Atrazine alters early sexual development of the South American silverside, *Odontesthes bonariensis*

Pedro Carriquiriborde [a], Juan Ignacio Fernandino [b,c], Carina G. López [b,c], Eduardo de San Benito [a], Juan Manuel Gutierrez-Villagomez [d], Diego Cristos [e], Vance L. Trudeau [d], Gustavo M. Somoza [b,c,*]

[a] *Centro de Investigaciones del Medioambiente (CIM, UNLP-CONICET), La Plata, Buenos Aires, Argentina*
[b] *Instituto Tecnológico de Chascomús (CONICET-UNSAM), Chascomús, Buenos Aires, Argentina*
[c] *Escuela de Bio y Nanotecnologías. UNSAM. Argentina*
[d] *Department of Biology, University of Ottawa, Ottawa, ON, K1S 6N5, Canada*
[e] *Instituto Nacional de Tecnología Agropecuaria, Centro de Investigación de Agroindustria (CIA-INTA), Castelar, Buenos Aires Argentina*

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ABSTRACT

Atrazine (ATZ) is a frequent contaminant in freshwater ecosystems within agricultural regions. The capacity of this herbicide to interfere with the vertebrate endocrine system is broadly recognized, but the mechanisms and responses usually differ among species. In this study, ATZ effects on hypothalamus-pituitary-gonadal (HPG) axis key genes expression and early gonadal development were evaluated in *Odontesthes bonariensis* larvae waterborne exposed during the gonadal differentiation period. Fish were treated to 0, 0.7, 7.0, and 70 μg ATZ/L at 25 °C from the 2nd to 6th week after hatching (wah), and a group was kept in clean water until the 12th wah. Parallelly, a group was submitted to 0.05 μg/L of ethinylestradiol (EE$_2$) as a positive estrogenic control. From each treatment, eight larvae were sampled at 6 wah for gene expression analysis and twelve larvae at 12 wah for phenotypic sex histological determination. The expression of *gnrh1, lhb, fshb*, and *cyp19a1b* was assessed in the head, and the ones of *amha, 11βhsd2*, and *cyp19a1a* in the trunk. Fish growth was significantly higher in fish exposed to 7 and 70 μg ATZ/L in the 6 wah, but the effect vanished at the 12 wah. The expression of *lhb* was upregulated in both sex larvae exposed from 7 μg ATZ/L. However, a dimorphic effect was induced on *cyp19a1a* expression at 70 μg ATZ/L, up or downregulating mRNA transcription in males and females, respectively. Delayed ovarian development and increased number of testicular germ cells were histologically observed from 7 to 70 μg ATZ/L, respectively, and a sex inversion (genotypic male to phenotypic female) was found in one larva at 70 μg ATZ/L. The *lhb* expression was also upregulated by EE$_2$, but the *cyp19a1a* expression was not affected, and a complete male-to-female reversal was induced. Further, EE$_2$ upregulated *gnrh1* in females and *cyp19a1b* in both sexes, but it did not alter any assessed gene in the trunk. In conclusion, ATZ disrupted HPG axis physiology and normal gonadal development in *O. bonariensis* larvae at environmentally relevant concentrations. The responses to ATZ only partially overlapped and were less active when compared to the model estrogenic compound EE$_2$.

1. Introduction

Atrazine (ATZ) is a pre-emergent herbicide broadly used worldwide for weed control, mainly in corn fields, and several studies have demonstrated that ATZ can reach surface waters. The maximum concentration measured ever in the US "Corn Belt" was 1074 μg/L, but average concentrations usually range between 3.5 to 8.6 μg/L (Battaglin et al., 2009; Kadoum and Mock, 1978). In Argentina, reported concentrations of ATZ in surface waters of the Pampa Region ranged between 0.02–28.0 μg/L, with detection frequencies varying from 28 to 100% (Bachetti et al., 2021; Corcoran et al., 2020; De Gerónimo et al., 2014; Garcia et al., 2019; Regaldo et al., 2018; Vera-Candioti et al., 2021). Such concentrations agree with a weight of evidence (WoE) based ecological risk assessment (ERA) that suggested 20 μg ATZ/L as a realistic concentration for conducting experimental testing (Van Der Kraak et al., 2014).

Disruption of the reproductive endocrine function has been reported as one of the significant biological effects induced by ATZ in vertebrates since it is an apical endpoint critical for population sustainability. In males, de-masculinization with testicular lesions associated with reduced germ cell numbers and partial or complete feminization has been observed across several vertebrate species (Hayes et al., 2011). An increase of the female to male ratio was found in zebrafish *Danio rerio* exposed to 2.2 and 22 μg ATZ/L related to the upregulation of gonadal aromatase (*cyp19a1a*) but not mediated by estrogen receptors (ERs) (Suzawa and Ingraham, 2008). A similar response was observed in the frog *Lithobates pipiens* exposed to 1.8 ATZ/L, but while brain ERα was up-regulated, brain aromatase mRNA levels were not affected (Langlois et al., 2010). In females, an increased number of previtellogenic oocytes, besides other histological lesions, was found in *Pimephales promelas* exposed to 5 and 50 μg ATZ/L compared to the control group, with a subsequent reduction of egg production and spawning (Tillit et al., 2010). Similar results were also observed in medaka, *Oryzias latipes* (Papoulias et al., 2014), zebrafish, *D. rerio* (Wirbisky et al., 2016), and African catfish, *Clarias gariepinus* (Opute et al., 2020). Ovarian degeneration was also reported in the bird *Coturnix coturnix* (Qin et al., 2015). In mammals, a reduction of primordial follicles number was observed in females mice treated from early development and impaired sexual maturation and subfertility in male rats exposed from gestation and lactation, respectively (Gely-Pernot et al., 2017; Pandey et al., 2021).

