EFFECTS OF 4*n*–NONYLPHENOL EXPOSURE ON THE REPRODUCTIVE BEHAVIOR AND TESTIS HISTOLOGY OF *Jenynsia multidentata* (ANABLEPIDAE: CYPRINODONTIFORMES)

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ABSTRACT

Increasing evidence has shown that wild populations of aquatic animals are exposed to chemical compounds that behave as hormone mimics and disrupt their reproductive physiology. Fish species are sensitive bioindicators of water quality. The one–sided livebearing fish *Jenynsia multidentata* has a wide neotropical distribution and has been used as an excellent model in both laboratory and field studies. The main goal of the present work was to assess the effects of subchronic 4*n*–nonylphenol (4*n*–NP) exposure on *J. multidentata*. Adult males were exposed to 0, 20 and 40 µg l^{-1} 4*n*–NP during 14 days. 17 β estradiol (E_2) was used as positive control. Reproductive behavior, somatic indices, testis and liver histology were evaluated. Our results suggest that 4*n*–NP has adverse effects on the reproductive behavior and histological characteristics in males of *J. multidentata*. Individuals exposed to 4*n* –NP show similar alterations to those recorded in E_2 treatments. Changes in the reproductive biology caused by exposure to xenoestrogens could potentially lead to long–term effects at population levels that may not always be immediately evident. To the best of the authors' knowledge, this is the first report that evaluates the effects of 4*n*–NP on the reproductive aspect of native fish species.

Key words:
Live–bearing fish, xenoestrogens, biomarkers.

EFECTOS DEL 4*n*–NONILFENOL SOBRE EL COMPORTAMIENTO REPRODUCTIVO E HISTOLOGÍA DEL TESTÍCULO EN *Jenynsia multidentata* (ANABLEPIDAE: CYPRINODONTIFORMES)

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RESUMEN

Una creciente evidencia científica demuestra que los organismos acuáticos son expuestos a diversos xenobióticos que pueden imitar la función de hormonas naturales, y, por lo tanto, alterar la fisiología reproductiva de los mismos. Los peces constituyen bioindicadores sensibles en la evaluación de la calidad de los recursos hídricos. *Jenynsia multidentata* (Jenyns, 1842) es un pez vivíparo de amplia distribución neotropical y ha sido utilizado como un excelente modelo experimental. El objetivo del presente trabajo fue evaluar los efectos de la exposición subcrónica del compuesto 4*n*–nonylphenol (4*n*–NP) en machos adultos de *J. multidentata*. Para ello los individuos fueron expuestos a 0, 20 y 40 µg l^{-1} 4*n*–NP durante 14 días. La hormona 17 β estradiol (E_2) fue utilizada como control positivo. El comportamiento reproductivo, índices somáticos, histología de gónadas e hígado fueron evaluados. Nuestros resultados sugieren que el 4*n*–NP afecta de manera adversa las variables anteriormente mencionadas. Las alteraciones registradas tras la exposición a 4*n*–NP fueron similares a las observadas para la hormona E_2. A nuestro entender, el presente trabajo constituye el primer reporte que evalúa los efectos del 4*n*–NP sobre parámetros reproductivos en una especie íctica autóctona de Argentina.

Palabras clave:
Peces vivíparos, xenoestrógenos, biomarcadores.

INTRODUCTION

A wide range of chemical compounds with adverse health effects on organisms are introduced into the aquatic environment. Endocrine–disrupting chemicals (EDCs) interfere with the endocrine system of wildlife and human populations. One group of EDCs includes xenoestrogens, which bind to estrogen receptors with a similar affinity to the endogenous estrogen hormone (17 β estradiol). They have the potential to exert effects at extremely low concentrations (Mills & Chichester, 2005) affecting animal development and reproduction.

