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Author: Ángela Gárriz Renata J. Menéndez-Helman Leandro A. Miranda

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Effects of estradiol and ethinylestradiol on sperm quality, fertilization, and embryo -larval survival of pejerrey fish (*Odontesthes bonariensis*).

Ángela Gárriz[a], Renata J. Menéndez-Helman[a,b], Leandro A. Miranda[a*]

lmiranda@intech.gov.ar

[a]Laboratorio de Ictiofisiología y Acuicultura. Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús, IIB-INTECH (CONICET-UNSAM).Intendente Marino Km. 8.200 (B7130IWA), Chascomús, Buenos Aires, Argentina
[b]Laboratorio de Enzimología, Estrés y Metabolismo – Facultad de Ciencias Exactas y Naturales (FCEyN, UBA-CONICET)

[*]Corresponding author.

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Highlights

Estrogenic compounds are present in surface waters as a consequence of sewage discharges.

Mixtures of E_2 and EE_2 affected pejerrey sperm motility and fertilization.

Mixtures of E_2 and EE_2 affected pejerrey embryo and larval survival.

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ABSTRACT

17β-Estradiol (E_2) and synthetic 17α-Ethinylestradiol (EE_2) are estrogenic compounds present in surface waters as a consequence of municipal sewage discharges. The aim of this study was to evaluate the effects of E_2, EE_2 and its mixtures on different reproductive parameters and embryo-larval survival in pejerrey fish (*Odontesthes bonariensis)*. In order to analyze the effect of these compounds on sperm quality, fertilization %, embryo-larval survival (%), and the point of no return (PNR), different assays were performed using concentrations 175, 350, 700 and 1400 ng/L of E_2; 22.5, 45, 90 and 180 ng/L of EE_2 and mixtures M1 (175 E_2 + 22.5 EE_2, ng/L), M2 (350 E_2 + 45 EE_2, ng/L), M3 (700 E_2 + 90 EE_2, ng/L) and M4 (1400 E_2 + 180 EE_2 ng/L). No significant differences in motility parameters were observed between E_2 and EE_2 treatments and the control group. However, a significant decrease in motility % was recorded for all mixtures tested compared with the control samples. For fertilization %, only sperm activated with M4 showed a significant decrease compared with the control group. In the case of embryo survival, there was only a significant decrease in the highest concentration of EE_2 compared with the control group. For the mixtures, M3 is the one that had the most adverse effect on embryo survival. In larval survival, there was a significant decrease in concentration 175 and 700 ng/L of E_2 compared with the control group. In EE_2 treatments, the ones with a significant reduction in larval survival were concentration 45 and 90 ng/L. And for the mixture treatments, M1, M3 and M4 had a significantly lower larval survival than the control group. In comparison to other treatments, M1 demonstrated a significant difference in PNR when compared with the control group. The results obtained demonstrated that the exposure to mixtures of E_2 and EE_2 affected fish sperm motility, fertilization % and, embryo and larval survival

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even at relevant environmental concentrations highlighting the necessity of considering the effects of pollutants mixtures in ecotoxicological studies.

Keywords: Embryo and larval survival; Estradiol; Ethinylestradiol; *Odontesthes bonariensis*; Sperm quality.

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1. Introduction

It is well known that the presence of estrogenic compounds in surface waters is the consequence of municipal sewage discharges (Arcand-Hoy and Benson, 1998; Larsson *et al*., 1999; Thorpe *et al*., 2003; Chang *et al*., 2008). 17β-estradiol (E_2) and the synthetic estrogen 17α-ethynylestradiol (EE_2), used in oral contraceptive and hormone replacement therapy (Kime, 2001), are excreted through the urine in an inactive conjugated form (Guengerich, 1990). Both estrogens can be reconverted to an activate form possibly by the action of certain bacteria present in sewage (Legler *et al.*, 2002)**.**

These estrogens are considered to be the primary pollutants contributing to the estrogenic activity in water bodies all over the world (Desbrow *et al*., 1998; Snyder *et al*., 1999; Sumpter and Jobling, 2013). New advancements in analytical methods have improved accuracy and detection limits for estrogenic compounds, allowing improvements of measuring techniques. Therefore, recent values for EE_2 concentrations in effluents and surface waters range approximately from 0.05 to 831 ng/L (Ternes *et al*., 1999; Baronti *et al.*, 2000; Kolpin *et al.*, 2002; Laurenson *et al.*, 2014).

This study was focused on the effects of E_2 and EE_2 concentrations found in Chascomús shallow lake where a pejerrey fish (*Odontesthes bonariensis)* population is well established. This shallow lake is located bordering the city with the same name in Buenos Aires province, Argentine (35°36'S 58°02'W). The city effluents are discharged in the "*Girado*" stream (35°38'S 58°0'W), which is connected with Chascomús lake and where pejerrey fish is also found (Colautti *et al.*, 2015). The morphological and physicochemical characteristics of this lake (Diovisalvi *et al.*, 2010) stimulate the presence and accumulation of this kind of environmental estrogens (Valdés *et al.*, 2014).

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A previous study reported that during summer months the concentration of E_2 and EE_2 in the "*Girado*" stream were 369 and 43 ng/L, respectively (Valdés *et al.*, 2014).

Although the levels of E_2 in effluents are relatively low, from <1 to 200-350 ng/L range (Desbrow *et al.*, 1998; Rodgers-Gray *et al.*, 2000; Laurenson *et al.*, 2014), they are still high enough to elicit adverse reproductive effects in fish (Halm et al., 2002; Kidd *et al.*, 2007; Rodgers-Gray *et al.*, 2001). The levels of E_2 found in water are higher than EE_2 (Kolpin *et al.*, 2002; Valdés *et al.*, 2014), and being that the toxicology of both compounds is similar, both act as sexual endocrine disruptors (ED) in different animal species (Vethaak et al., 2006).

