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Recovery of the Reproductive Capability Following Exposure to 4-*tert*-Octylphenol in the Neotropical Cichlid Fish *Cichlasoma dimerus*

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Abstract Here, we analyzed the recovery of the reproductive capability in male *Cichlasoma dimerus* after exposure to sublethal concentrations of 4-*tert*-octylphenol (OP), a demonstrated estrogenic chemical. Adult fish were exposed to 0, 30, 150 and 300 µg/L OP during 60 days and subsequently transferred to OP-free water for another 60 days. At 150 and 300 µg/L, absence of fertilized spawnings were recorded during the first 4 weeks following OP exposure, which could be explained by the impairment of testis architecture recorded at the highest OP concentrations. The restoration of the testicular organization began by day 14 in OP-free water, when the germinal epithelium re-started to proliferate. Testicular functionality was recovered by day 28, yielding fertilized eggs and viable F1 embryos. These results show that pathological features induced in the testes of *C. dimerus* by OP exposure are not permanent since fish recover their fertilization capacity after an adequate depuration period.

Keywords Teleosts · Cichlids · Xenoestrogens · Octylphenol · Recovery · Reproductive capability

The alkylphenols (APs) such as 4-*tert*-octylphenol (OP) are weak estrogenic chemicals that may induce endocrine disruption in teleost, raising concern over their potential negative impacts on wild populations. Laboratory studies have shown that exposure of male fish to APs results in induction of circulating vitellogenin, inhibition of testicular growth, reduced testosterone levels, impaired sexual behavior, reduced sperm quality and fertility rate, and formation of intersex gonads (Jobling et al. 1996; Gray et al. 1999; Gronen et al. 1999; Knörr and Braunbeck 2002; Rasmussen et al. 2005; Balch and Metcalfe 2006; Roggio et al. 2012; Maltais and Roy 2014).

The South American cichlid fish *Cichlasoma dimerus* is a perciform teleost representative of the ichthyic fauna in the La Plata River basin (Argentina). In a previous study in which males were exposed to waterborne OP, we reported the presence of vitellogenin both in plasma and surface mucus as well as the induction of testicular damage (Rey Vázquez et al. 2009).

Determining whether the impairment of the reproductive function produced by xenobiotic exposure is permanent or reversible is important for evaluation of risks at population levels in aquatic ecosystems. Few studies have assessed the reversibility of histopathological and functional features of fish male gonads after depuration.

The aim of this study was to analyze the recovery of the reproductive capability after exposure to sublethal concentrations of OP in males of *C. dimerus*. Testicular histology as well as successful spawnings were assessed by an experimental design of exposure and subsequent depuration in clean water.

Materials and Methods

The adult specimens of *C. dimerus* used in this study were captured in Esteros del Riachuelo, at Corrientes, Argentina (27°25′S, 58°15′W) at the onset of reproductive season, that extends from September to March, when fish spawn on average every 29.4 days under laboratory conditions (Rey

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Vázquez et al. 2012). A total of 72 fish (60 males and 12 females) were kept in aquaria at 26.5 ± 1°C, pH 7.3, with 12:12 h photoperiod. Fish were fed once a day with pelleted commercial food and were allowed to acclimate to captivity conditions for a month prior to the onset of experiments.

The test substance, OP (>97 % pure) was obtained from Sigma. Exposure concentrations were selected from our published studies (see Rey Vázquez et al. 2009). Prior to the onset of exposure, for each assayed concentration, 5 males and 1 female fish were transferred to each 50 L glass tank under the same physical conditions and alimentary ration. Animals were allowed to acclimate to the new experimental conditions for a week before the experiment was started. Fish were exposed to nominal concentrations of 0 µg/L OP (solvent control, ethanol 0.005 %), 30 µg/L OP, 150 µg/L OP and 300 µg/L OP. Three replicate tanks were established for each treatment group. Exposure to OP was for 60 days under semi-static conditions, water changes being made twice a week. Stock solutions were prepared once a week by dissolving OP in 100 % ethanol and stored in the darkness at 4°C. During each renovation of 80 % water, small aliquots of the stock solution were added to filtered tap water in order to obtain the desired concentrations. At the end of the 60 day treatment, aquaria contents were renewed for OP-free water, with water changes made twice a week for another 60 days. During the 60-day depuration period, male reproductive capability was assessed by registering successful spawnings, as evidenced by egg fertilization and hatching. In addition, one male of each tank was sacrificed at 7 day intervals (on days 0, 7, 14 and 21 of the depuration period), and their gonads were processed for histological analysis. The remaining fish, a pair per aquarium, were kept and evaluated for successful spawnings until the end of the experiment on day 120.