Among xenoestrogens, alkylphenol polyethoxylates (APEOs) are used as nonionic surface active agents in a wide variety of industrial and agricultural products, including paints, pesticides, detergents and cosmetics (Ying *et al*., 2002). The degradation of APEOs in the environment produces alkylphenols, such as 4*n*–Nonylphenol (4*n*–NP). This product is a ubiquitous contaminant in the aquatic environment and it is characterized by its high toxicity and resistance to biodegradation (Ministry of the Environment, Government of Japan, 2001; Tsuda *et al.,* 2001).

The potential threat of xenoestrogens to animal reproduction posed the necessity to identify and develop bioindicators and biomarkers to monitor their effects. Fish are sensitive biondicators of aquatic environment quality and are useful models to understand the mechanisms of external perturbations that could disrupt essential biological functions in other vertebrates (Kime, 1999; Jalabert *et al.,* 2000). Exposure of fish males to 4*n*–NP or other alkylphenols has been reported to cause a decrease in testicular size, testicular fibrosis, apoptosis of germ and Sertoli cells, ovotestes, vitellogenin induction, and changes in reproductive behavior patterns (Miles–Richardson *et al.,* 1999; Dréze *et al*., 2000; Metcalfe *et al.,* 2001; Nakamura *et al.,* 2002; Kinnberg *et al*, 2003; Weber *et al.,* 2003, Rey Vázquez *et al*., 2009). Several studies on the effects of 4*n*–NP have been conducted on different fish species (Mills & Chichester, 2005). Nevertheless, there is a gap of knowledge about the effects of this compound on South American native fish.

The one–sided livebearing fish, *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes) is a conspicuous species in the Neotropical region (Malabarba *et al*., 1998). It has been used as laboratory fish model to evaluate the effects of xenobiotics through different biomarkers (Cazenave *et al.,* 2008; Amé *et al.,* 2009; Ballesteros *et al.,* 2009). In particular, the reproductive behavior and histological characteristics have been pointed out as useful biomarkers (Hued *et al.,* 2012; Guyón *et al.,* 2012). Males have a coercive behavior (Bisazza *et al.,* 2000) and the testis structure has been classified as restricted lobular type (Martinez & Monasterio de Gonzo, 2002).

The aim of this study was to evaluate changes in testicular histology and in male reproductive behavior of the native fish, *J. multidentata* in response to subchronic exposure (14 days) to 4*n*–NP.

MATERIALS AND METHODS

REAGENTS

Ultrapure water (<5mg/L TOC) was obtained from a purification system (Arium 61316–RO plus Arium 611 UV). 4*n*–Nonylphenol (4N–NP) and 17β–Estradiol (E_2) were obtained from Sigma–Aldrich Argentina (99.9 % and 98 % purity, respectively). E_2 was used as a positive control to examine whether the effects of 4N–NP corresponded to the effects of natural estrogen. The stock solutions (1 mg ml^{-1}) were prepared by dissolving each reagent in acetone (Merck Chemist) and stored at 20°C. Aliquots of these solutions were used to prepare the 4*n*–NP and E_2 working solutions by diluting them in ultrapure water.

FISH

Adult males of *J. multidentata* were caught by a backpack electrofisher from a reference site in the San Antonio River, Córdoba, Argentina (64°32¨W 31°28¨S) (Hued & Bistoni, 2005) and transported to the laboratory in 20–L water tanks. Fish were acclimated for two weeks under controlled laboratory conditions (21ºC and 12:12h light–to–dark cycle). During the acclimatization period fish were fed twice a day with commercial fish food. Females used in the behavioral analysis were captured at the same site.

EXPOSURE CONDITIONS

Fish were exposed to 20 µg l^{-1} and 40 µg l^{-1} of 4*n*-NP (1) during 14 days in a 10 L aerated glass aquarium. Two concentrations of E_2 were used as positive control: 100 ng l^{-1} and 1000 ng l^{-1}, and acetone alone (0.005 % v/v) as solvent control. Five fish were exposed to each concentration and four replicates were made for each treatment (n =20). Male standard length (mean ± SD) was 27.09 $\pm$ 3.65 mm and mean weight was 0.43 $\pm$ 0.17 g Males were fed and kept under the same conditions during the exposure and acclimatization periods. Water was replaced partially twice a week and completely once a week.