It has been reported that low concentrations of EE_2 (between 2 and 100 ng/L) are linked to the formation of ova-testis and fibrosis in sheepshead minnow (*Cyprinodon variegatus*; Zillioux *et al.,* 2001); reduction of growth and fecundity rate or to a complete lack of testicular development, in fathead minnow (*Pimephales promelas*; Scholz and Gutzeit, 2000) and medaka (*Oryzias latipes*; Lange *et al.*, 2001). As well, in adult males of *J. multidentata* exposed to 75 and 150 ng/L of EE_2 a significant decrease in the gonadal somatic index (GSI) and several alterations in the reproductive behavior were observed (Roggio *et al.*, 2014). This study also showed no significant differences in VSL, VCL and linearity between treatments and the control group (Roggio *et al.*, 2014). Similar results have been observed in sperm velocity when males of *J. multidentata* were exposed to E_2 (50–250 ng/L) for 28 days, coupled with an increase of behavioral sexual activity at a single concentration of 50 ng/L (Guyón *et al.*, 2012).

Although both sexes are affected by the presence of estrogens in the environment, males exhibit the highest grade of adverse effects compared to females, such as vitellogenin induction, increase of aromatase activity, ova-testis development and an increase of apoptosis in germ cells (Sumpter, 1995; Balch *et al.*, 2004; Caspillo *et al.*,

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2014). In most teleost species fertilization is external (Schoenfuss *et al.,* 2009) and the sperm are immotile until they make contact with water, where the sharp drop in osmolality activates them (Takai and Morisawa, 1995). Once activated, the sperm are motile for a few minutes (Morisawa and Suzuki, 1980; Billard *et al.,* 1995). During this time, they must enter the egg via the micropyle (Kime and Nash, 1999). It is because of the nature of this fertilization that the first minute after induction of motility is crucial for success (Casselman *et al.*, 2006; Hara *et al.*, 2007) and, the sperm must swim fast enough and in the right direction to find the micropyle (Rurangwa *et al.*, 2004). Thus, sperm motility is a reliable indicator of fertilization success in fishes (Rurangwa *et al.*, 2001; Fitzpatrick *et al.*, 2009), and the presence of any contaminant in the water that affects this direction may be harmful to the fertilization process (Montgomery *et al.*, 2012).

Moreover, early life stages are considered to be particularly vulnerable to xenoestrogens, mainly during morphological sex differentiation (Vizziano *et al.*, 1996). This has caused a tendency to focus on intersexuality as a critical endpoint of these kinds of toxicological evaluations. However, this endpoint may not always be the most sensitive to estrogen exposure and even earlier signs may exist, since the effect could happen before these stages are completed (Schultz *et al.*, 2003).

Recently, there has been more interest to investigate the combined or synergic effects of groups of endocrine disruptor chemicals. In this sense, zebrafish (*Danio rerio*) fry exposed to a mixture of 2 mM EE_2 and 50 mM DBP (dibutyl phthalate) for 95 days posthatching, showed alterations in the morphology of the gonad, liver and gills. However, when pollutants were tested separately compared to pollutants tested in combination, these alterations were absent. This indicates that the mixture is more

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harmful on the development of zebrafish (Chen *et al.*, 2015). As well, Silva and collaborators (2012) found that the exposure to a xenoestrogens mix, present in the Douro River (Portugal), caused a decrease of the relative volume of spermatozoa in zebrafish, which consequently can adversely affect breeding (Silva *et al*., 2012).

Pejerrey fish (*Odontesthes bonariensis),* the model species used in the current study, is an emblematic species of continental waters of the Pampa region, Argentine (Somoza *et al*., 2008). This fish is very sensitive to different contaminants (Carriquiriborde *et al*., 2008) and was demonstrated that E_2 administration at dosages of 20 and 50 mg/kg in the diet, produced 100% females (Strüssmann *et al.*, 1996). In addition, Pérez et al. (2012) reported that there was a feminization process driven by EE_2 in pejerrey larvae during the first 6 weeks after hatching. In this context, the aim of this study was to evaluate the effects of E_2, EE_2 and its mixture, even at environmentally relevant concentrations, on the first steps of reproductive process in pejerrey fish (*Odontesthes bonariensis)*; evaluating sperm quality, fertilization % and embryo-larval survival.

2. Materials and methods

2.1. Chemicals and treatment solutions

A stock solution of 100 μg/L of E_2 and EE_2 (Sigma–Aldrich; St. Louis, MO, USA) was prepared by dissolving the reagent in ethanol; and the treatment solutions were prepared by diluting these stocks with ground water (pH 7.97; osmolarity 222.7 mOsm/L; Alkalinity 611.3 mg $CaCO_3$/L; Hardness 202.7 mg $CaCO_3$/L). The final concentrations were: 175, 350, 700 and 1400 ng/L (corresponding to 0.64, 1.28, 2.57 and 5.14 nM respectively) for E_2; and 22.5, 45, 90 and 180 ng/L (corresponding to 0.08,

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0.15, 0.30 and 0.61 nM respectively) for EE_2. Also, mixtures of both estrogenic solutions were prepared as a combination of each concentration mentioned above: M1 (175 ng/L E_2 and 22.5 ng/L EE_2), M2 (350 ng/L E_2 and 45 ng/L EE_2), M3 (700 ng/L E_2 and 90 ng/L EE_2) and M4 (1400 ng/L E_2 and 180 ng/L EE_2). Some of these concentrations are environmentally relevant as it was mentioned in the introduction (see Valdés *et al.*, 2014). All the treatment solutions were prepared immediately before the moment of the analysis.