Multiple mechanisms of action have been proposed for ATZ on the vertebrate hypothalamic-pituitary-gonadal (HPG) axis. Suppression of several HPG axis-related genes (i.e., *gnrh, lh*) with induction of gonadal aromatase was proposed for explaining demasculinization and feminization of vertebrate male gonads (Hayes et al., 2011). Similarly, in mammalian models, atrazine was able to affect gonadotropin-releasing hormone (GnRH) release and the luteinizing (LH) and follicle-stimulating (FSH) hormones surge (Stradtman and Freeman, 2021). Therefore, different mechanisms could be acting in different vertebrate species and would explain the diversity of sensitivity in tissues to ATZ effects.

The pejerrey (*Odontesthes bonariensis*, Valenciennes, 1835), a South American silverside, is a native fish of the meridional sector of the Rio de la Plata Basin and is highly valued for the quality of the flesh and its recreational fishery (Somoza et al., 2008). This species is particularly abundant in shallow lakes of the Pampa, the main agricultural district of Argentina. It has also been demonstrated to be very sensitive to environmental pollutants, such as heavy metals, pesticides, and pharmaceuticals (Carriquiriborde et al., 2009; Carriquiriborde and Ronco, 2006; Pérez et al., 2012). This species has no clear secondary sexual characters and is considered a differentiated gonochoric species (Ito et al., 2003). As earlier described in its close relative, *O. hatcheri* (Hattori et al., 2012), in *O. bonariensis* sex is also determined by the presence of the *amhy* gene (XY or XX genotype) (Hattori et al., 2019). This gene is a paralog of the autosomal gene of the anti-Mullerian hormone (*amha*), located in the Y chromosome, that in normal conditions drives testicular development (Yamamoto et al., 2014).

Previous studies in *O. bonariensis* have suggested that the central nervous system plays a pivotal role in maintaining the hypothalamus-pituitary-gonadal axis active during the gonadal differentiation period through Gnrh neurons (Miranda et al., 2003). The early immunodetection of both gonadotropins, follicle-stimulating (Fsh) and luteinizing (Lh) hormones, from 2 wah to just before the gonadal differentiation, would suggest they play a role during this process (Miranda et al., 2001; Shinoda et al., 2010). The expression of brain aromatase (*cyp19a1b*), regulated through estrogen receptors, is activated in the brain during the early larval period before gonadal development (Strobl-Mazzulla et al., 2008). At the gonadal level, several genes have been identified to play a key role during sex differentiation. In *O. hatchery*, for example, the early expression (<6 d after fertilization) of *amhy* drives a later dimorphic expression pattern of specific genes. There is a higher expression of *cyp19a1a* and forkhead box protein L2 (*foxl2*) in females, and higher

expression of the anti-Mullerian hormone (*amha*) gene in males (Hattori et al., 2013). In *O. bonariensis*, the exposure to an environmental stressor, like temperature, can modify the expression pattern of these genes, inducing the expression of *cyp19a1a* at female-promoting temperatures (Fernandino et al., 2008b). Moreover, *cyp19a1a* and 11 β-hydroxysteroid dehydrogenase 2 (encoded by *11βhsd2*) are two key enzymes involved respectively in the biosynthesis of estradiol and 11-ketotestosterone (11-KT) in the gonads, driving either the ovarian or testicular differentiation (Fernandino et al., 2012; Zhang et al., 2018). Importantly, in addition to temperature, other environmental factors such as pollutants (i.e., endocrine-disrupting chemicals, EDCs) can affect gonadal differentiation in *O. bonariensis*. For example, exposure to both natural (17β-estradiol; E₂), or synthetic (17α-ethinylestradiol; EE₂) estrogens during the labile period of gonadal differentiation skews sex ratios to 100% females, with the induction of *cyp19a1a* and inhibition of *11βhsd2* (Pérez et al., 2012). Within this framework we report on modulation of specific HPG axis-related genes in relation to altered gonadal histology and sex ratios in *O. bonariensis* larvae exposed to environmentally relevant concentrations of ATZ during the gonadal differentiation period.

2. Materials and methods

2.1. Chemicals

Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) active ingredient (97% purity) was obtained from Gleba S.A. (Argentina). EE₂ Parafarm® (98% purity) was purchased at Droguería Saporiti (Argentina). Methanol and acetonitrile solvents were HPLC grade from J.T. Baker®.

2.2. Test organisms

Newly hatched *O. bonariensis* larvae were obtained from the Estación Hidrobiológica de Chascomús (Ministerio de Asuntos Agrarios de la Provincia de Buenos Aires) and transported to the Centro de Investigaciones del Medioambiente (CIM, CONICET-UNLP) aquatic facilities in polyethylene bags provided with oxygen. Larvae were maintained during two weeks in a 2000 L closed circulating system with dechlorinated and charcoal-filtered City of La Plata tap water (hardness 250 mg CaCO3/L; pH 7,8; dissolved oxygen 9.0 mg/L). The temperature was set at 25 ± 1 °C and photoperiod 16 h light (L): 8 h darkness (D). Larvae were fed with live *Artemia* spp. and fish larvae commercial food BB Shulet® four-times a day. Fish were handled in accordance with the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals (https://www.ufaw.org.uk/) and internal institutional regulations (CICUAL protocol # 006–26–17).

2.3. Experimental design

Two-weeks after hatching the larvae were waterborne exposed to 0.0 (control), 0.7, 7.0, 70 μg ATZ/L during the gonadal differentiation period (2nd to 6th wah) at 25 °C. This temperature does not induce sex reversal, producing approximately 50:50 male/female ratio (Strüssmann et al., 1997). The experimental design also included an estrogenic feminizing positive control group (0.05 μg EE₂/L). Each treatment was duplicated, placing 50 larvae per tank (100 larvae per treatment). At the 6th wah (end of the ATZ exposure), 8 fish per tank were sampled for gene expression and genotypic sex determination (See below). The remaining fish were grown in clean water until the 12th wah (time needed for histological gonadal differentiation), and 15 fish per tank were sampled for assessment of genotypic and phenotypic (gonadal histology) sex identification.