REPRODUCTIVE BEHAVIOR

At the end of the exposure period, each male was transferred to a 5–L aquarium and paired with an unexposed female for 24 hours. Male sexual display was recorded using a video recorder (Panassonic; mod. 3244) and by direct observation for 15 min in the morning and 15 min in the afternoon (due to the diurnal habits of *J. multidentata*, Cazenave *et al.*, 2008). Based on the normal reproductive behavior described by Bisazza *et al*. (2000), the following variables were estimated:

- *Persecution time (PT)*: period of time (in seconds) that a male spends in persecuting a female to make contact with her gonopore.

- *Number of persecutions (P):* number of times that a male persecutes a female in an attempt to make contact with her gonopore.

- *Copulatory attempts (CA):* number of times that a male enlarges its gonopodium to make contact with the female gonopore.

- *Number of copulations (C):* number of times that a male makes direct contact through its gonopodium with the female gonopore.

The following ratios were then calculated: CA/PT, CA/P, C/PT, C/P (successful persecution) and C/CA. The last parameter was used as an estimation of mating success (Pilastro *et al*., 1997; Bisazza *et al.,* 2000).

SOMATIC CONDITION AND HISTOLOGICAL ASSESSMENT

Immediately after recording reproductive behavior, each male was euthanized by immersion in tricaine metanosulfonate overdose (500 mg l^{-1} MS222). Total weight and standard length were recorded to determine the Fulton Index (K) for each fish (Anderson & Newman, 1996). Testis and liver were excised and weighed in order to calculate the Gonadosomatic Index (GSI) and Hepatosomatic Index (HSI) (Goede & Borton, 1990), and fixed in 10 % formalin. Tissue samples were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections of 4 to 6–µm thick were stained with hematoxylin and eosin (H&E) for examination under a light microscope.

STATISTICAL ANALYSES

Descriptive statistics of the reproductive behavior and somatic variables measured (mean, standard deviation, range) were calculated. Normal variables were analyzed with one–way analysis of variance (ANOVA) to evaluate the differences between treatments followed by the DGC test *a posteriori* (Infostat 2003). Variables with non–normal distribution were analyzed with the Kruskal–Wallis Analysis, followed by the Dunn test *a posteriori*. The level of significance was $p<0{\cdot}05$.

RESULTS

SURVIVAL

All of the control and 4*n*–NP and E_2 exposed fish survived to the end of the experiment.

REPRODUCTIVE BEHAVIOR

The behavioral parameters recorded in the present work did not show significant differences in each treatment group between morning and afternoon. Therefore all the data was combined and analyzed together.

One hundred percent of the control males were sexually active in the presence of the female. However, the percentage of males exposed to 4*n*–NP and E_2 showed a lower percentage of sexual activity, decreasing 30 % at 20 µg l^{-1} 4*n*–NP and 20 % in the other treatments.

The behavioral parameters are shown in Table 1. Several variables were affected by estrogen exposure, showing a significant decrease relative to the control group. Both CA and C decreased significantly in all treatments relative to the control group, but no differences were recorded in PT and P among males exposed to 4*n*–NP and E_2.

The number of copulas related to time of persecution (C/TP) was lower in the highest concentration of 4*n*–NP. This pattern was recorded in males exposed to both concentrations of E_2. The proportion of persecutions that ended in copula (C/P) (successful persecutions) decreased significantly in males exposed to 4*n*–NP and E_2 relative to the control group. On the other hand, the relationship between copulatory attempts and number of persecutions (CA/P) was significantly different only in males exposed to both concentrations of E_2. Finally the proportion of copulatory attempts that ended in copula (C/CA) (successful attempts) was lower in males exposed to 4*n*–NP and 100 ng E_2 l^{-1}.