Two control groups were done, one with ground water, and the other one containing the solvent vehicle (ethanol) at the highest concentration used in the preparation of treatment solutions (0.7µL/50 ml water). In all the reproductive parameters analyzed in both controls, there were no significant differences, so, the graphics below will only show the control group containing just water.

2.2. Analysis of sperm quality

In October, during the spawning season (Elisio *et al.*, 2014), five sexually mature pejerrrey males were selected from indoor tanks (3000 L) at the *Instituto de Investigaciones Biotecnológicas - Instituto Tecnológico de Chascomús* (*Chascomús, Buenos Aires, Argentine*) aquaculture facilities. Before manipulation, the fish were anesthetized by immersion in a 100ppm benzocaine solution for five minutes approximately. The total sperm was stripped from each male, by abdominal massage, collected with a syringe, drawn into a capped tube and kept on ice until used. Every contaminated sample with urine or blood was discarded. To record the videos, 1 µl of semen was activated by mixing with 1800 µl of each treatment solutions, at room temperature (22 °C). After gentle stirring, 10 µl of this dilution was immediately loaded into a Neubauer chamber and covered with a coverslip (24 mm x 24 mm), previously

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pre-coated with 1% polyvinyl alcohol solution and dried at 60 ºC to avoid the adhesion of spermatozoa (Kime *et al.,* 1996; Chalde *et al.*, 2014). Videos of each sperm sample were captured using a Basler 602fc camera (Ahrensburg, Germany) attached to a trinocular Olympus CX 41 microscope at 10X magnification. The videos were recorded during 35 seconds at a rate of 100 frames/sec in format .avi using the software AMCAP (Basler Vision Technologies, Ahrensburg, Germany). The videos were edited with the software VIRTUALDUB-1.9.0 (virtualdub.org) and exported as a sequence of images in format .jpg. The images corresponding to 1 second of video were edited in the software IMAGEJ (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) and compiled using the application CASA (Computer Assisted Sperm Analyzer; University of California and Howard Hughes Medical Institute, USA). The sequences analyzed were the 10, 20 and 30 seconds post activation (pa). The parameters analyzed were: Motility (%), VCL (Curvilinear velocity), VAP (Average path velocity) and VSL (Straight line velocity); using the configuration of the CASA system already described in Chalde *et al*. (2014). All these parameters are known indicators of fertilization success in different fish species (Fitzpatrick *et al*., 2009).

2.3. Fertilization assays

Eggs from three mature females were stripped and samples of approximately 100 eggs were fertilized *in vitro* with a pool of sperm of ten males, previously activated with the following solutions: water (control), 1400 or 14000 ng/L of E_2, 180 or 1800 ng/L of EE_2, and the mixture of 1400 ng/L E_2 and 180 ng/L EE_2 (M4), the highest mixture already used for the analysis of the sperm quality parameters. Each egg sample was hydrated with 15 ml of each treatment solution by duplicate; and then incubated at 24ºC

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for 24 hrs. At that moment, the fertilization % was assessed, following procedures described in Chalde *et al*. (2011).

2.4. Analysis of embryo-larval survival

Advanced embryos, with ocular pigments (Chalde *et al.*, 2011), were selected out of the embryo stock obtained from the broodstocks kept at the *"Instituto de Investigaciones Biotecnológicas - Instituto Tecnológico de Chascomús"* aquaculture facilities. The embryos (n=630) were separated in groups of 15 and were placed in plastic containers (50 ml). All the exposures were done in triplicate, and the concentrations used were the same mentioned for the sperm quality analysis. The total volume of each solution (30 ml) was changed for fresh treatment solution daily, at the same time, for a total of 24 days (an average of 8 days for the embryos and 16 days for the larvae).

Embryo survival was recorded daily, and dead organisms were removed. The larvae were separated and maintained in different containers by the day that they hatched. The estrogens exposure continued and larvae survival and the point of no return (PNR= days at which 50% of starved larvae died) were assessed.

All these experiments were performed in a growth chamber (GC 300, JEIO TECH, Korea) with controlled temperature (22ºC) and photoperiod (12L:12D).

2.5. Data analysis

To obtain a single value per parameter per fish for each sperm sample, the values of the sperm quality parameters were averaged from two recorded video sequences. For sperm quality analysis (n=5), fertilization % (n=3) and PNR the results are presented as mean ± SEM. Normal distribution for data was analyzed by the Shapiro–Wilk test, and

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the Levene test was used to check the homogeneity of variance. One-way analysis of variance (ANOVA) was performed for normally distributed variables, followed by *post hoc* Tukey's HSD test ($P < 0.05$) to evaluate the differences between treatments and control. All statistical analyses were performed using GraphPad Prism 5.0 Software.

3. Results

3.1. Sperm quality

The CASA parameters for the control group used in this study were: 65.37 ± 2.50 % (Mean ± SEM) of motile sperm; VCL = 161.10 ± 6.16 µm/sec; VAP = 119 ± 4.71 µm/sec and VSL = 74.53 ± 5.86 µm/sec, at the initial time (sec 10 pa). As it is known, all of these sperm quality parameters significantly decreased during the first minute post-activation. This decrease was 23.38, 65.72, 70.51 and 37.24 for sperm motility VCL, VAP and VSL at second 30 pa, respectively. No significant differences in these parameters were observed between E_2 or EE_2 treatments and the control group (Fig. 1 and 2), even though the motility showed a tendency to decrease in the treated groups (Fig. 1A and 2A). The motility between the different estrogens mixtures and control samples was significantly different. The motility (%) was lower in M2 and M3 compared with the control sample, at second 10 pa. The same was observed at second 20 pa but it included M4 as well. And, at the last post activation time analyzed (second 30 pa), the percentage of motile sperm was lower in M3 in contrast to the control (Fig. 3A).