All experiments were conducted in accordance to international standards on animal welfare (Canadian Council on Animal Care 2005) and local institutional regulations.

For light microscopy (LM), pieces of testes were fixed in Bouin's liquid, embedded in paraffin, sectioned at 6 µm and stained with Hematoxylin-Eosin or Masson's trichrome. Photomicrographs were taken with a Nikon-Microphot FX microscope. For transmission electron microscopy (TEM), ultrathin sections were obtained according to Rey Vázquez et al. (2009) and examined and photographed with a Philips EM 301 microscope.

In order to evaluate the decrease of OP concentration in assay water, actual OP concentrations were measured from the 150 and 300 µg/L treatments. Water samples were taken every 24 h during the last week of the exposure period and treated by reverse phase HPLC coupled to fluorescence detection according to Rey Vázquez et al. (2009).

Results and Discussion

C. dimerus has an unrestricted lobular testis. On days 0, 7, 14 and 21 of the depuration period, a normal testicular architecture was observed in control males as well as in 30 µg/L OP-exposed males, in which OP treatment produced no apparent effects under microscopy evaluation (Fig. 1a–d). Testes maintained their structural integrity and accordingly, their functionality was evidenced by successful spawning events (Fig. 4). In testes of fish treated with the higher OP concentrations, structural and functional