Variables	Control	20 µg L^{-1}NP	40 µg L^{-1}NP	100 ng $L^{-1}E_2$	1000 ng $L^{-1}E_2$
PT	43.80 ± 6.86**A**	31.87 ± 8.84**A**	35.89 ± 4.45**A**	33.21 ± 11.44**A**	27.55 ± 8.87**A**
P	18.20 ± 2.35**A**	17.10 ± 4.12**A**	15.85 ± 5.20**A**	11.50 ± 4.63**A**	12.21 ± 4.12**A**
CA	17.60 ± 2.15**A**	5.92 ± 7.19**B**	4.62 ± 3.27**B**	5.64 ± 2.77**B**	8.91 ± 2.39**B**
C	4.85 ± 0.85**A**	1.21 ± 2.46**B**	1.47 ± 1.15**B**	0.87 ± 0.61**B**	0.86 ± 0.41**B**
NP/PT	0.50 ± 0.05**A**	0.64 ± 0.06**A**	0.91 ± 0.15**B**	0.79 ± 0.13**B**	0.74 ± 0.06**B**
CA/TP	0.47 ± 0.04**A**	0.42 ± 0.07**A**	0.73 ± 0.25**A**	0.39 ± 0.07**A**	0.53 ± 0.10**A**
C/PT	0.08 ± 0.01**A**	0.04 ± 0.02**B**	0.02 ± 0.01**B**	0.02 ± 0.01**B**	0.04 ±0.02**B**
CA/P	1.09 ± 0.11**A**	0.71 ± 0.15**B**	0.74± 0.14**B**	0.52 ± 0.07**B**	0.75 ± 0.14**B**
C/P	0.18 ± 0.03**A**	0.09 ± 0.04**B**	0.05 ± 0.03**C**	0.04 ± 0.02**C**	0.06 ± 0.02**C**
C/**C**A	0.17 ± 0.03**A**	0.10 ± 0.04**B**	0.05± 0.03**C**	0.06 ± 0.03**C**	0.09 ± 0.03**B**

Tabla 1. Reproductive behavioral variables registered in *Jenynsia multidentata* males exposed to sublethal concentrations of 4*n*–NP and E_2 for 14 days. Mean ± EE; *n*= 15. References: (PT) Time of persecution; (P) number of persecutions; (CA) copulatory attempts; (C) copulas. Capital letters indicate significant differences among treatments ($p<0.05$).

SOMATIC CONDITION AND HISTOLOGICAL ASSESSMENT

The average, maximum and minimum values of somatic indices in control and treatment groups are shown in Table 2. Only males exposed to 1000 ng l^{-1} E_2 showed a significant increase of K regarding other treatments. On the other hand, the GSI decreased significantly in both treatments of 4*n*–NP and E_2 and the HSI increased significantly at the 40 µg l^{-1} 4*n*–NP and 100 ng $L^{-1}E_2$ compared with the control group.

Testis from control fish contain regularly organized cysts with all spermatogenetic stages (Fig.1a–b). The testicular structure corresponds to the lobular restricted type. Each lobule contains isogenic germ cells enclosed by Sertoli cells forming organized cysts, from immature to mature cysts, arranged from the periphery to the efferent ducts. Spermatogonias (Sg) are restricted to the testis periphery whereas cysts with spermatocytes (Sc), spermatids (*St*) and mature spermatozoa are arranged toward the central duct. Males exposed to 4*n*–NP and E_2 showed a clear disarrangement and loss of cyst structure, where the cyst walls are not recognizable (Fig 1c–f). Apoptotic bodies were also observed inside Sertoli cells. These bodies correspond to phagocytized degenerated germ cells (Fig 1c, e and f). A decrease in spermatocyte cysts was registered in all treatments, being more evident at 1000 ng L^{-1} E_2 (Fig. 1f).

Histological examination of the liver revealed progressive adverse effects on the parenchyma structure following exposure to increasing 4*n*–NP and E_2 concentrations. The liver of control males showed a homogenous parenchyma structure (Fig. 2a, 2d) whereas males exposed to both 4*n*–NP and E_2 showed a loss of hepatic structure, a generalized hydropic degeneration (cells appeared swollen), vascular congestion, sinusoid dilatation and numerous hepatocytes contained pyknotic nuclei (Fig. 2b, c, e, f). Focal necrosis was registered at 1000 ng L^{-1} E_2 it (Fig. 2f).

