Effects of the Synthetic Estrogen 17α-Ethinylestradiol on Aromatase Expression, Reproductive Behavior and Sperm Quality in the Fish Jenynsia multidentata

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Effects of the Synthetic Estrogen 17α-Ethinylestradiol on Aromatase Expression, Reproductive Behavior and Sperm Quality in the Fish *Jenynsia multidentata*

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Abstract The synthetic estrogen 17α-ethynylestradiol (EE_2) has been increasingly detected in sewage effluents in the last two decades. The aim of the present study was determined if EE_2 exposure adversely affected reproduction in internally fertilizing fish species *Jenynsia multidentata*. Sexual behavior, brain and gonadal aromatase expression as well as sperm quality were evaluated. The brain aromatase expression, reproductive behavior, spermatozoa viability and gonadosomatic index were sensitive biomarkers of EE_2 effects on this species. The condition factor, hepatosomatic index, gonadal aromatase expression, sperm count and sperm velocities were unaltered after EE_2 exposure. The present work highlights the importance of using a combination of several biomarkers to study the effects of estrogenic compounds, especially when trying to link these results to potential population-level effects.

Keywords Endocrine disruption · Xenoestrogen compounds · One-sided livebearing fish · Sexual activity · Aromatase expression · Sperm parameters

M. A. Roggio · N. F. Guyón · A. C. Hued · M. A. Bistoni
Fac. Cs. Ex. Fís. y Nat., Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, CP X5000JJC Córdoba, Argentina

A. C. Hued · M. E. Valdés · M. A. Bistoni (✉)
Instituto de Diversidad y Ecología Animal (IDEA, CONICET), Córdoba, Argentina
e-mail: mbistoni@efn.uncor.edu

M. V. Amé
Departamento de Bioquímica Clínica-CIBICI, Fac. Cs. Químicas, Universidad Nacional de Córdoba, Medina Allende y Haya de la Torre, CP X5000HUA Córdoba, Argentina

M. E. Valdés
Fac. Cs. Químicas, Universidad Nacional de Córdoba, Medina Allende y Haya de la Torre, CP X5000HUA Córdoba, Argentina

L. C. Giojalas
Centro de Biología Celular y Molecular, Edificio de Investigaciones Biológicas y Tecnológicas, Universidad Nacional de Córdoba, Avda. Vélez Sarsfield 1611, CP X5016GCA Córdoba, Argentina

D. A. Wunderlin
Departamento de Química Orgánica-ISIDSA-ICYTAC, Fac. Cs. Químicas, Universidad Nacional de Córdoba, Av. Juan Filloy s/n, CP 5016 Córdoba, Argentina

Many natural and anthropogenic chemicals discharged into freshwater and estuarine systems are capable of disrupting the endocrine system of aquatic organisms. One kind of endocrine disrupting chemicals (EDCs) are estrogens (either natural or synthetic), which bind to estrogen receptors and are able to induce biological changes affecting the animal development and reproduction.

The 17α-ethynylestradiol (EE_2), an active component of oral contraceptive pills, has been widely chosen to evaluate the effect of estrogenic compounds on reproduction. This is due to its presence in the environment as well as its confirmed effects on the reproductive endocrine system via estrogen receptor-mediated pathways (Metcalfe et al. 2001). The steroid's estrogenic potency (up to 2–5 times higher than the endogenous 17β-estradiol) in combination with its environmental persistence and the ability to bioconcentrate in fish tissue (Nash et al. 2004) make EE_2 a potential risk to fish and other aquatic organisms. EE_2 is widely distributed in surface waters and sewage effluents with detectable concentrations ranging from <1 to 831 ng/L (Desbrow et al. 1998; Wise et al. 2011). Even at low concentrations, EE_2 affects different aspects of fish reproductive biology (Söffker and Tyler 2012).

Reproductive behavior has been demonstrated to be a sensitive indicator of endocrine disruption (Clotfelter et al.

