5.**A.** Box plots indicating the activity of LDH released to the supernatant from thicklip grey mullet (*C. labrosus*) ovarian tissue explants after 48 and 120 hours of culture. Box-plots represent the data within the 25th and 75th percentiles, indicating the median with a line. Whiskers include all data except outliers, which are identified by dots (defined as any data between 1.5 and 3 box lengths). LDH cytotoxicity was measured using the Pierce LDH Cytotoxicity Assay Kit. Cont represents control treated only by growth media, while E1, E2 and E3 represent tissue explants incubated with different concentrations of 17β-estradiol (E1=10 pg mL^{-1}, E2=100 pg mL^{-1}, E3=1000 pg mL^{-1}).
B and **C**. Represent the same data as in A and the % of cytotoxicity (red lines) in comparison to the maximal LDH release and the release in the control group at time 48 H (B=48 H; C=120 H).

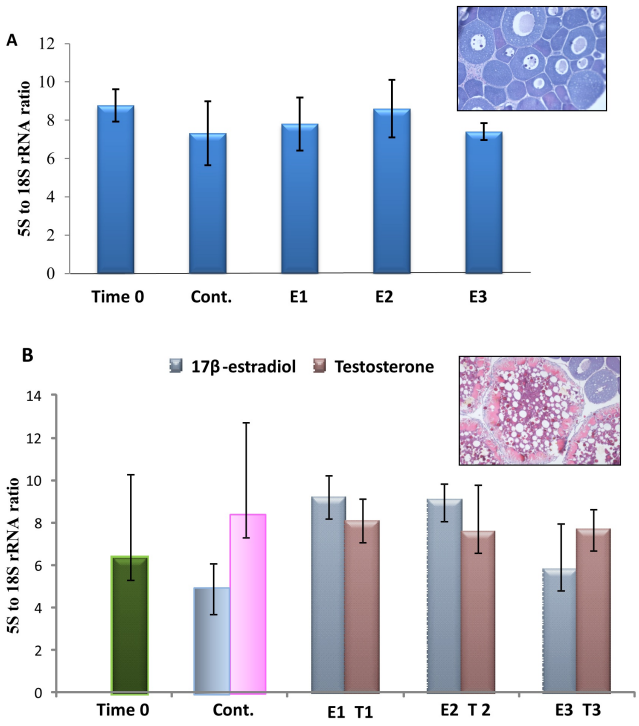

Figure 6. The $\log_2$ 5S to 18S rRNA ratio of ovarian tissue explants from *C. labrosus* in previtellogenesis (A, n=5) and in vitellogenesis (B, n=2) incubated with different concentrations of 17β-estradiol (E1 = 10 pg mL^{-1}, E2=100 pg mL^{-1}, E3=1000 pg mL^{-1}) and testosterone (only during vitellogenesis, T1=10 ng mL^{-1}, T2=100 ng mL^{-1}, T3=1000 ng mL^{-1}). Time 0 belongs to the ovary sample directly after dissection and before *in vitro* culture. Cont represents control explants cultured for 5 days.

DISCUSSION

In vitro organ culture is becoming an important methodological approach in predictive Ecotoxicology to forecast ecological risk from the use of certain chemicals (Villeneuve and Garcia-Reyero 2011) through the manipulation of isolated organs over a more or less extended time period. In the present study a simple protocol to maintain thicklip grey mullet gonad explants, in two different developmental stages, under culture conditions for up to 5 days was elaborated. The viability of the cultures was studied through histological analysis of the preservation of cell and tissue morphology, a cell integrity and viability assay, analysis of cell proliferation under incubation with steroid sex hormones, and the analysis of the transcriptional levels of estrogen synthesis and oocyte development related genes.