Indexes	Control	20 µg L^{-1}NP	40 µg L^{-1}NP	100 ng $L^{-1}E_2$	1000 ng $L^{-1}E_2$
K	1.98 ± 0.18**A**	2.08 ± 0.14**A**	2.24 ± 0.17**A**	2.10 ± 0.17**A**	2.21 ± 0.03**A**
GSI	2.60 ± 0.31**A**	1.20 ± 0.24**B**	1.09 ± 0.24**B**	1.58 ± 0.23**B**	1.23 ± 0.15**B**
HSI	1.74 ± 0.18**A**	1.94 ± 0.27**A**	2.41 ± 0.25**B**	2.51 ± 0.17**B**	1.96 ± 0.14**A**

Tabla 2. Somatic indexes measured in *Jenynsia multidentata* males exposed to sublethal concentrations of 4*n*–NP and E2 for 14 days. Mean ± EE; *n*= 15. References: (K) Fulton Index; GSI: Gonadosomatic index and HSI: Hepatosomatic index

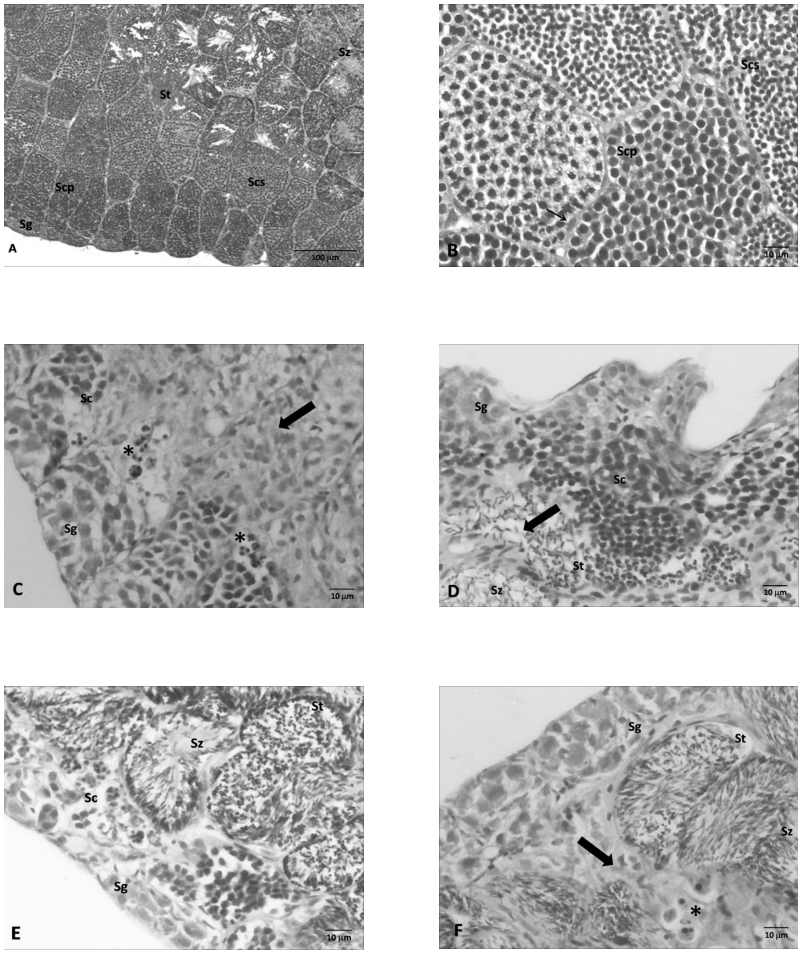

Figure1. Light microphotographs of testis of *Jenynsia multidentata*. **(A)** The testicular structure corresponds to the lobular restricted type (100x); **(B)** a well–defined cyst structure in control fish; **(C)** male exposed to 20 µg NP L^{-1} showing a clear loss of cyst structure (thick arrows) and the presence of apoptotic bodies; **(D)** at 40 µg NP L^{-1} a disarrangement of cyst structure and spermatogonial hypertrophy was evidenced; **(E)** male exposed to 100 ng L^{-1} E_2 showing apoptotic