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3.2. Fertilization assay

The groups exposed to E_2 or EE_2 and the mix of both estrogens showed lower fertilization percentages than the control group (87.31 ± 3.54 %). However, only significant differences were found between M4 (1400 ng/L E_2 and 180 ng/L EE_2) and the control group (Fig. 4).

3.3. Embryo-Larval survival

The embryo survival (until hatching) was reduced in E_2, EE_2 and the mixtures groups compared to the control group (Fig 5 A, B, C), but was significant in the highest concentration of EE_2 (180 ng/L) compared with the control group (Fig. 5B). Between the mixture treatments, M3 had the most adverse effect on embryo survival (Fig 5C).

The hatching % ranged from 75.56 to 84.45 % for the E_2 group; 63.34 to 93.33 % for the EE_2 group; and from 73.33 to 86.67 % for the mixture group. There was no significant difference compared with the control group (82.22 ± 2.22 %).

In the larval survival, a significant decrease was observed at a concentration of 175 and 700 ng/L of E_2 compared with the control group (Fig. 6A). In EE_2 treatments, there was a significant reduction in larval survival at a concentration of 45 and 90ng/L (Fig. 6B). The mixture treatments (M1, M3, and M4) had a significantly lower larval survival than the control group (Fig 6C). In addition, the time of the PNR obtained from the estrogen mixtures was lower than the one obtained from the control group (8.33 ± 0.67 days), but was only significantly different in M1 treatment (Table 1).

During the exposure, larvae were observed under a stereomicroscope (Nikon SMZ800, Tokyo, Japan) and there were no abnormalities that could be related to the exposure to these kinds of contaminants.

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4. Discussion

The current study revealed that E_2 and EE_2, at environmentally relevant concentrations, did not affect sperm quality and fertilization % in pejerrey when tested separately. However, when these two estrogens were combined, they had the potential to reduce sperm motility thus negatively affect the % of fertilization of this fish species.

It is known that sperm motility duration varies among fish species, being the rapid exhaustion of intracellular adenosine triphosphate (ATP) as one of the reasons for the short duration of motility (Iwamatsu et al., 1993). Thus, the energy necessary for motility is provided by the hydrolysis of ATP which is catalyzed by dynein ATPase (Burness *et al*., 2004) and, usually, it is rapidly exhausted in fish sperm. Different pollutants could interfere with this process. For example, *in vitro* sperm incubations with different concentrations of genistein (a soya phyto-oestrogen) demonstrated a significant negative relationship between the concentrations of this estrogen with ATP content and sperm motility of channel catfish (*Ictalurus punctatus*) and walleye (*Sander vitreus*; Green *et al.*, 2008). As well, the assessment of motility provides a reliable estimate of the quality of sperm and the fertilization rate (Kime *et al.*, 2001). Experiments in adult males of medaka demonstrated that sperm velocity increased with a rise in the duration of EE_2 exposure (above 60ng/L), while the fertilization rate was reduced (Hashimoto *et al.*, 2009). However, the activation of sperm in water with EE_2 (10 or 100 ng/L) did not affect sperm motility in *Beta splendens* (Montgomery *et al.*, 2012).

Additionally, analysis of sperm motility in rainbow trout fish and danube bleak (*Chalcalburnus chalcoides*) showed that gametes are very sensitive to environmental variables immediately after release and that external substances such as dinitrophenol

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and potassium cyanide could easily penetrate the sperm membrane (Lahnsteiner *et al.*, 1999). In other fish species, such as *J. multidentata,* adults exposed to environmentally relevant concentrations of E_2 (50, 100 and 250ng/L) over 28 days caused alterations in the reproductive biology at the molecular level, causing a strong enhancement of *cyp19a1b* expression at all concentrations assayed; but there was no effect in sperm motility, velocity or viability (Guyón *et al.*, 2012). Nevertheless, the same study using EE_2 (10, 75 and 150ng/L) did not show any significant differences in several sperm motility parameters (VSL, VCL and Linearity) or in viability between treatments and the control group, however, a significant decrease was recorded in the percentage of motile live spermatozoa and an increase in immotile live and dead spermatozoa at the highest concentration tested (150 ng/L). This reduction implies that a lower percentage of spermatozoa could be reaching the oocyte reducing the fertilization success (Roggio *et al.*, 2014). In the current study, it was not possible to find any adverse effects on the quality of pejerrey sperm exposed either to E_2 or EE_2. Although, when these two pollutants are mixed, they negatively affected the sperm motility and consequently the fertilization %. The results obtained with CASA system demonstrated that this kind of analysis of sperm quality is a useful tool in the early identification of potential stressors or pollutants and can be utilized as a bioindicator for contamination in fish populations.

It has been reported that the administration of 20 to 50 mg/kg of E_2 in the pejerrey diet produced 100% females (Strüssmann *et al.*, 1996). Since this finding, a single study has been conducted where pejerrey larvae were fed with artificial food containing EE_2 (0.1 and 1.0 mg) during the sex determination window, and a feminization process could be observed, at both the morphological and the molecular level (Pérez *et al.*, 2012).