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2004) and additionally, it provides an important measure of neuroendocrine function in fish (Gore 2008). Another important target for EDCs is brain aromatase (*cyp19a1b*), which catalyses the conversion of androgens into estrogens. It has been observed that changes in the expression of this enzyme after chemical exposure lead to altered reproductive behavior in male fish (Guyón et al. 2012). Finally, the sperm quality, which plays a key role in the fertilization process, could be a useful tool in the early identification of potential stressors of a natural population, particularly when they are not so harmful as to be detected by acute toxicity tests (Kime and Nash 1999). The one-sided livebearing fish, *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes) has emerged as an effective field and laboratory model to identify the endocrine-disrupting potential of xenoestrogen compounds. This is due to its ability to adapt to a wide variety of environments (Hued and Bistoni 2005), its sexual dimorphism and its reproductive behavior which involves internal fertilization and coercion (Bisazza et al. 2000).

The aim of the present study was to evaluate the effects of EE_2 on reproductive aspects of *J. multidentata*, through the assessment of different biomarkers. This is the first report that evaluates the effects of EE_2 on *cyp19a1* gene expression, male sexual behavior, sperm count, motility and viability and establishes potential connections between changes in sexual behavior and aromatase expression.

Materials and Methods

17α-Ethynylestradiol (EE_2) (99.1 % purity) was obtained from Sigma-Aldrich. The EE_2 stock solution (6.7×10^{-4} M) was prepared in acetone (Merck Chemist).

Eighty adult males of *J. multidentata* (mean standard length 29.01 ± 2.33 mm and mean body weight 0.49 ± 0.13 g) were collected from an unpolluted site (Yuspe River, Córdoba, Argentina) using a backpack electrofisher, during a reproductive season (February 2011). Fish were acclimatized for 30 days at 21 ± 1°C under a constant 12:12 h light:dark photoperiod and fed twice a day with commercial fish pellets (TetraMin®).

Five adult males were exposed together in a glass aquarium containing 5 L of water supplied with 0 (control group), 10, 75 and 150 ng/L EE_2, during 28 days. Four replicates were made for each treatment. Taking into account the half-life of EE_2 (33 ± 13 h) (Nagpal and Meays 2009), the test solutions were renewed daily. The concentration of EE_2 in water solutions was verified by high performance liquid chromatography coupled to mass spectrometry using a triple quadrupole analyzer, with an electrospray ionization source operated in positive mode (HPLC–ESI–MS/MS) after sample extraction (Yan et al. 2009; Vulliet et al. 2008). Recovery percentages were evaluated from spiked aquarium water samples with 50 ng/L EE_2. The recovery percentage obtained was 59 % affording a quantification limit of 5 ng/L. Samples were collected before and after water replacement.

The reproductive behavior was evaluated in 50 % of males for each concentration and control group. After 28 days of exposure, each male was transferred to a 3 L glass aquarium and then paired with an unexposed female to record sexual behavior. Ten minutes after female introduction, male sexual activity was recorded for 20 min using a video recorder (Sony CCD TR818). Based on the normal reproductive behavior described by Bisazza et al. (2000), the following variables were estimated: Persecution time (PT), number of persecutions (P), number of copulatory attempts (CA) and number of copulations (C). Sustained copulations, defined as the direct contact through the gonopodium with female gonopore in a prolonged way for at least 5 s were also registered. To evaluate the efficiency of male sexual behavior, the following relations were calculated: CA/PT, CA/P, C/PT, C/P and C/CA. In those individuals where no sign of sexual behavior was observed, the female was removed and replaced by another one, up to four times.

Sperm parameters were evaluated in all males: exposed (E) and not exposed (NE) to stimulus female. For sperm collection all males were anaesthetized in a water solution of MS-222 (5 g/L) (Tricaine methanesulfonate; Sigma-Aldrich). In order to obtain the sperm samples and to asses sperm movement the method described in Roggio et al. (2012) was employed. The sperm kinetic parameters calculated were: (1) straight line velocity (VSL) (μm/s): straight distance traveled by the spermatozoon from the beginning to the end of its track over time; (2) curvilinear velocity (VCL) (μm/s): length of the spermatozoon track over measurement time and (3) linearity (LIN): the quotient between VSL and VCL which indicates the grade of straightness of a track (expressed in percentage), where values near 100 % represent a linear movement and values near 0 %, a more erratic path.