bodies enclosed in Sertoli cells; and **(F)** individual exposed to 1000 ng L-1 E2 showing a loss of cyst structure and a decrease in number of spermatocytes (**B–F**: 400x). References: Sc, spermatocytes; Scp, primary spermatocytes; Scs,secondary spermatocytes; Sg, spermatogonias; St, spermatids; Sz, spermatozoa; asterisks show apoptotic bodies; thick arrows show loss of cyst structure. H& E stain.

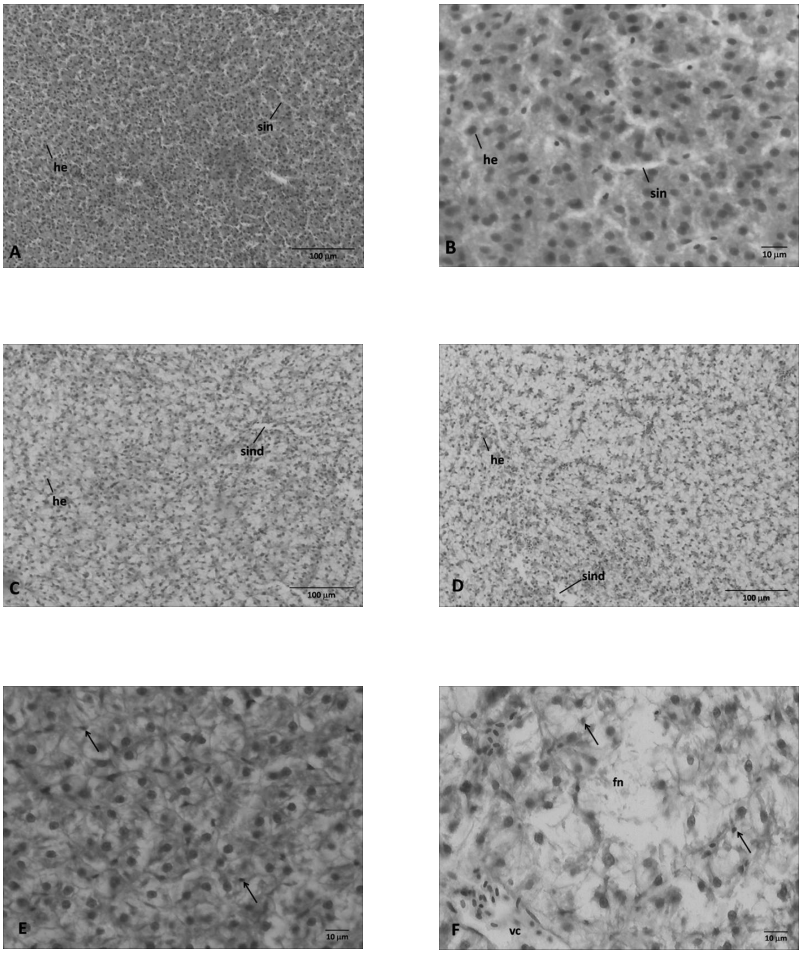

Figure2. Light microphotographs of liver from *Jenynsia multidentata*. **(A–B)** A well–defined parenchyma structure in control fish (100x and 400x respectively); **(C–D)** males exposed to 4*n*–NP and E_2 respectively, showing a clear loss of parenchyma structure and generalized hydropic degeneration (100x); **(E)** male exposed to the highest concentration of 4*n*–NP showing pyknotic cells and **(F)** male exposed to the highest concentration of E_2 showing focal necrosis and pyknotic cells (**E–F**: 400x). References: hd, hydropic degeneration; he, hepatocytes; fn, focal necrosis; sin, sinusoids, sind, sinusoid dilatation; vc, vascular congestion; arrows show pyknotic nuclei. H& E stain.