It is well known that early life stages of several fish species are sensitive to pollutants. Particularly, Brion *et al.* (2004) demonstrated that early life stages of

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zebrafish are sensitive to low concentrations of E_2 (100ng/L) during the period of sex differentiation, showing an alteration of the normal process and an abnormal vitellogenin induction. During which there were no significant effects on fish survival for any of the exposure regimes. Meanwhile, the present study demonstrated that the highest concentrations of E_2, EE_2 and their combination were harmful for embryo survival. Similar results have been observed in rainbow trout embryos exposed to 10 and 100 ng/L EE_2 (Schultz *et al.*, 2003), zebrafish exposed to 15 ng/L of EE_2 (Andersen *et al.*, 2003) and medaka fish exposed to 5 µg/L of E_2 (Koger *et al.*, 2000).

The hatching % could be affected when embryos are exposed to different environmental estrogens. For example, sheepshead minnow exposed to 200 ng/L or higher concentrations of EE_2 produced a decrease in both, reproductive and hatching success (Zillioux *et al.*, 2001), as well as in medaka exposed to 60 ng/L EE_2 (Hashimoto *et al.*, 2009). But in fathead minnow, there was no significant evidence of a dose response in hatching success or larvae survival versus EE_2 exposure. In the current study, no differences in hatching % were recorded, but the embryo survival was affected by the different EDs exposures. However, for larval survival this effect was not so marked. A remarkable result was that the environmental concentration of EE_2 (45ng/L) found in Chascomús lake, significantly affected pejerrey larval survival. Besides, the mix of the concentrations of E_2 and EE_2 above and below the environmental relevant concentrations (M1 and M3), had adverse effects on the larval survival, too.

In previous studies, full or partial life-cycle tests in different fish species showed alterations such as edema, damage to gill epithelia (Zillioux *et al.*, 2001), or physical deformities such as anal protrusion, distended abdomens and bleeding (Lange *et al.*, 2001). This was not the case in the present study, where there were no morphological alterations in the embryos or larvae.

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In conclusion, the results obtained demonstrated that the exposure to mixtures of E_2 and EE_2 affected fish sperm motility, fertilization success, and embryo and larval survival, even at relevant environmental concentrations. This study intends to highlight the need to consider the effects of pollutants mixtures in ecotoxicological studies.