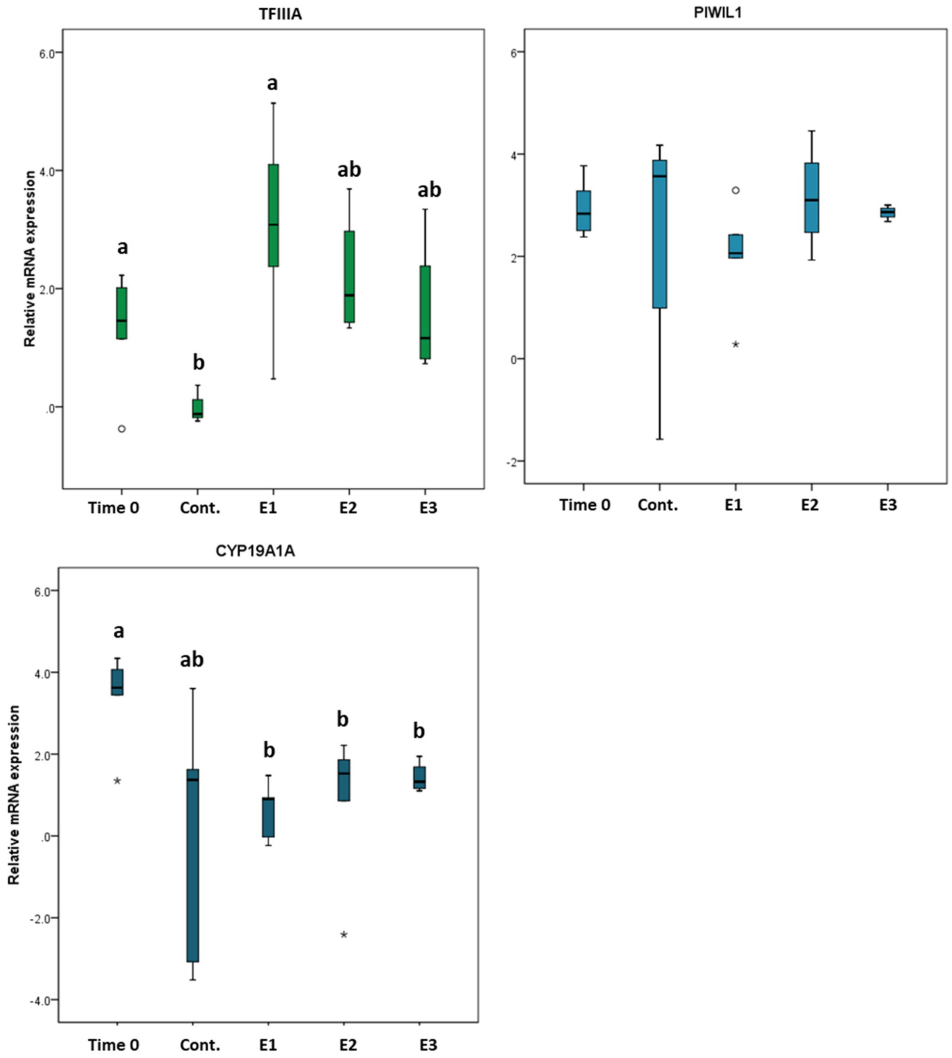

Figure 7. Box plots representing gene transcription levels across five conditions corrected by the amount of starting cDNA and normalized to the average of control group of thicklip grey mullets in previtellogenesis (n=5). Box-plots represent the data within the 25th and 75th percentiles, indicating the median with a line. Whiskers include all data except outliers, which are identified by dots (defined as any data between 1.5 and 3 box lengths) and asterisks (extreme outliers). Time 0 represents ovaries directly after dissection, Cont represents control group after 5 days of in vitro culture, while E1, E2 and E3 represent tissue explants incubated with different concentrations of 17β-Estradiol (E1=10 pg mL^{-1}, E2=100 pg mL^{-1}, E3=1000 pg mL^{-1}). Different letters indicate significant differences between groups at $p<0.05$.

Explants Morphology in Thicklip Grey Mullet Under Culture

Histological analyses of the gonad explants under culture in the present study showed that the structure of oocytes, more so in the case of previtellogenic oocytes than in the case of vitellogenic ones, was maintained nearly unaltered. Nevertheless, ovarian morphology after in vitro culture displayed some prominent alterations related to the culture conditions irrespective of the incubation with external steroid hormones in comparison with the gonad morphology after direct dissection of the test fish. Disruption of the stromal connective tissue surrounding the oocytes was evident both in mature gonads containing vitellogenic oocytes and in previtellogenic ovaries. Furthermore, a considerable thickening of the follicular cell layer, with aggregation of stromal connective cells, was noted around the vitellogenic oocytes in the ovaries of mullets captured in April. Such alterations were not reported in brown trout