Fifteen minutes after sample collection, spermatozoa viability was estimated for each male according to World Health Organization (1999). One hundred cells were randomly chosen in order to register the number of unstained mobile and immobile cells and stained spermatozoa (died cells). From this, the percentage of spermatozoa viability was estimated for each male.

The sperm sample was tenfold diluted with distilled water to immobilize the cells. An aliquot was place in an "improved Neubauer chamber" haemocytometer and counting sperm by duplicate. The total amount of spermatozoa was expressed by microlitre of sample.

Fish were sacrificed by breaking the spinal cord and then measured (standard length) and weighted in order to estimate the Foulton condition factor (K) (Anderson and

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Newman 1996). Liver and gonads were excised and weighted to calculate hepatosomatic index (HSI) and gonadosomatic index (GSI) respectively (Goede and Barton 1990).

For *cyp19a1* mRNA quantification, brain and gonads were snap-frozen in liquid nitrogen and stored in RNA*later* (QIAGEN) at −80°C until analysis. Non-specific reverse transcription was performed from individual tissue total RNA according to Ame et al. (2009). Quantitative PCR was performed to amplify and measure the transcript abundance of *cyp19a1a* in testis, *cyp19a1b* in brain and β-actin in both as reference gen (Ame et al. 2009). Primers sequences for *cyp19a1* mRNA quantification were obtained from Sigma-Aldrich (cyp19a1a forward: 5′-CAAAGGG CACAAATATCATCTTGA-3′ reverse: 5′-CGGGCTGGT GGAAGAACTC-3′; cyp19a1b forward: 5′-GAAACATCA TTAACAAAACTGAGAAACTG-3′ reverse: 5′-GAGAG CTCCCCATGGTTCTG-3′). Relative gene expression to *J. multidentata* β-actin was analyzed by using the standard curve method (Larionov et al. 2005).

Statistical analyses were carried out using Infostat Software Package (InfoStat 2002). The variables were analyzed through one-way analysis of variance (ANOVA) followed by the DGC (Di Rienzo, Guzmán and Casanoves) post-test. Correlations between sperm count and number of copulations were tested using Pearson correlation tests.

Results and Discussion

The stability of EE_2 in solution was in accordance with previously reported half live (Nagpal and Meays 2009). After 24 h of exposure EE_2 residual levels were between 15 and 55 % for the higher concentrations tested (75 and 150 ng/L; Table 1). Residual levels at 10 ng/L EE_2 were below the limit of detection. Even when decay in EE_2 concentration was measured, the nominal values are used throughout the paper.

No males died in the control group during the experimental time. In the 10, 75 and 150 ng/L EE_2 treatment groups, one, three and two males died, respectively. Fish exposed to 75 and 150 ng/L EE_2 showed a significant GSI decrease respect to control group and those exposed to 10 ng/L (Table 1). These results indicate a testis reduction which is consistent with other authors who obtained similar results after EE_2 exposure (concentrations ranging 5–300 ng/L EE_2) (Martyniuk et al. 2006; Velasco-Santamaria et al. 2010). On the other hand, no significant differences in K as well as in HSI were observed among treatments (Table 1). This is in concordance with the results registered for *Danio rerio* exposed to 1, 10, and 100 ng/L EE_2 for 14 days (Versonnen and Janssen 2004) and for *Zoarces viviparous* exposed to 5 ng/L for 21 days (Velasco-Santamaria et al. 2010). Nevertheless, the absence of differences is in disagreement with other published studies. Zha et al. (2007) have registered increase in HSI values, while others authors have described a significant decrease (Saaristo et al. 2010).