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Figure Captions

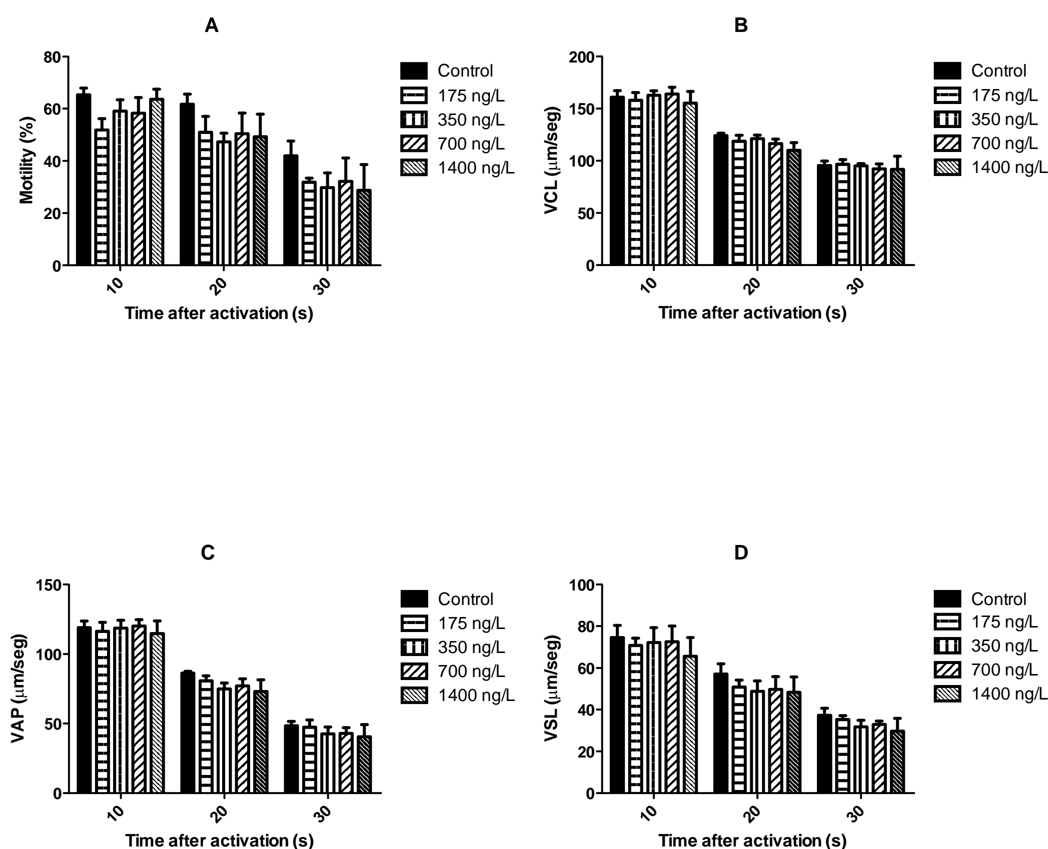

Fig. 1. Effects of different concentrations of E_2 on the sperm quality of pejerrey fish (*O. bonariensis)*. (A) Motility %; (B) VCL (Curvilinear velocity); (C) VAP (Average path velocity); (D) VSL (Straight line velocity). Values are means ± SEM (n = 5).
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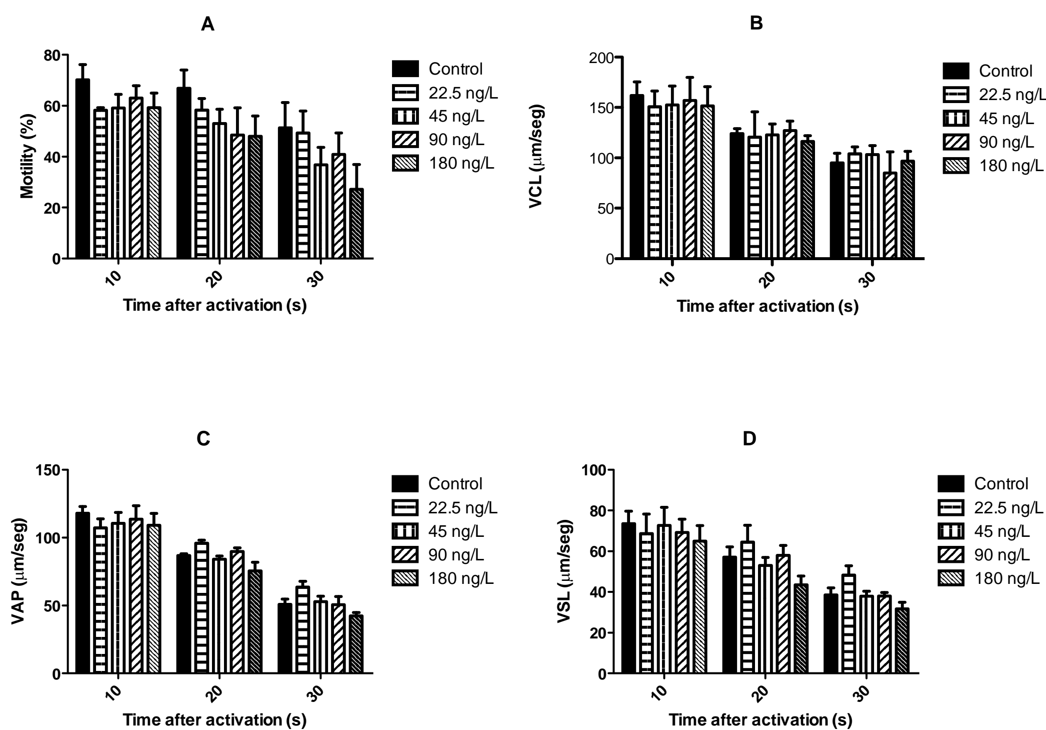

Fig. 2. Effects of different concentrations of EE_2 on the sperm quality of pejerrey fish (*O. bonariensis)*. (A) Motility %; (B) VCL (Curvilinear velocity); (C) VAP (Average path velocity); (D) VSL (Straight line velocity). Values are means ± SEM (n = 5).
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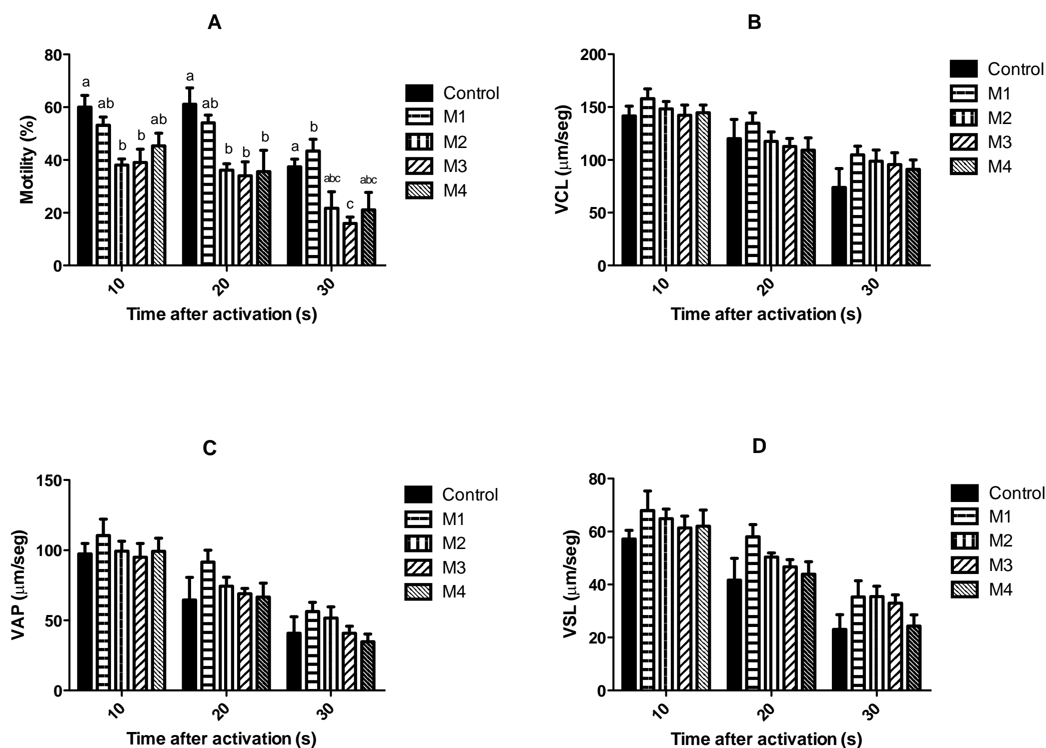