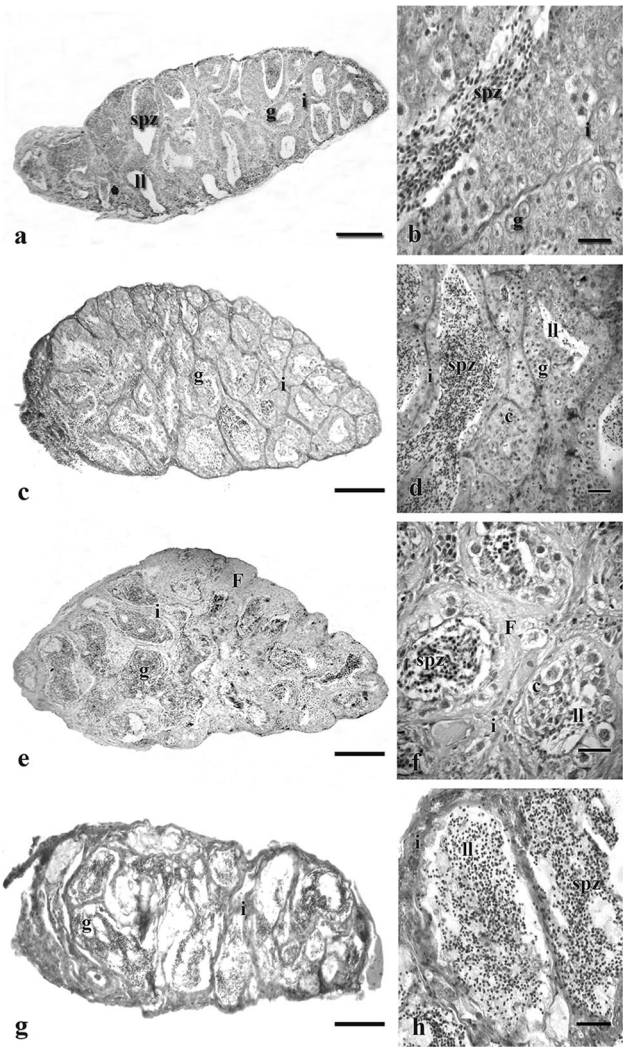

Fig. 1 Testis morphology in *C. dimerus* at the end of the OP treatment. LM. **a**, **b** Cross section and detail of the unrestricted lobular testis of a control male. **c**, **d** Testis of a 30 µg/L OP-exposed male showing a normal lobular architecture. **e**, **f** In testes of 150 µg/L OP-treated males, interstitial fibrosis became apparent. **g**, **h** Males from the 300 µg/L OP treatment exhibited a disarrangement of the lobular organization and an abnormal spermatogenesis with absence of cysts. *c* cyst, *F* interstitial fibrosis, *g* germinal compartment, *i* interstitial tissue, *ll* lobular lumen, SPZ sperm. *Scale bars* 100 µm (**a**, **c**, **e**, **g**); 20 µm (**b**, **d**, **f**, **h**)

alterations were observed. In 150 µg/L OP-exposed males, an increased fibrosis was evidenced within the interstitial tissue. Although testes preserved the lobular structure, the germinal epithelium (GE) looked disorganized (Fig. 1e, f). Testes of fish treated with 300 µg/L OP appeared as sacks of fibrous interstitial tissue filled with sperm; absence of cysts as well as a disarranged lobular structure were observed (Fig. 1g, h). Under TEM, these testes resembled those at the regression class of a normal reproductive cycle (Fig. 2a), typical of non-sexually active males, in which the lobular configuration is temporally altered and the discontinuous GE is composed mainly of Sertoli cells and dispersed spermatogonia that persist from the previous maturation class. Also, the presence of residual sperm and granulocytes is common (Rey Vázquez et al. 2012). However, testes of 300 µg/L OP-treated males showed differences with those corresponding to a normal regression class. The GE acquired a squamous appearance given that nuclei of Sertoli cells appeared strongly flattened and strikingly isolated; also few spermatogonia were found scattered throughout the remnants of the GE (Figs. 1g, h, 2b).

On the other hand, the abundance of sperm observed within the testes of fish exposed to 300 µg/L OP, seemed to be higher than the amount of residual sperm in testes at the regression class, probably due to the inability to release it. Interestingly, Rasmussen and Korsgaard (2004) reported that treatment with OP altered the seminal fluid quality and its biochemical composition in the eelpout, *Zoarces viviparus*. They argued that Sertoli cells were disrupted in its function and produced deficiency of seminal fluid because these cells are involved in the regulation of sperm release among other main functions.

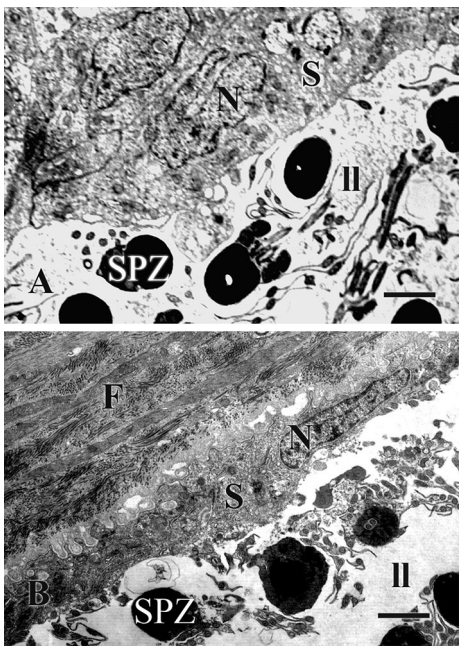

Fig. 2 Detail of the GE in control and OP-treated males. TEM. **a** Discontinuous GE in a control fish (regression class). **b** Remaining tissue of the GE and increased interstitial fibrosis in males exposed to 300 µg/L OP. *F* interstitial fibrosis, *ll* lobular lumen, *N* nucleus, *S* sertoli cell, *SPZ* sperm. *Scale bar* 1 µm

The impairment of gonadal morphology produced by OP exposure might be mediated by effects on estrogen receptors (ERs). Rasmussen et al. (2005) demonstrated that an antiestrogen such as ZM 189.154 can abolish some of the alterations induced by OP in the testes of *Z. viviparus*, providing evidence that some of these effects may be mediated by ERs. Alternatively, these pathological effects may be due to an impaired expression of ERs, which was reported in *C. dimerus* under OP exposure (Genovese et al. 2014).