DISCUSSION

Reproduction is considered to be a key parameter to evaluate the harmful potential of endocrine disrupters. It is well–known that sex hormones play an important role in the expression of sexual behavior in fish (Arcand–Hoy & Benson, 1998), and reproductive success depends on the ability to perform the appropriate sexual behavior. It has been suggested that changes in sexual behavior following exposure to EDCs could be useful biomarkers to assess estrogenic effects and could predict effects at the population level (Bayley *et al.,* 1999; Bjerselius *et al*., 2001; Doyle & Lim, 2005).

Control males showed the same reproductive behavior pattern described by Bisazza *et* al. (2000), with coercive copulatory attempts through gonopodial thrusting. When a male meets a female, he moves immediately behind her and then darts forward along her side in an attempt to bring forward his gonopodium. When his body is in close contact with the female, he rotates the gonopodium towards the female and tries to insert his gonopodium into her gonopore, repeatedly.

A significant decrease in most behavioral parameters was recorded after exposure to 4*n*–NP and E_2, suggesting that 4*n*–NP mimics the E_2 activity. Although exposed and non–exposed males spent the same time persecuting females, males treated with 4*n*–NP and E_2 showed a decrease in the number of copulatory attempts and copulas compared to non–exposed males. After 4*n*–NP and E_2 exposure a decrease of 50 % or more was recorded in the percentage of successful persecution (C/P) compared to control males. On the other hand, the effectiveness of copulatory attempts (C/CA) was lower in all treatments compared to the control group. Bisazza *et al.* (1996) highlighted the importance of copulatory attempts in viviparous fish behavior because only a small proportion of CA was successful. Therefore, any reduction in male sexual activity of *J. multidentata* could reduce the ability of males to impregnate females. Therefore, our results indicate that xenoestrogen exposure has a negative impact on the reproductive behavior of *J. multidentata*.

Several studies have shown the adverse effects of alkylphenols and E_2 on fish reproductive behavior, showing a generalized decline in male reproductive performance (Bayley *et al.,* 1999; Bjerselius *et al.,* 2001; Oshima et al. 2003). Doyle & Lim (2005) demonstrated a lower number of approaches and copulatory attempts in males of eastern mosquitofish *Gambusia holbrooki* exposed to 20, 100 and 500 ng l^{-1} E_2 for 84 days. Effects of 4*n*–NP on fish reproduction have also been documented. Schoenfuss *et al.* (2008) reported that male *Pimephales promelas* exposed to a low concentration of 4*n*–NP exhibited an apparently beneficial effect on their nest holding ability (an excitatory response), whereas males exposed to high 4*n*–NP concentrations (6 to 60 µg l^{-1}) suffered adverse consequences. Cardinali *et al*. (2004) showed that newly born guppies (*Poecilia reticulata)* exposed to 100 µg l^{-1} 4*n*–NP for 90 days did not approach females and assumed sigmoid display. The alterations registered by the mentioned author are in agreement with our results.

The Gonadosomatic index (GSI) has been pointed out as a general measure of gonadal maturation based on the hypothesis that larger gonads indicate higher development (Schweer, 1992). Therefore, reduced testicular size could be an indication of hampered fertility (Jobling *et al.,* 1996). In the present study the GSI decreased significantly in all treatments. Males exposed to 4*n*–NP and 1000 ng L^{-1} E_2 showed approximately a 50 % reduction in the GSI compared to the control group. Similar results have been reported in males of rainbow trout *Oncorhynchus mykiss,* southern platyfish *Xiphophorus maculatus* and *P. reticulata* exposed to different concentrations of 4*n*–NP (Jobling *et al.,* 1996; Christiansen *et al.,* 1998; Cardinali *et al.,* 2004) and in common carp, *Cyprinus carpio* and goldfish *Carassius auratus* exposed to E_2 (Gimeno *et al.,* 1998; Bjerselius *et al.,* 2001).