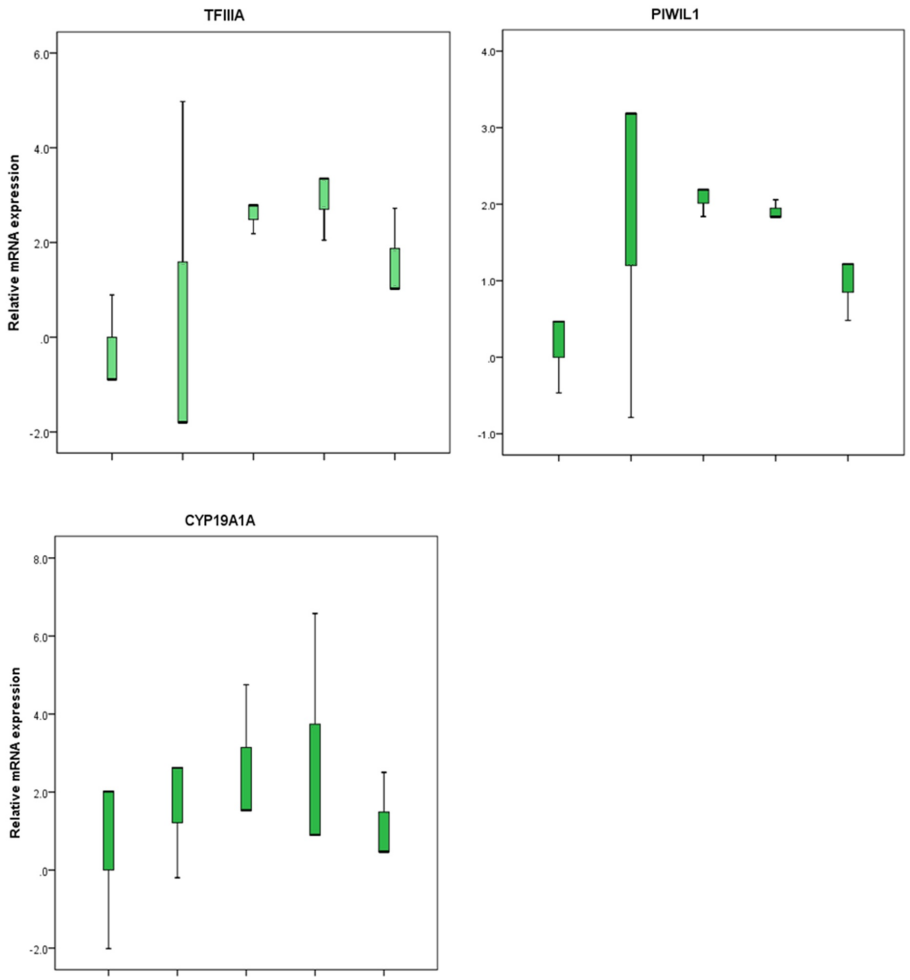

Figure 8. Box plots representing gene expression analyses across five conditions corrected by the amount of starting cDNA and then normalized to the average value of the control group in the ovaries of thicklip grey mullets in vitellogenesis (n=2). Box-plots represent the data within the 25th and 75th percentiles, indicating the median with a line. Whiskers include all data except outliers, which are identified by dots (defined as any data between 1.5 and 3 box lengths) and asterisks (extreme outliers). Time 0 represents ovaries directly after dissection, Cont represents control group after 5 days of *in vitro* culture, while E1, E2 and E3 represent tissue explants incubated with different concentrations of 17β-Estradiol (E1=10 pg mL^{-1}, E2=100 pg mL^{-1}, E3=1000 pg mL^{-1}). There are no significant differences between groups at $p<0.05$.

ovary explants but, testis explants in the same species suffered from germ cells degeneration and Sertoli cells vacuolation after 8 days in vitro culture (Pereira et al. 2007). Similarly, zebrafish testis explants showed seriously disrupted morphology following 7-day incubation with basal medium (Leal et al. 2009).

We observed only slight morphological differences in both previtellogenic and vitellogenic oocytes after 5 days in vitro culture in comparison to the time 0 structures, but vitellogenic oocytes showed a small deterioration of the vitelline envelope under culture. These observations might demonstrate that gonadal maturation stage represents an important factor with regard to the resilience of fish ovarian tissue to culture conditions as well as to its sensitivity to steroid hormone exposure. Thus, in vitro gonad explants studies using wild sentinel fish species (especially in species with seasonal synchronous developing ovaries) should take into account the gonadal developmental stage of the fish and should be treated separately, as this could considerably affect the viability of the culture and the representativeness of the endpoints measured.