Experiments were conducted in 60 L stainless-steel tanks filled with 50 L of dechlorinated City of La Plata tap water filtered through activated charcoal. A working solution of ATZ (10 mg a.i./L) in distilled

water was freshly prepared every renewal day and the appropriate volume added to each test tank. Media renewal was performed every 48 h during the 4 weeks of exposure. Fish were fed on a mixture of commercial fish food (BB Shulet®) and live food (*Artemia* spp. *nauplii*) until satiation every day. Room temperature was set at 25 °C and photoperiod 16:8 h (L:D). Gentle aeration was delivered into the tanks through a 0.2 ml glass pipettes at a flow rate of 60 bubbles/min. The average water pH was 7.2 ± 0.5 and oxygen 8.0 ± 1.3 mg/L.

2.4. Chemical analysis

The concentration of ATZ in the different treatment tanks was determined by LC-MS using a Waters ACQUITY® UPLC system equipped with an autosampler module and coupled to a Single Quadrupole Detector. Samples of water from each experimental tank were collected for ATZ analysis just after renewing the test media (t0) and before the next renewing (t48). Water samples were immediately frozen at -20 °C until analysis. Before instrumental analysis, samples were filtered through a syringe filter (13 mm Nylon/GF 0.45 μm) into the chromatographic vials. Injection volume was 10 μl. Chromatographic separation was achieved on an X-Select® CSH C18 column (Waters) 75×4.6 mm and 3.5 μm at 35 °C column using 0.1% formic acid aqueous phase and 50:50 acetonitrile/methanol organic phase at a flow of 0.2 μl/min. Atrazine was detected by electrospray ionization (ESI) in positive mode. Chromatograms were analyzed using the MassLynx 4.1 software. Quantification was achieved using a five-point external calibration curve (0 to 50 μg/L) using the certified ATZ Pestanal™, analytical standard (Sigma-Aldrich). The limit of detection of the method was 0.5 μg/L.

2.5. Genetic sex determination

Each fish sampled at the 6th and 12th wah was analyzed for identifying its genotypic sex. A sample from the caudal fin of each animal was subjected to DNA analysis for the presence/absence of the *amhy* gene. For this purpose, genomic DNA was extracted using conventional saline buffer extraction (Aljanabi and Martinez, 1997). PCR analysis was then performed to analyze the presence of *amhy* together with *β-actin* gene as a DNA loading control (Sarida et al., 2019). All *amhy*-positive fish were scored as XY and all *amhy*-negative as XX (Hattori et al., 2018; 2019). The PCR products were analyzed on a 1% agarose gel.

2.6. Phenotypic sex and gonadal histology analysis

The phenotypic sex of each fish larvae sampled at the 12th wah was histologically identified. At the time of sacrifice, larvae were fixed in Bouin's fluid overnight at 4 °C. Then, they were washed twice in 70% ethanol and kept there at 4 °C until paraffin embedding. The trunk was included in paraffin and the whole section, holding the gonads, was cut in 5 μm-thick slices, spaced every 100 μm, using a Leica RM2235 microtome. Slices were serially mounted onto histological slides.

In addition to phenotypic sex, ovarian development stage (ODS) and testis germ cell number (TGCN) were histologically assessed. Different developmental stages are found along the gonad (Supplementary Figure 1), therefore both features were assessed in the five successive slices where the gonad showed the most advanced development degree (most distinctive and indicative sector). The ODS was divided into three classes (pictures inserted in Fig. 1): 1) linear arrangement with growing somatic-cell-lips in borders, 2) somatic-cell-lips bent forming an open ovarian cavity, 3) closed ovarian cavity. Testis germ cells were identified by the distinctive characteristics of the nucleus and counted only if the nuclei were clearly visible in the histological section. The double count was prevented by discarding away 100 μm between analyzed sections. Three classes were considered for the TGCN (pictures inserted in Fig. 2): 1) no germ cells, 2) one germ cell, 3) two or more germ cells.
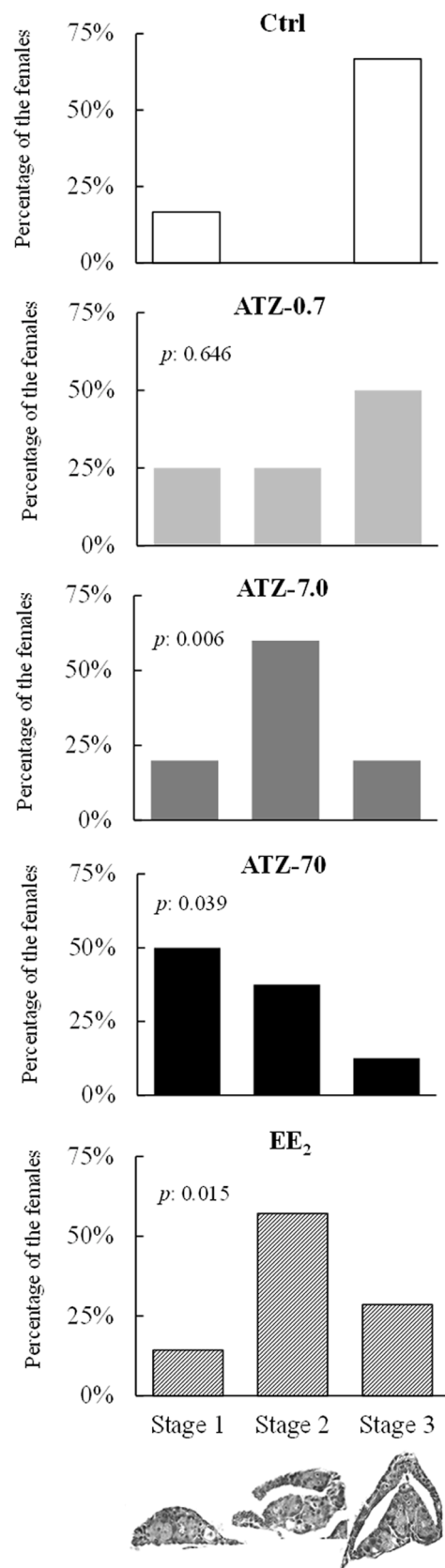