Estrogen exposure affected several reproductive behavioral parameters. All the control males were sexually active in the presence of the female, whereas the proportion of males which exhibit sexual behavior decreased after EE_2 exposure: 60 % at 10 ng/L, 70 % at 75 ng/L and 50 % at 150 ng/L. Moreover, 90 % of control males began female approaches immediately after the female was introduced into the glass aquarium. This situation was not observed in fish exposed to EE_2, where it was necessary a female replacement in 33 % of the cases at the lowest concentration and 60 % at 75 and 150 ng/L EE_2. On the other hand, it is noteworthy that the proportion of males which showed sustained copulations was 60 % for the control group, 20 % at 10 ng/L EE_2 and none for the higher concentrations of EE_2. Most of the reproductive variables analyzed decreased significantly in males exposed to EE_2 (Table 1). PT, CA and C values were significantly lower at all the concentrations tested. It is noticeable that the highest reduction was registered at 75 ng/L EE_2. The relations among the four variables showed a significant decrease in C/PT, CA/P and C/P at all concentrations. At 75 and 150 ng/L EE_2 the copulation attempt effectiveness (C/CA) decreased more than 50 % respect to the males exposed to 10 ng/L and control group.

Since aromatase expression was not affected by previous reproductive behavior ($F = 0.53$; $p = 0.5$), all males were assessed all together. No significant effect of EE_2 on the transcription abundance of *cyp19a1b* gene was registered at the lowest concentration tested (10 ng/L EE_2). However, exposure to 75 ng/L EE_2 caused a 3.75-fold significant increase of brain *cyp19a1b* gene expression, compared to the control group. This difference was not so noticeable at 150 ng/L EE_2, where a 1.92-fold significant increase of brain *cyp19a1b* gene expression was observed (Fig. 1A). Conversely, no effect on gonadal *cyp19a1a* gene expression was registered after EE_2 exposure (Fig. 1B). An upregulation of *cyp19a1b* expression and/or brain aromatase activity by estrogenic compounds has been demonstrated in other fish species (Lyssimachou et al. 2006). However, these compounds have failed to modulate the expression of the ovarian aromatase (*cyp19a1a*). This could be explained by the lack of the consensus estrogen response element (ERE) in the promoter region of the gonadal variant as it has been already observed in some teleost species (Tchoudakova et al. 2001). In our study we registered a clear *cyp19a1b* increase at 75 ng/L EE_2, and a decrease at the highest concentration tested (150 ng/L), although it remained above control values. According to

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Table 1 Mean values and standard deviation of biomarkers measured in *J. multidentata* males exposed to different concentrations of 17α-ethynilestradiol during 28 days

	Control	10 ng/L	75 ng/L	150 ng/L
Somatic indexes				
K	1.95 ± 0.14	1.98 ± 0.03	1.99 ± 0.03	1.89 ± 0.04
HSI	2.47 ± 0.64	2.59 ± 0.20	2.39 ± 0.13	2.53 ± 0.25
GSI	2.74 ± 0.76[a]	2.55 ± 0.17[a]	1.90 ± 0.12[b]	1.18 ± 0.17[c]
Behavioral parameters				
PT	72.40 ± 6.80[a]	36.80 ± 10.89[b]	20.80 ± 9.04[b]	42.2 ± 11.34[b]
P	23.83 ± 1.96	18.60 ± 5.53	10.80 ± 4.22	17.60 ± 3.97
CA	45.60 ± 8.78[a]	18.00 ± 16.35[b]	5.40 ± 0.81[c]	18.20 ± 6.76[b]
C	17.60 ± 5.0[a]	3.25 ± 1.31[b]	0.80 ± 0.37[b]	2.80 ± 1.59[b]
P/PT	0.34 ± 0.04	0.55 ± 0.07	0.49 ± 0.01	0.45 ± 0.04
CA/PT	0.62 ± 0.09	0.42 ± 0.07	0.34 ± 0.09	0.37 ± 0.07
C/PT	0.24 ± 0.05[a]	0.14 ± 0.05[b]	0.07 ± 0.02[b]	0.05 ± 0.02[b]
CA/P	1.98 ± 0.51[a]	0.85 ± 0.22[b]	0.58 ± 0.14[b]	0.87 ± 0.20[b]
C/P	0.80 ± 0.30[a]	0.32 ± 0.16[b]	0.11 ± 0.04[b]	0.13 ± 0.06[b]
C/CA	0.37 ± 0.04[a]	0.33 ± 0.19[a]	0.15 ± 0.06[b]	0.13 ± 0.05[b]
Sperm parameters				
Motility				
VSL (μm/s)	100.81 ± 4.32	105.42 ± 3.57	102.54 ± 3.87	109.37 ± 2.88
VCL (μm/s)	102.97 ± 4.54	108.50 ± 3.57	106.94 ± 4.44	106.11 ± 7.79
LIN (%)	99.95 ± 0.05	99.66 ± 0.21	98.89 ± 0.54	99.49 ± 0.35
Viability (%)				
MLS	82.09 ± 2.66[a]	75.35 ± 4.34[a]	76.16 ± 4.55[a]	65.34 ± 3.77[b]
ILS	12.11 ± 2.61[a]	18.85 ± 3.06[a]	17.31 ± 3.20[a]	24.09 ± 3.99[b]
DS	5.84 ± 0.74[a]	6.15 ± 1.23[a]	6.53 ± 2.49[a]	10.57 ± 1.56[b]
Count (cell/μL)				
$Count_{NE}$	2,387 ± 564	2,199 ± 744	2,221 ± 78	1,983 ± 629
$Count_{E}$	893 ± 101	1,290 ± 187	1,803 ± 782	1,704 ± 595
EE_2 concentration (ng/L)				
Before renewal	<LOD	<LOD	11 ± 4	83 ± 10
After renewal	<LOD	5 ± 6	64 ± 8	127 ± 15