The appearance of such morphological alterations after in vitro culture could be due to suboptimal conditions for the ovary tissue explants. In fact, floating agarose organ culture system that sustains the explants

so it is not immersed in the growth media (Miura et al. 1991) was not used in the present study. Instead, the tissue explants were submerged in the growth media. This technique involves placing gonad fragments on top of a nitrocellulose filter on top of agarose cylinders that are immersed in the growth media (Miura et al.1991, Leal et al.2009). Agarose cylinder is usually produced from agarose solution using 96 well plates as mould. The advantage of this structure is that it allows the explants to be well ventilated while receiving medium and test chemicals uniformly as they float in the wells containing the growth medium. Additionally, no incubation chamber could be employed in the present study to fine adjustment of temperature (at18ºC), humidity control, and atmospheric air circulation, as it is done in all studies involving gonad explants in vitro culture (eg. Leal et al. 2009). Additionally, other sample processing effects might have arisen during the experimental protocol such as those derived from cutting gonads into smaller pieces. Moreover, paraffin embedding approach was undertaken instead of metacrylate embedding (Bizarro et al. 2014) this resulting in suboptimal ovarian (especially mature one) histological sample quality. Despite this, there was no difference observed in regard to RNA integrity and abundance between the intact ovary at time 0 and tissue explants after 5 days culture, as confirmed from electrophoresis by Agilent Bioanalyzer 2100. Thus, we believe that the culture conditions described in the present study optimally maintained the morphology and function (see below) of oocytes, but it was suboptimal in regard to preserving the supporting somatic cell structure and thus it needs further improvement for future mechanistic studies.

We did not observe any histological alterations in regard to E2 and T treatments or in regard to change in oocyte size or type composition along culture. The in vitro incubation of eel (*Anguilla australis*) ovary explants with 11-ketotestosterone has been reported to result in an increase in the diameter of previtellogenic oocytes (Rohr et al. 2001, Lokman et al. 2003, 2007), while E2 did not have any effect on the oocyte size (Lokman et al. 2003, 2007). These results indicate that the growth of previtellogenic ooocytes in teleost fish may be regulated by androgen steroid hormones through lipid accumulation into the oocyte (Lokman et al. 2007). In this study, we observed a thickening of the follicular cell layer of vitellogenic oocytes despite the effect was not quantified. This should be examined in future studies, since this could provide important insight into the effects of EDCs on the reproductive development of thicklip grey mullet populations. Alternatively, it could be just an effect of the disruption in the connective cells, it produces an aggregation onto follicular cells.

Cell Viability Assay and Thicklip Grey Mullet Ovarian Explants

The viability of the explants incubated in medium culture with or without 17β-estradiol in previtollgenic oocytes captured in May-June did not show any significant alteration, in regard to any significant increase in LDH enzyme activity released to the medium,when comparing the situation after 48 and 120 h of incubation period. This should indicate that thicklip grey mullet ovary explants did not suffer any significant loss of cell viability in the period elapsing in between the 48 and the 120 h of culture. Yet, viability tended to decrease and the quantity of LDH released was higher after 120 h of culture. Incubation with the highest concentration of E2 (1000 pg mL^{-1}) produced higher, although not significant, cytotoxic effect in ovarian explants in both time points, cytotoxicity being very high in all groups at 120 h. High viability, greater than 80%, as measured by MTT assay, has been reported, for fathead minnow ovary explants under in vitro culture in L-15 medium for 28 days (Johnston et al. 2014). As to the effects of E2 in culture, incubation of Human Eyelid-Derived Adipose Stem Cells (hEASCs) with low concentrations of E2 (10 nM-10 μM) for 24 hrs did not result in insignificant changes in cell survival, but higher doses of E2 (100 μM) induce toxicity after 24 and 48 hrs incubation (Zhou et al. 2014). Related to the LDH test employed hereby, it has not been previously employed in fish gonad explants but we can mention that Gerbron et al. (2010) studying liver explants in roach (*Rutilus rutilus*) observed no significant changes in cell viability after 3 days in vitro culture, although after 96 and 120 h significant amount of LDH were released to the medium. The cytotoxicity percentage of 32% at 120 h control culture in comparison to the control condition at 48 h relative to highest LDH possible activity release reveals quite severe disruption of the somatic cell structure of the ovary. This was slightly increased to 40% in the highest E2 concentration. We interpret that the metabolically dormant oocytes have no meaningful LDH activity so the enzyme should be released from the somatic cells. Incubation temperature has a profound effect on tissue viability of gonad explants in in vitro culture. In this way, lower temperatures improved the viability of fathead minnow ovary explants due to