Histological changes in gonads can be predictive of the reproductive success of the species under study and thus the population fitness (Van der Ven *et al.,* 2003). In the present study we showed several effects of 4*n*–NP and E_2 on the testicular structure of fish. A loss in cyst structure was recorded in all treatments. These alterations are in agreement with those recorded in viviparous eelpout *Zoarces viviparous* injected intraperitoneally with 100 µg l^{-1} week $^{-1}$ 4*n*–NP (Christiansen *et al.,* 1998) and *X. maculatus* exposed to 80 and 280 µg l^{-1} ^{4}*n*–NP (Kinnberg *et al.,* 2000). Similar results have been reported after E_2 exposure (*P. reticulata*; 10 and 15 ng L^{-1}) during 3.5 months (Nielsen & Baatrup, 2006) and *Danio rerio* exposed to 1, 10 and 100 nM E_2 during 21 days (Van der Ven *et al.,* 2003). The mechanism(s) by which 4*n*–NP affects testicular structure is still unknown. However, a possible explanation for these changes is an effect on Sertoli cells. These cells constitute the cyst wall where spermatogenesis takes place (Grier, 1981) and phagocytize discarded organelles (residual bodies) and cytoplasm during spermiation or phagocytize germ cells that degenerate in the normal course of spermatogenesis (Hunter & Donaldson, 1983). However, in the present work the multiple apoptotic bodies observed in Sertoli cells of immature cysts could be the result of the adverse effect of 4*n*–NP and E_2, which indicates the increase in cellular apoptosis or necrosis of degenerated germ cells. These alterations have also been registered in testis of fathead minnow *P. promelas* exposed to E_2 (Miles–Richardson *et al.,* 1999), *Z. viviparous* exposed to 4*n*–NP and E_2 (Christiansen *et al.,* 1998) and Japanese medaka *Oryzias latipes* and *D. rerio* exposed to 4*n* –NP (Weber *et al.,* 2002; Weber *et al.,* 2003). The clear loss of cyst structure in *J. multidentata* exposed to 4*n* –NP and E_2 is coincident with the decrease registered in GSI values. This decrease could be the result of the testis structure disorder together with an increase of cell apoptosis or necrosis registered in all treatments.

The liver analysis showed a significant increase in the HSI values in males exposed to 40 µg l^{-1} of 4*n*–NP and 100 ng l^{-1} of E_2. This result could be an adaptive response to increase the capacity of the liver to detoxify foreign compounds (Goede & Barton, 1990). A similar variation of HSI was registered in newly born guppies exposed to a sublethal

concentration of 4*n*–NP for 90 days (Cardinali *et al*., 2004) and in field studies, in sites contaminated with alkylphenols (Orlando *et al.* 1999).

Liver histopathology is employed as a sublethal test for evaluating the toxic effects of xenobiotic compounds. The liver is considered the target organ of chemically induced tissue injuries due to its function of xenobiotic biotransformer and its central role in the circulatory system (Hinton *et al*., 2001). When toxic compounds exceed the detoxification level of the liver, high concentrations of a toxicant cause modifications to the normal hepatic structure. In the present work the hepatocytes of control males were homogeneous both in size and cytoplasm density. On the contrary, the liver histological assessment of exposed males revealed dose–related damages, varying from hydropic degeneration at low concentrations to focal necrosis at the highest concentrations. A loss of hepatic tissue structure and the presence of pyknotic nuclei for all the treatments was also observed. These changes could affect the normal liver function. Similar histological alterations in liver have been pointed out by several authors (Ballesteros *et al*., 2007, Yang *et al*., 2008, Hued *et al*., 2012) who evaluated the effects of different toxics on liver histology.

In summary, the remarkable changes observed in reproductive behavior and histological changes induced by 4*n*–NP suggest that these parameters are sensitive and potentially useful biomarkers for the exposure to environmentally relevant estrogenic compounds. According to our results, 4*n*–NP produces similar effects on the natural hormone E_2, indicating that 4*n*–NP exerts a clear estrogenic action on males. This compound has a negative influence on the male reproductive biology of *J. multidentata* and could potentially lead to long–term effects on wild populations that may not always be immediately evident.

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