The values are expressed as means ± SDs.

Different letters indicate significant differences among treatments ($p < 0.05$)

K condition factor, *HIS* hepatosomatic index, *GSI* gonadosomatic index, *PT* persecution time (s), *P* number of persecutions, *CA* number of copulatory attempts, *C* number of copulation and different relations calculated among these variables, *VSL* straight line velocity, *VCL* curvilinear velocity, *LIN* Linearity, *MLS* motile live spermatozoa, *ILS* immotile live spermatozoa, *DS* dead spermatozoa ($n = 20$), $Count_{NE}$ sperm count of males were not exposed to female ($n = 10$), $Count_{E}$ sperm count of males were exposed to female ($n = 10$)

other authors, this may be due to alternative regulatory pathways of brain aromatase that are probably triggered by levels of estrogens above a certain threshold after which no longer effects are observed (Melo and Ramsdell 2001). Therefore at certain levels EE_2 could elicit responses that do not behave in a dose–response manner and the effects at low doses do not predict the ones at high doses (Vandenberg et al. 2012).

While a detailed description of the role of aromatization process on the regulation of fish sexual behavior is still not completely understood, it has been observed that alterations in brain aromatase expression or activity during exposure to EDCs results in impaired sexual behavior in male fish (Guyón et al. 2012). Our results showed impairment in male reproductive behavior after EE_2 exposure (Table 1). It is noticeable that at the higher concentrations (75 and 150 ng/L EE_2) it was necessary a 60 % of female replacement to cause a male stimulation. Moreover, sustained copulations were not registered at these concentrations. The most deleterious effect of EE_2 on

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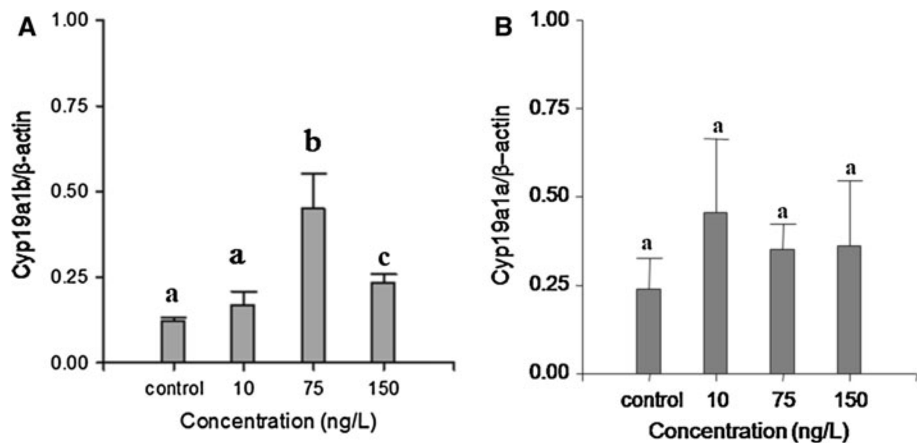