reduced bacterial and fungal growth and decreased cellular metabolism (Johnston et al. 2014). Normally, donor fish spawning temperature range in the wild is recommended as the temperature to incubate explants maintaining relevant cellular functions (Johnston et al. 2014), so we assumed that 18ºC was an optimal culturing temperature for thicklip grey mullet. Instead, culture conditions did not allow the most effective monitoring of temperature conditions in the medium, and this should be considered for future analysis.

The Effect of Steroid Hormones on the 5S/18S rRNA Index

Recent field-based studies have shown that 75% of the total RNA content in the ovary of thicklip grey mullet ovaries corresponds to 5S rRNA (Diaz de Cerio et al. 2012, Ortiz-Zarragoitia et al. 2014). Considerable oocyte specific accumulation of 5S rRNA was first time reported in anuran frogs *Xenopus laevis*(Van den Eynde et al. 1989) but is seems to be something general in all teleost fish species (Ortiz-Zarragoitia et al. 2014, Rojo-Bartolomé et al.2014). The production of 5S rRNA is so high that it can be studied through a simple electrophoresis of total RNA extracted from the ovaries, revealing a very potent 120 bp band with very faint bands corresponding to the largest rRNA molecules; 18S and 28S rRNA. qPCR analysis has also confirmed that the transcription level of 5S rRNA is much higher in ovaries than in testis during the whole annual reproductive cycle of mullets in the Basque coast (Diaz de Cerio et al. 2012, Ortiz-Zarragoitia et al. 2014). On the other hand, intersex individuals found in the polluted harbor of Pasaia due to environmental exposure to xenoestrogens showed intermediate 5S rRNA expression profiles revealing that the oocyte and not the somatic cells was the site of 5S rRNA production in the ovary (and in the intersex testis). Therefore, 5S rRNA has been suggested to be as a powerful biomarker to molecularly identify sex and reproductive endocrine disruption in fish (Diaz de Cerio et al. 2012, Ortiz-Zarragoitia et al. 2014).

In the present study, the results of the electrophoresis of ovarian total RNA showed strong production of 5S rRNA, irrespective of the maturity stage of the gonad, both before and after the culture of the ovarian explants. Based on these data 5S/18S rRNA index was calculated, and no significant changes were observed under the culture conditions. Ovary explants in previtelogenic stage incubated with lowest E2 concentration (10 and 100 pg mL^{-1}) showed non-significant slightly higher 5S/18S rRNA index compared to other groups. These findings indicate that the accumulation of 5S rRNA was not affected during culture and proving, as the histological analysis did, that the morphology and function of the oocytes was preserved under the employed culture conditions. We do not know whether what we are quantifying is 5S rRNA accumulated in the oocytes already before dissection of the gonads, or whether any new 5S rRNA was being transcribed during the 5 days of culture.

In the case of vitellogenic oocytes 5S/18S rRNA index showed a considerable fluctuation among the treatment groups, but we should understand that we only quantified two individuals so this may be related to the inter-individual differences. On the other hand, no differences were observed among the groups of the ovarian tissue explants (containing vitellogenic oocytes) incubated with testosterone. The transcription of 18S and 28S rRNA profoundly increases with respect to that of 5S rRNA as vitellogenesis advances towards oocyte maturation. In fact, 5S rRNA transcription could be considered a marker of previtellogenic oocytes (Rojo-Bartolomé et al. 2014). In this study, we observed that the 5S/18S rRNA index was higher in ovaries from animals captured in May and June (previtellogenic oocytes) than in the two females studied in April (Vitellogenic). This strong expression of 5S rRNA as in thicklip grey mullets has also been reported for other species such as zebrafish Danio rerio, European hake *Merluccius merluccius* (Diaz de Cerio et al. 2012), anchovy *Engraulis encrasicolus* or megrim *Lepidorhombus whiffiagonis* (Rojo-Bartolome et al. 2014) where the index always exhibited highest value during previtellogenic stages, due to active transcription of 18S rRNA during vitellogenesis (Rojo-Bartolome et al. 2014). Still the 5S/18S rRNA ratio was very high in the ovaries of April, and as it was revealed from the histological analysis prominent clusters of previtellogenic oocytes were present in these ovaries. It is very possible that the two females that were studied in April were females that had skipped spawning. Consequently, they should be undergoing resorption of vitellogenic oocytes that should have been spawned at sea during January-March, while preparing already for the next reproductive season initiating another gametogenic cycle. Thus, the index has revealed itself as an important tool that can identify different stages of the ovarian development based on the relative amount of 5S to 18S rRNA accumulated in oocytes, also under ovarian explant culture conditions.