Fig. 1. Frequencies of ovarian developmental stages in 12 wah larvae of *O. bonariensis* exposed to sublethal concentrations of ATZ or EE$_2$ during the gonadal differentiation time period.
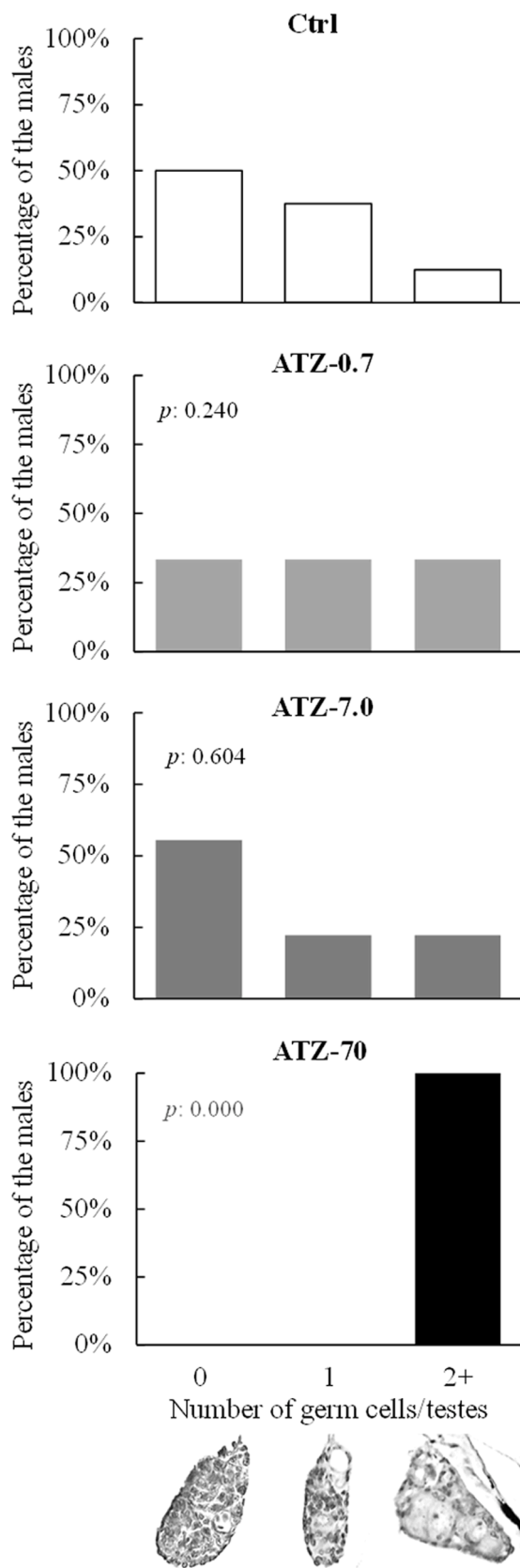
Fig. 2. Frequencies of testicular developmental stages (given by the germinal cell number) in the 12 wah larvae of *O. bonariensis* exposed to sublethal concentrations of ATZ or EE₂ during the gonadal differentiation time period.

2.7. Gene expression

For gene expression analysis, 8 fish larvae per treatment were sampled at the 6th wah. Fish were anesthetized in ice-cold water and the standard length was measured. The head, trunk, and caudal fin were dissected, and immediately placed RNAlater™, kept at room temperature overnight, and then frozen at −80 °C until processing. Gene expression was assessed according to Vera-Chang et al. (2019). Briefly, total RNA from each sample was isolated by the TRIzol™ method using a ball bearing Retsch® (04,182) mixer mill and followed by RNA purification using the RNeasy Plus Mini kit (Qiagen) including on-column DNase digestion step. Obtained RNA was quantified using a Nano-Drop™ 2000c (Thermo Scientific) and integrity was assessed running the samples in 1% agarose gel. cDNAs were synthesized using a Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR. Real-time PCR was performed on a Rotor-Gene Q 2PLEX HRM (Qiagen) using the Rotor-Gene SYBR® Green PCR Kit (Qiagen).

The expression of nine genes was assessed. In the larval head, gonadotropin-releasing hormone (*gnrh1*), the β subunits of luteinizing hormone (*lhb*) and follicle-stimulating hormone (*fshb*), brain aromatase (*cyp19a1b*), and elongation factor-1 (*ef1*). In the trunk, the genes of the autosomal anti-Mullerian hormone (*amha*), the Forkhead box protein L2 (*foxl2*), ovarian aromatase (*cyp19a1a*), 11 β-hydroxysteroid dehydrogenase (*11βhsd2*), and β-actin (*bact*). Primers sequences (Supplementary Table 1) were obtained from our previously validated studies (Fernandino et al., 2008b, 2012; Hattori et al., 2012; Miranda et al., 2007; Strobl-Mazzulla et al., 2008) and sequencing was performed at Eurofins Genomics (Louisville, USA). Melting-curve analysis was conducted at the end of the qRT-PCR protocol between 60 and 95 °C with an interval of 0.5 °C. Samples were run in technical triplicates, and their absolute abundance was calculated based on standard curves using Rotor-Gene Q Series Software 2.0.3 (Qiagen). The mRNA abundance was normalized using the NORMA-GENE algorithm (Heckmann et al., 2011).

2.8. Statistical analysis

Student's *t*-test was used for the comparison of ATZ concentrations between time 0 and 48 h for each testing media renewal interval. ANOVA was used to assess ATZ and exposure effects on studied variables followed by LSD post-hoc test for assessing differences among treatments. Normality and homoscedasticity assumptions for each variable were tested with Shapiro-Wilk and Levene's tests, respectively. Those variables that did not meet the assumptions (i.e., gene expression) were log-transformed. The Chi-square test was used for assessing the effect of ATZ on the sex ratios, comparing the number of males to females obtained by the phenotypic (gonadal histology) respect to the genotypic (sex maker genes PCR) analysis within the same treatment. The Chi-square test was also applied for the statistical comparison of the ODS and TGCN among the control group and the ATZ treatments.