Fig. 1 Cyp19a1 gene expression relative to β-actin in *J. multidentata* males exposed to different concentrations of 17α-ethynilestradiol by quantitative real-time PCR method. **A** Cyp19a1b expression in brain. **B** Cyp19a1a expression in testis (n = 20). Data are indicated as mean ± SD. *Different letters* indicate significant differences among tested groups ($p < 0.05$)

behavior was observed at 75 ng/L, in coincidence with the peak of brain aromatase expression. Several studies have documented the adverse effects of exogenous chemicals with estrogenic activity on fish reproductive behavior, showing a generalized declination in male reproductive performance and a behavioral demasculinization (Martinovic et al. 2007). Melo and Ramsdell (2001) reported a sexual dimorphism in the localization of brain aromatase activity in *Oryzias latipes*, in accordance with the suggested role of aromatase in the regulation of male or female sex-specific behaviors. They also demonstrated that after E_2 exposure, male brain phenotype suffered a feminization which included elevation in total levels of aromatase activity and increased activity in brain areas in which females usually show higher levels. Following the proposal of those authors, our results in *J. multidentata* showed that EE_2 treatment strongly induced expression of the *cyp19a1b* gene and those higher aromatase levels could probably cause male brain feminization of aromatase distribution, which is reflected in the registered reduction in male sexual behavior. The impairment of sexual behavior that we registered in *J. multidentata* after the exposure to environmentally relevant EE_2 concentrations pose a risk to the successful reproduction of this species, since *J. multidentata* presents a coercive behavior and therefore males take an active role in reproduction.

Spermatozoa motility and viability were not affected by previous reproductive behavior; therefore, males exposed and not exposed to females were assessed together for statistical analyses. No significant differences in VSL, VCL and linearity were registered between treatments and between treatments and control group. These findings support the results from a previous study conducted by Guyón et al. (2012) where males of *J. multidentata* exposed 28 days to natural hormone E_2 (50–250 ng/L) did not showed changes in sperm velocity.

Spermatozoa viability was negative affected by EE_2 exposure. At the highest concentration tested (150 ng/L) it was registered a significant decrease in the percentage of motile live spermatozoa (MLS) and an increase in immotile live (ILS) and dead (DS) spermatozoa, respect to other concentrations and control group (Table 1). This reduction implies that a lower percentage of spermatozoa could be displacing to the oocyte and therefore it reduces the fertilization success. According to these results the sperm viability could be considered a key parameter in both ecological and toxicological environmental evaluation and a "quick response tool" in the presence of potentially hazardous events that may adversely affect an aquatic ecosystem (Fabbrocini et al. 2010).

The analysis of variance revealed an interaction between sperm count and the previous sexual activity. Control males who exhibited sexual behavior had a lower sperm count than those unexposed to females (Table 1). This result is correlated with the highest percentage of copulations recorded in this group ($r^2 = -0.75$; $p = 0.003$). This negative correlation was not registered at any EE_2 concentration evaluated due to the lower percentage of copulations registered in these individuals (including sustained copulas). Considering these results, in order to establish the specific effect of EE_2 on sperm count we only considered those individuals who were not exposed to a female. The analysis of variance showed that sperm count of individuals unexposed to a female was not affected by EE_2 exposure.

In summary, the remarkable changes observed in reproductive behavior at the lower EE_2 concentration suggest that male sexual activity is the most sensitive and potentially useful biomarkers for exposure to environmentally relevant estrogenic compounds. Among other parameters, *cyp19a1b* gene expression, GSI and sperm viability seemed to be good biomarkers of EE_2 exposure. Our results also suggest that *J. multidentata* could be considered as a useful model for examining the effects of estrogenic compounds. The present work highlights the importance of using a combination of several biomarkers when studying effects of estrogenic compounds, and especially when extrapolating effects of EDC on wild fish populations.

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