Fig. 3. Effects of different concentrations of E_2 and EE_2 mixtures on the sperm quality of pejerrey fish (*O. bonariensis)*. (A) Motility %; (B) VCL (Curvilinear velocity); (C) VAP (Average path velocity); (D) VSL (Straight line velocity). M1: 175 ng/L E_2 and 22.5 ng/L EE_2; M2: 350 ng/L E_2 and 45 ng/L EE_2; M3: 700 ng/L E_2 and 90 ng/L EE_2; M4:1400 ng/L E_2 and 180 ng/L EE_2. Values are means ± SEM (n = 5). Significantly different values ($p \leq 0.05$) are indicated with different letters.
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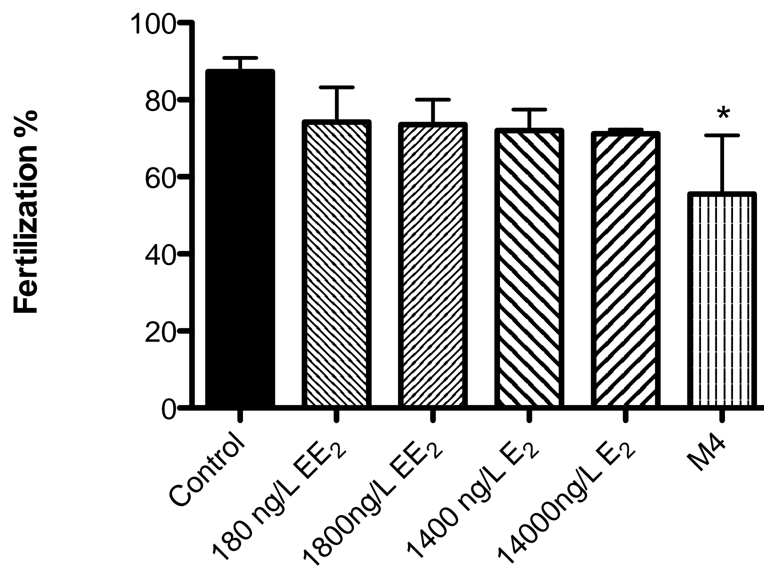

Fig 4. Fertilization % after sperm activation with water prepared with different concentrations of E_2, EE_2 and the mix of both estrogens (M4:1400 ng/L E_2 and 180 ng/L EE_2). Values are means ± SEM (n = 3). * Significant differences compared to the control group ($P \leq 0.05$).
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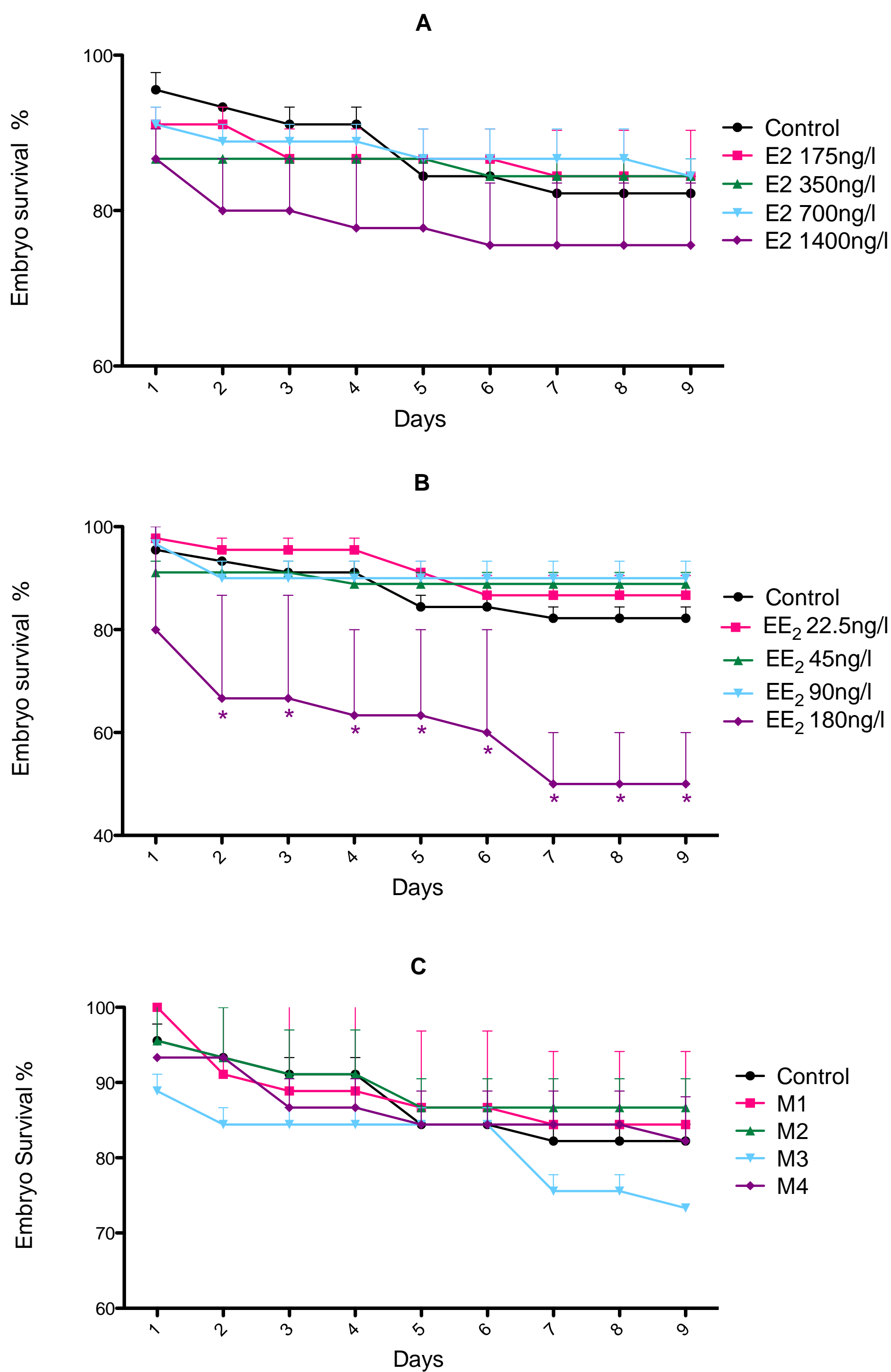

Fig 5. Effects of different concentrations of E_2 (A), EE_2 (B), and the mix of both estrogens (C) on embryo survival (%) of pejerrey fish (*O. bonariensis*). Values are means ± SEM (n=3). * Significant differences compared to the control group ($P \leq 0.05$). Size: Single column.