Gene Transcription Levels in Thicklip Grey Mullet Ovarian Explants

cyp19a1a transcript levels

cyp19a1a transcription could be effectively quantified through qPCR in thicklip grey mullet ovarian explants (both, in early gametogenesis and in vitellogenesis) kept in culture for 5 days. Only 2 females were analysed during the April sampling, so nothing statistically relevant can be said according to the effects of the culture conditions. Cyp19a1a was significantly down-regulated in ovaries containing previtellogenic oocytes after 5 days of culture, both under control conditions and under incubation with E2. This could be another proof of the effect of the culturing conditions on the follicular cells surrounding the oocytes and responsible for aromatase expression and estradiol production. The disruption of ovarian somatic cells was very pronounced as observed in the histological analysis, and as the cell viability decreases and the ovarian somatic cells are destroyed a reduction of *cyp19a1a mRNA* transcript levels should be expected, as cyp19a1a is expressed mainly in the follicular cells lining the vitellogeneic oocytes during vitellogenesis (Devlin and Nagahama, 2002). Very interestingly, and although studying testis explants of zebrafish, Leal et al. (2009) also observed a down-regulation of the steroidogenic system, in this case measured in terms of 11-KT production and *cyp17a1* transcript levels. They considered this to happen very rapidly after initiating culture conditions, within 2 days, and resulting mainly in a severe disruption of the development of spermatogonia, spermatocytes, and spermatids in zebrafish testis explants under basal conditions.

Similar results to ours have been described for other fish in which ovarian explants have been cultured in vitro culture. For instance, Vang et al. (2007) found that treating salmon previtellogenic oocytes with of EE2 (10 nM) significantly decreased the transcription levels of *cyp19a1a* after 3 days in vitro culture. On the contrary, significantly increased *cyp19a1a* transcript levels were observed in isolated marine medaka ovarian follicular cells in vitro (not tissue explants) after 2 days of exposure to 20 nM and 200 nM of E2, but not when exposed to a higher concentration of 2 μM (Tsea et al. 2013). On the other hand, in vivo E2 treatment has also been described to result in a significant reduction in *cyp19a1a mRNA* expression levels in rainbow trout ovary (Nakamura et al. 2009) or in rare minnows exposed to EE2 (Wang et al. 2010). Instead, no significant changes in expression levels were reported in fathead minnow ovaries after exposure to 14 ng mL^{-1} of E2 for 14 days (Filby et al. 2007).

We would like to point out that the transcriptional levels of *cyp19a1a* showed a slight increase after incubation of ovaries with vitellogenic oocytes with T (remember the impossibility to run a statistical analysis with these samples due to n=2), which was more pronounced at low concentrations (10 ng mL^{-1}). A similar androgen hormone 17α-methyltestosterone produced elevated *cyp19a1amRNA* expression in Atlantic cod ovary explants with previtellogenic oocytes, after 1 day of incubation (Kortner and Arukwe 2007). Opposite effect with a significant decrease was observed after 5 and 10 days of exposure (Kortner and Arukwe 2007). The size of previtellogenic oocytes in the explants of the same species significantly increased after in vitro exposure to the androgens 11-ketotestosterone (11-KT) and T for 5 and 10 days, although the effect was stronger for 11-KT (Kortner et al. 2008).This seems to indicate that androgens play a pivotal role regarding early oocyte growth control in teleost fish. This is very important for future studies regarding intersex testis, as the development of oocytes in a natural testosterone producing cellular environment, could be studied using ovary, testis and intersex testis explants, externally supplemented with hormones.