3. Results

Average concentrations of ATZ measured in each tank at 0 and 48 h were not significantly different either at the beginning or at the end of the testing media renewal interval (Supplementary Table 2). The overall average concentrations per treatment were 0.73 ± 0.05, 6.90 ± 0.28 and 74.9 ± 4.73 µg/L, thus is good agreement with the nominal concentrations of 0.7, 7.0, and 70 µg/L, respectively. ATZ was not detected in any of the control group tanks. Water analyses showed that the ATZ was stable between testing media renewals and concentrations were close to the nominal values (differences <10%).

Although no mortality was observed for any treatment, growth of *O. bonariensis* larvae was significantly affected by ATZ during the exposure period (2nd to 6th wah). The standard length of larvae exposed to 7 and 70 µg/L was significantly higher than of the control group (Table 1). The same response was observed in the EE₂ group, showing a

Table 1

Effect of ATZ exposure on growth and sex ratio of *O. bonariensis*.

Treatment	Conc. (µg/L)	6th wah (end of exposure) SL (mm) Mean	±	SE (n = 14)		Genotypic sex Female	Male	12th wah (differentiation period) SL (mm) Mean	±	SE (n = 16)		Genotypic sex Female	Male	Phenotypic sex Female	Male		Sex Reversal
Ctrl	0.00	16.7	±	0.13	a	62%	38%	25.2	±	0.12	a	43%	57%	43%	57%	a	0%
EE₂	0.05	18.2	±	0.11	b	50%	50%	24.1	±	0.05	a	50%	50%	100%	0%	b	100%
ATZ	0.70	17.4	±	0.18	a,b	50%	50%	23.4	±	0.15	a	43%	57%	31%	69%	a	0%
	7.00	18.8	±	0.13	b	50%	50%	24.0	±	0.12	a	36%	64%	36%	64%	a	0%
	70.00	18.4	±	0.08	b	38%	62%	23.9	±	0.12	a	57%	43%	64%	36%	a	14%

wah: weeks after hatching; SL: standard length; different letters mean statistical differences (SL, One-way ANOVA or Sex percentages, Chi-square, $p<0.05$).

significant increase in the standard length comparing to the control group. However, these differences were no longer apparent at 12th wah, six weeks after transferring fish into clean water.

The percentage of genotypic sex for all larvae sampled at week 6 was 50% female 50% male and deviations obtained in some treatments (i.e., controls and ATZ-70) were not significantly different from that proportion (Table 1). The proportion of genotypic sex for all larvae sampled in week 12 was 46% female and 54% male, not significantly different from the expected 50%. Deviations from that proportion obtained in some treatments were not statistically significant. The percentage of phenotypic sex at the 12th wah (Table 1 and Supplementary Figures 2, 3, 4, 5, and 6) was significantly different from the respective genotypic sex proportion only for EE₂ treatment ($p = 0.012$), which presented 100% feminization. In the ATZ treatments, the Chi-square test gave no statistical differences. However, 14% sex inversion was observed in the highest concentration, with an increase of the percentage of phenotypic females.

Significant effects of ATZ were observed for ODS and TGCN. In the case of the ODS (Fig. 1), a dose-dependent delay of the ovarian development was observed. While in the control larvae the predominant stage was the "closed ovarian cavity", in the larvae exposed to 70 µg/L the most frequent stage was the "linear arrangement with growing somatic cell in the lips". A control-like profile was observed in larvae exposed to the lowest ATZ concentration (0.5 µg/L). An intermediate situation was observed in the larvae exposed to 7 µg/L, showing as the dominant stage the "somatic cell lips bent forming an open ovarian cavity". Such an intermediate profile also was characteristic of the positive control group larvae, exposed to 0.05 µg/L of EE₂.

The TGCN was also significantly affected by ATZ (Fig. 2), but only in larvae exposed to the highest tested concentration (70 µg/L). The effects of the herbicide were characterized by inducing an increase in the testicular germ cell number. While at the control and the lower ATZ concentration groups the most frequent number of germ cells per section was zero, at the 70 µg ATZ/L treatment always two or more germ cells were counted per section.

The gene expression analysis of all selected genes in the head and trunk of the 6th wah *O. bonariensis* larvae are shown in Supplementary Figure 7. In the head, only *lhb* was significantly affected by ATZ (two-way ANOVA, $p = 0.021$). The expression was induced in a concentration-dependent manner independently of the genotypic sex of the fish (Fig. 3). The levels of increase were 2.3-, 4.4-, and 4.0-fold compared to the control group in fish exposed to 0.7, 7.0, and 70 µg/L ATZ, respectively.

A similar response (7.6-fold induction) of *lhb* was observed in the heads of fish exposed to EE₂ (two-way ANOVA; $p = 0.000$). However, in this case, the expression of *gnrh1* (two-way ANOVA; $p = 0.013$) and *cyp19a1b* (two-way ANOVA; $p = 0.000$) were also significantly affected by the xenoestrogen, upregulating the expression of the first only in females and of the second in both sexes (Fig. 4). The strongest response to the EE₂ observed in the brain was for *cyp19a1b* with 10.4-fold induction independently of the genotypic sex.