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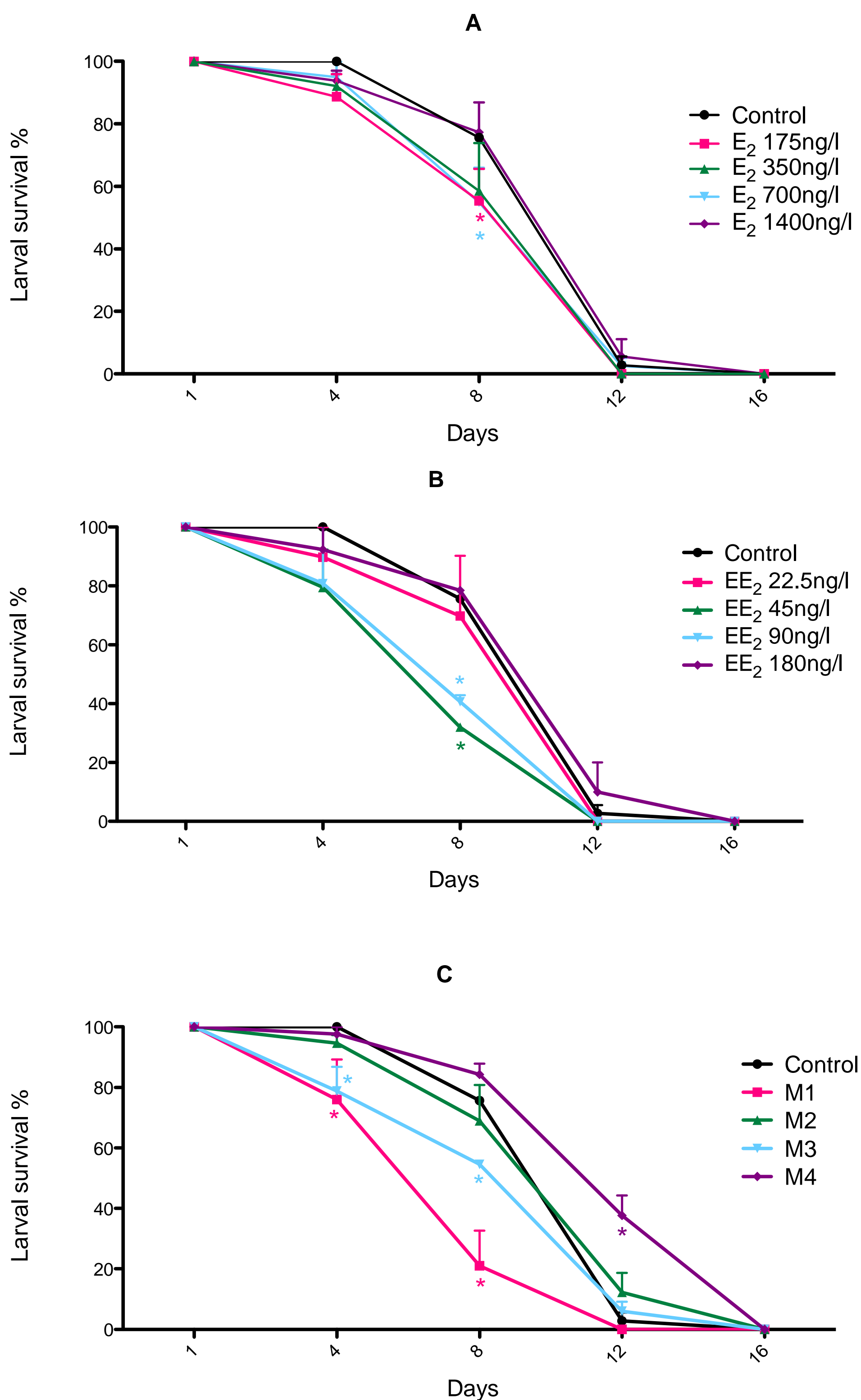

Fig 6. Effects of different concentrations of E_2 (A) EE_2 (B), and the mixtures of both estrogens (C) on larval survival (%) of pejerrey fish (*O. bonariensis*).Values are means ± SEM (n=3). * Significant differences compared to the control group ($P \leq 0.05$). Size: Single column.

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Tables

Table 1. Point of no return (PNR) of pejerrey (*O. bonariensis)* larvae exposed to different concentrations of EE_2, E_2 and the Mixtures of both estrogens (M). Values are means ± SEM (n=3). * Significant differences compared to the control group ($P \leq 0.05$). Size: Single column.

Point of no return (PNR)	
Treatments	Mean ± SEM (days)
Control	7.66 ± 0.66
E_2 175ng/l	6.66 ± 0.88
E_2 350ng/l	6.66 ± 1.45
E_2 700ng/l	5.33 ± 0.88
E_2 1400ng/l	7.33 ± 0.88
EE_2 22.5 ng/l	6.67 ± 1.45
EE_2 45ng/l	7.00 ± 0.00
EE_2 90ng/l	6.00 ± 1.15
EE_2 180ng/l	7.66 ± 0.67
M1	3.33 ± 0.67 *
M2	7.33 ± 1.67
M3	4.33 ± 0.33
M4	9.00 ± 0.58