tfiiia and piwil1 transcription levels

In eukaryotic cells 5S rRNA is produced when RNA polymerase III is activated via the general transcription factor IIIA (TFIIIA) (Szymanski 2003 cited in Diaz de Cerio et al. 2012). TFIIIA protein in turn, is able to bind to newly produced 5S rRNA to help in its accumulation and stockpiling in the cytosol in the form of small ribonucleoprotein particles, this means that the transcription profiles of *tfiiia* and 5S rRNA should be very similar (Szymanski 2003). In this way, the transcription levels of tfiiia and 5S rRNA have been shown to display the same profile in *C. labrosus* ovaries along the reproductive cycle in the Basque coast. *tfiiia* is also very strongly transcribed in the ovaries in contrast to the testis, and its transcription levels are increased in intersex testis with a profile in between normal testis and ovaries (Diaz de Cerio et al. 2012). Thus, *tfiiia* transcription levels, as it is the case for 5S rRNA, can be considered good biomarkers to molecularly sex fish and to follow the growth and development of oocytes from

one stage to the next as vitellogenesis advances (Rojo-Bartolomé et al. 2014).

As it comes to piwil1 (*ziwi*in zebrafish), this is a germline stem cell marker gene, since during adulthood, it is expressed exclusively in the gonads of zebrafish, more strongly in testis (Houwing et al. 2007), within the proliferating oogonia or spermatogonia (Leal et al. 2009). In zebrafish ovary *piwil1* is also expressed in early stage I and II oocytes (Tan et al. 2002). The transcription of piwil1 in *C. labrosus* was stronger in testis than in the ovary, in ovaries the transcription being at its highest during early vitellogenesis where the ovaries contain plentiful oogonia and stage I and II oocytes (Diaz de Cerio et al. 2012).

As said before, we wanted to study whether ovary explant germ cells would proliferate under incubation to steroid hormones in in vitro culture conditions. Thus, tfiia and piwil1 transcription levels were quantified as important oocyte differentiation molecular markers in previtellogenic and vitellogenic ovaries. After 5 days of culture *tfiiia* was significantly down-regulated, down-regulation that was rescued by incubation with externally supplemented estradiol, in the three concentrations tested, but especially in the lowest concentration, with a three-fold increase in transcript levels with 10 pg mL^{-1} E2. *tfiiia* transcript levels being markers of oocyte development and differentiation, mainly during early gametogenesis, reveals the level of germ line differentiation that is made possible by estrogen. If the culture conditions, as we have seen resulted in a down-regulation of *cyp19a1a*, this should obviously result in a reduced production of estradiol and a reduced transcription of *tfiiia*. Instead, the administration of external E2 during the 5 days of incubation allows maintaining the transcription levels of *tfiiia*. Finally, no regulation was observed in *piwil1* transcription levels under culture condition that would signify that there was not a proliferating effect of estradiol on the mitotically dividing oogonia, and this was reflected in an unmodified *piwil1* transcription profile.

CONCLUSIONS

Based on the results obtained the following conclusions can be drawn: (1) The ovary explant culture conditions employed in this study preserved the morphology of oocytes for 5 days, but slight disruption of the stromal connective tissue and the follicular cells surrounding the oocytes. (2) The viability of cells in the explants showed a decreasing trend with the corresponding increase of LDH release after 120 h of culture period in comparison to 48 h. Percentage of cytotoxicity reached high levels at 120 h in all cases showing that culture conditions were suboptimal for the maintenance of ovarian somatic cell viability. (3) Strong 5S rRNA production was observed in all the ovaries studied, a production that was not altered after culturing ovarian explants for 5 days. As a result, 5S/18S rRNA index did not change significantly under culture, but it was higher in the ovaries showing only previtellogenic oocytes than in those containing vitellogenic oocytes. (4) As a consequence of follicular cell disruption *cyp19a1a* transcription levels were significantly down-regulated in ovaries containing previtellogenic oocytes after 5 days of culture, both under control conditions and under incubation with 17β-estradiol. (5) In previtellogenic ovary explantsfrom thicklip grey mullets captured in May–June, *tfiiia* transcription levels were significantly reduced relative to the time 0, but exogenous supply of 17β-estradiol at the three concentrations tested rescued *tfiiia* transcription activity. The transcriptional levels of *piwil1* were not modified by the culture conditions.

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Authors' contributions: Assefa WW conceived the study, organized the field work for data collection, laboratory analyses, data analyses and writing of the draft manuscript; Ibon Cancio designed the study, correcting the first draft, preparing graphs; Both authors reviewed, edited and approved the final manuscript for submission.

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