In the trunk, a dimorphic gene expression pattern was observed for *cyp19a1a* (Fig. 5, Student's t-test; $p = 0.003$). Although no significant effect of ATZ exposure alone was observed on the expression of
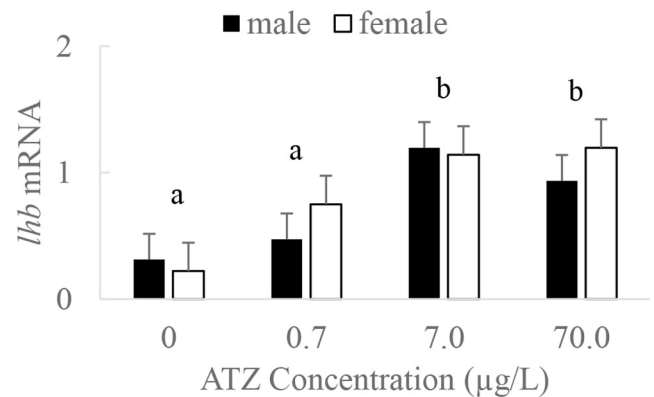

Fig. 3. Luteinizing hormone β (*lhb*) mRNA levels in the heads of *O. bonariensis* exposed to sublethal waterborne concentrations of ATZ. Different letters indicate statistically significant differences in gene expression among treatments ($p<0.05$).

cyp19a1a, the interaction between the herbicide and the fish sex was significant (two-way ANOVA, $p = 0.022$), down and upregulating the expression in females and males, respectively. The post-hoc analysis showed significantly higher expression levels (3.1-fold) of *cyp19a1a* in males exposed to 70 µg ATZ/L with respect to the control group (Fig. 5). No significant effects ($p>0.05$) of EE₂ were observed on gene expression in the trunk of the 6th wah *O. bonariensis* larvae (Supplementary Figure 7).

4. Discussion

The Argentinian Pampa is one of the most important agricultural regions of the world producing large quantities of soybean (16.1 million ha in 2021/22) and corn (10.3 million ha in 2021/22) (MAGyP, 2022). ATZ is the main herbicide used for corn, but it is also "illegally" used in chemical fallow for soybeans in this region. In recent years, several studies have reported high frequencies (33–100%) and maximum concentrations between 1.73 to 15.98 µg/L of the herbicide in surface waters of the core agriculture area, and concern has arisen about the potential risk it could pose to the aquatic ecosystem (Bachetti et al., 2021; Regaldo et al., 2018; Vera-Candioti et al., 2021).

Although several studies have assessed the biological effects of ATZ on different vertebrate species (Hanson et al., 2019), and most of them indicating potential endocrine disruption (Foradori et al., 2018; Graceli et al., 2020; Hayes et al., 2011; Opute et al., 2020; Stradtman and Freeman, 2021; Wirbisky and Freeman, 2015), little is known about the effects on fish species inhabiting this region of the world. In particular, adverse effects of this herbicide on the reproductive system of many fish have been demonstrated, though reported responses vary between species (Papoulias et al., 2014; Richter et al., 2016; Suzawa and Ingraham, 2008; Tillitt et al., 2010; Wirbisky et al., 2016; Yang et al., 2021). In the present study, waterborne ATZ exposure during the gonadal differentiation period modified the expression of some key genes (i.e., *lhb,*
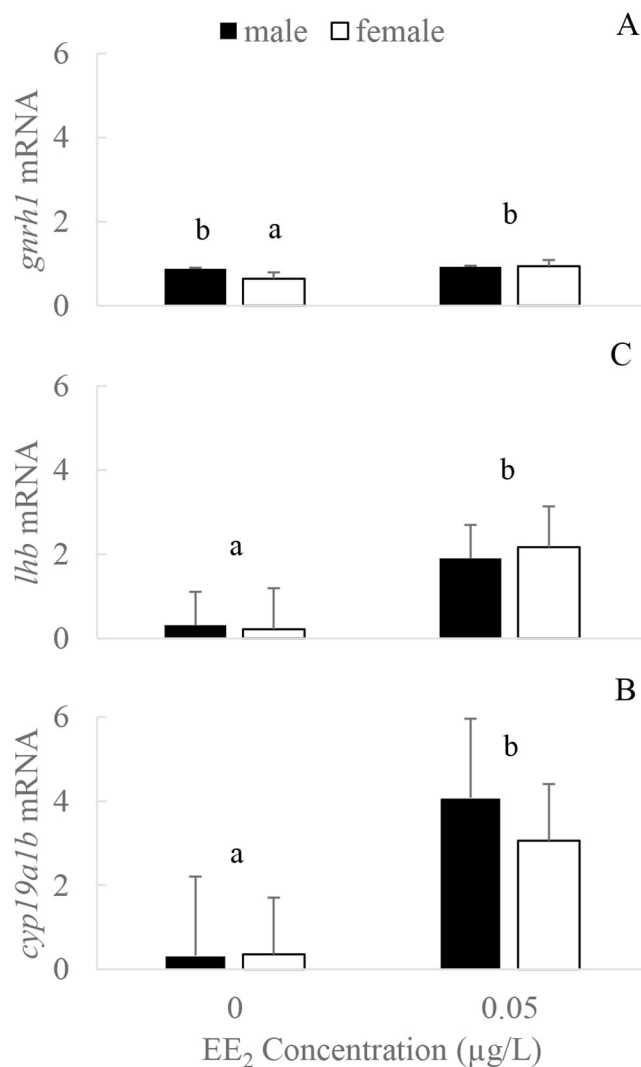
Fig. 4. Levels of gonadotropin-releasing hormone (*gnrh1*), brain aromatase (*cyp19a1b*), and luteinizing hormone β (*lhb*) mRNA levels in the heads of *O. bonariensis* larvae exposed to a sublethal waterborne concentrations of the model xenoestrogen EE$_2$. Different letters indicate statistically significant differences in gene expression among treatments (*p*<0.05).
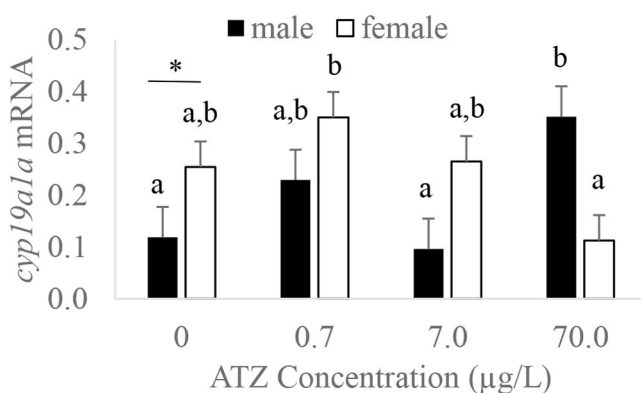

Fig. 5. Gonadal aromatase (*cyp19a1a*) mRNA levels in the trunk of *O. bonariensis* waterborne exposed to sublethal concentrations of ATZ. Different letters show statistically difference among treatments (Two-way ANOVA; *p*<0.05). Asterisk indicates statistically significant differences in gene expression among sex in the control group (Student-T test; *p*<0.05).

cyp19a1a) of the HPG axis and altered normal gonadal development of *O. bonariensis*. However, a defined feminizing effect was not detected, since no intratesticular oocytes were observed and only one sex inversion was registered.

The endocrine disrupting effects of ATZ in fish, amphibians, and crustaceans are often associated with de-masculinization or even male-to-female reversal (Hayes et al., 2011). In the present study, although *O. bonariensis* larvae complete feminization was found in the EE$_2$ exposed group, no partial or complete feminization or significant differences in sex ratio were observed at any ATZ treatment. A similar response was observed in *Micropterus salmoides* larvae exposed to 10 ng/L of EE$_2$ and up to 100 µg/L of ATZ (Leet et al., 2020). Taken together, these results would indicate that ATZ, at the studied developmental stage, exhibits a low capacity to feminize gonads of pejerrey when compared with a strong estrogenic compound such as EE$_2$.

On the other hand, exposure of ATZ induced alterations in gonadal development as revealed by the histological analysis. Females presented less developed ovaries, with no ovarian cavity, as ATZ concentration increased, compared to control larvae, which mostly presented a closed ovarian cavity. In larvae exposed to EE$_2$, an intermediate developmental stage (open ovarian cavity) was the most frequent stage observed. In genetically male larvae, no testicular oocytes were identified in fish exposed to the herbicide, However, an increase in the germinal cell number was observed in the testes of the fish at the highest concentration. The consequences of gonadal alterations during development on the reproductive performance are unknown, but evidentially it is important to note that the developmental changes persisted for at least 6 weeks after removal of the herbicide.

De-masculinization, attributed to a reduction of androgens and the increase in estrogens, has been proposed as the major action of ATZ on the gonad of male vertebrates (Hayes et al., 2011). For example, the induction of testicular oocytes was reported in adult males of *P. promelas* exposed to 5 µg/L ATZ (Tillitt et al., 2010). In goldfish, *Carassius auratus*, ATZ exposure reduced plasma levels of testosterone and 11-KT and increased levels of E$_2$, inducing disruption of the organ histoarchitecture without intratesticular oocytes formation (Spanò et al., 2004). In the present study, an increase of the germ cell number per cross section was found in genetically male larvae exposed to the highest dose of ATZ. It is important to note that a higher number of germ cells in females than in males was previously reported during the process of sex differentiation of *O. bonariensis* (Ito et al., 2005). An increase in germ cell number by intermittent proliferation, but no cystic proliferation, was described to be necessary for a male to female sex inversion in medaka, *O. latipes*, showing that germ cells have an essential role in feminizing the gonad (Arias Padilla et al., 2021; Nishimura et al., 2018). In consequence, results obtained in the present study would suggest an incipient feminization process induced by ATZ. However, the potency of the herbicide would be weak compared to that of EE$_2$, one of the most potent EDCs that cause a complete feminization. A similar effect was also reported in larvae exposed to E$_2$ (Fernandino et al., 2008a). In this context, in the future, it would be important to evaluate the effects of early exposure of ATZ in reproductive parameters of adult pejerrey.

The alteration of gene expression in response to ATZ exposure during the sex differentiation period was characterized by the induction of *lhb* in the head of both genetic sexes and *cyp19a1a* in the trunk of genetic males. In rats, it is known that the exposure to ATZ can reduce LH serum levels (Cooper et al., 2000). However, an increase of plasmatic LH with downregulation of gene expression in the pituitary and upregulation in the ovary was found in quails exposed to the herbicide, suggesting differences among taxonomic groups (Qin et al., 2015). Mutational studies in medaka (*O. latipes*) provided evidence that Lh plays an essential role only in ovulation, while in mammals LH, stimulated by GnRH, plays an important role in the folliculogenesis (Takahashi et al., 2016). In zebrafish females, *lhb* expression in the pituitary is upregulated during the transition from primary growth (PG) to previtellogenic (PV) follicles in the ovary (Chen and Ge, 2012). In the present study, *lhb* upregulation

in the head of ATZ-exposed genotypic male larvae was accompanied by an increase in the number of germinal cells in the gonad, which could indicate an incipient testicular feminization. Differently, in genotypic females, it was linked to a retarded gonad development manifested by an incompletely closed ovarian cavity. Analogous *lhb* upregulation was observed in the head of larvae exposed to EE$_2$, but it was together with an increase of the *cyp19a1b* expression and the complete feminization of the gonads. Downregulation of pituitary LH mRNA caused an increase in circulating LH levels and abnormal ovarian development in quails exposed to ATZ (Qin et al., 2015), showing that complex feedback mechanisms would be acting between the direct site of action of the herbicide and the response at the gonad.

A significant induction of brain aromatase in response to EE$_2$ (5 ng/ L), but not to ATZ (1 and 100 μg/L), was also observed in the brain of *Lithobates catesbeianus* tadpoles (Gunderson et al., 2011). The response of *cyp19a1b*, but not *cyp19a1a*, variant following EE$_2$ exposure was consistent with the presence of an estrogen-responsive element (ERE) in the *cyp19a1b* in fish (Kazeto et al., 2003; Zhang et al., 2012). The lack of response of *cyp19a1b* to ATZ suggests that the mechanism of action of the herbicide would not be through the estrogen receptors. This result is consistent with the mechanism of endocrine gene networks activation proposed for ATZ via non-steroidal NR5A nuclear receptors (Suzawa and Ingraham, 2008). Despite this, some common disruptive neuroendocrine pathways along the hypothalamus-pituitary axis between ATZ and EE$_2$ would be expected, considering the similar induction of *lhb* expression.

In *O. bonariensis* gonads, a dimorphic expression of *amha, 11βhsd2, cyp19a1a* was found during the gonadal differentiation period, with an upregulation of *amha* and *11βhsd2* at male-promoting temperature (29 °C) and *cyp19a1a* at female- promoting temperature (17 °C), regardless of the genotypic sex (Zhang et al., 2018). In the present study, conducted at an intermediate temperature (25 °C), no dimorphic expression was observed for *amha* and *11βhsd2*, but differences were found for *cyp19a1a* and *foxl2*, with higher levels in the genotypic females. Differences in *foxl2* were not statistically significant, probably due to the limitation of assessing it in the trunk instead of directly in the gonad. However, the same dimorphic expression of these two genes was previously observed in 4 weeks-old larvae *O. hatcheri* (Hattori et al., 2012). The lack of differences in *amha* and *11βhsd2* could be a consequence of the intermediate temperature used in this study, as was previously observed in pejerrey (Fernandino et al., 2008a; 2012). In the trunk of larvae exposed to ATZ, a significant increase in the *cyp19a1a* expression was observed, characterized by a shift in the expression levels given by a strong upregulation of the gene in genotypic males, and a slight downregulation in genotypic females, at the highest concentration treatment. Differently, none of the assessed genes in the gonads were responsive to EE$_2$ exp+osure. In a transcriptomic study, a partially overlapped response in the gene expression was found in genes linked with reproductive development and function (*star, cyp11a2*, and *cyp1a*) at the testes of early larvae of *Micropterus salmoides* exposed to ATZ and EE$_2$ (Leet et al., 2020). In particular, modulation of the steroidogenic acute regulatory protein gene (*star*) would indicate that ATZ could act upstream in the steroidogenic biosynthetic pathway regarding those genes assessed in the present study.

The effect of ATZ on growth was characterized by an increase of about 12% in body length at the 6th wah, compared to the control group. A similar response was observed in fish exposed to the EE$_2$. In both cases, the effect on growth was no longer apparent at the 12th wah, after 6 weeks in ATZ-free water. That would indicate that not only the effect was caused by the chemicals, but also it that was reversible. A broad spectrum of responses has been reported for ATZ on fish growth (Hanson et al., 2019). In most cases, fish length or weight were not affected or reduced, especially when ATZ tested concentrations were relatively high (Brain et al., 2021). Only a few studies have reported an increase in the growth of some species as a response to the herbicide. For example, weight increase was found at emergence in *Oncorhynchus nerka*

larvae exposed to 25 μg ATZ/L from fertilization to emergence (Du Gas et al., 2017). A comparable response to that observed in our model species was reported in tadpoles of the south American toad, *Rhinella arenarum*, exposed to 1, 10, and 100 μg ATZ/L, and 100 μg E$_2$/L, showing higher weights in ATZ and E$_2$ treatments compared to the control (Brodeur et al., 2013). The role of E$_2$ in shaping body growth and sexual characteristics has been widely recognized and could act to modulate the pituitary-hepatic growth hormone/insulin-like growth factor 1 pathway (Canosa et al., 2007; Leung et al., 2004; Zou et al., 1997). It is also known that the mitogenic action of E$_2$ is mediated through estrogen receptors (ERs) by inducing the mitogen-activated protein kinase, so showing crosstalk between E$_2$ and growth factor-signaling pathways (Kato et al., 1998). On the other hand, the action of ATZ on growth is less clear. It has been proposed the herbicide would have the capacity to bind the growth hormone release hormone receptor (Fakhouri et al., 2010). This mechanism would explain the reduction of growth by inhibiting gene expression of growth hormone. Non-monotonic responses have been described for the action of ATZ on amphibian development, usually delayed or accelerated at high or low doses, respectively (Trudeau et al., 2020). Such as response could be explained by crosstalk and feedback between growth factors and nuclear receptor signaling (Picard, 2003).

Regarding the environmental relevance of the findings, the USEPA has established a chronic ATZ LOEC of 0.5 μg/L based on reproductive effects (Farruggia et al., 2016) and later a chronic LOEC of 1 μg/L related to decreasing egg production in freshwater Japanese medaka fish (USEPA, 2019). The lowest observed effect concentration (LOEC) obtained in the present study was 7 μg/L. This value is in the same order of magnitude as these LOEC and surface water ATZ concentrations recently reported in the Pampas region (Bachetti et al., 2021; Garcia et al., 2019; Vera-Candioti et al., 2021). Our results alert the potential risk ATZ poses to normal gonadal development of *O. bonariensis* when the herbicide reaches surface waters during the reproductive season when larvae would be may be exposed during sensitive gonadal development stages.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

PC, JIF, VLT and GSM were responsible for conceptualization, experimental design, data analysis, and manuscript writing. PC and ESB performed experimental trials. PC, JIF and JMGV conducted PCR primer design and assays. CGL performed gonadal histology and DG ATZ chemical analysis.

Author statement

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Supplementary Material

The Supplementary Material for this article can be found online at:
Supplementary Table 1. Primer sequences
Supplementary Table 2. Actual concentrations of ATZ in testing media
Supplementary Table 3. Gene expression statistical analysis
Supplementary Figure 1. Illustrative gonadal developmental stages
Supplementary Figure 2. Gonad histology in control group
Supplementary Figure 3. Gonad histology in ATZ0.7
Supplementary Figure 4. Gonad histology in ATZ7.0
Supplementary Figure 5. Gonad histology in ATZ70
Supplementary Figure 6. Gonad histology in EE20.05
Supplementary Figure 7 a, b, c, d. Gene expression

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106366.

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