In the control and 30 µg/L OP treatments, the reproductive activity was the expected according to the season when the experiment was performed and fish performed regular spawnings both during the exposure and depuration periods (Fig. 4). In contrast, no spawnings of the females were recorded during the exposure period in the 150 and 300 µg/L OP treatments (Fig. 4). In our previous study, this had already been observed, even when histology of ovaries of OP-treated females did not differ from that of control females (Rey Vázquez et al. 2009). Then, the lack of spawnings might be explained by chemical exposure affecting courtship activities and other reproductive behaviors typical of cichlid fishes. However, pair formation and successful reproduction were observed following transfer to OP-free water (Fig. 4).

Histological examination of the testes of fish transferred to clean water after being treated with the higher OP concentrations showed a remarkable progressive recovery of the organ architecture (Fig. 3). Animals exposed to 150 µg/L OP showed a gradual decrease in testicular fibrosis from the seventh day post-treatment until full recovery, which was observed in samples taken by day 14 (Fig. 3, left column).

In males treated with 300 µg/L OP, the recovery of the unrestricted lobular organization was observed on day 14 after transfer to OP-free water. The GE progressively retrieved its original organization by proliferation from the scarce Sertoli cells and spermatogonia remaining after OP exposure. Testis architecture was completely recovered by day 21 (Fig. 3, right column). Although the histological damage was higher in 300 µg/L than in 150 µg/L OP-exposed males, and consequently, a longer time until full recovery was registered in the former group, males of both groups exhibited a normal looking testis after 3 weeks in clean water (Fig. 3). For both OP concentrations, the restoration of testicular functionality was evidenced on day 28 of the depuration period, when the first fertilized spawnings and hatched eggs were recorded. From this day,

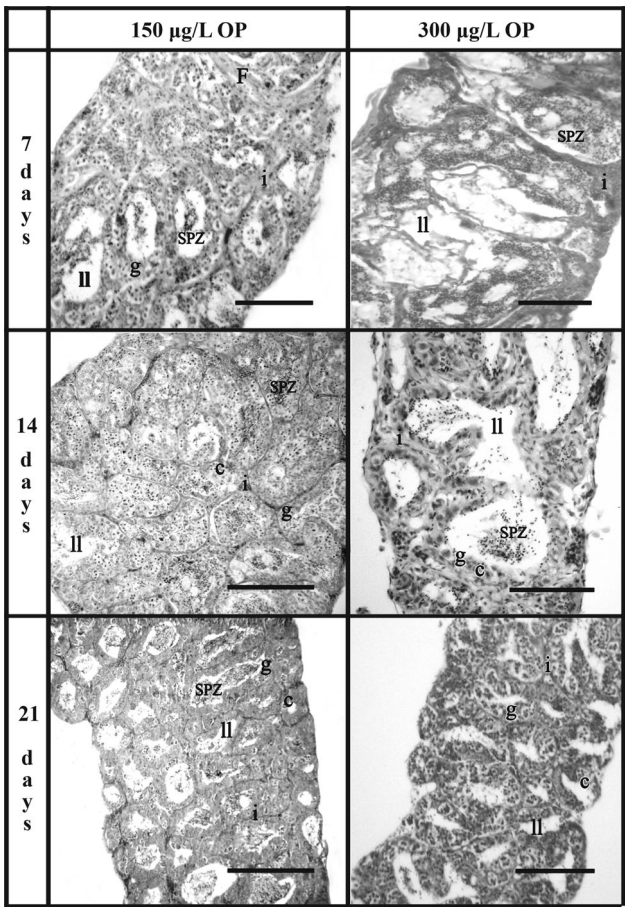

Fig. 3 Sections of testes of *C. dimerus* exposed to 150 µg/L OP (*left*) and 300 µg/L OP (*right*) showing histological recovery after 7 (*above*), 14 (*middle*) and 21 (*below*) days following transfer to clean water. *c* cyst, *F* interstitial fibrosis, *g* germinal compartment, *i* interstitial tissue, *ll* lobular lumen, *SPZ* sperm. *Scale bar* 100 µm

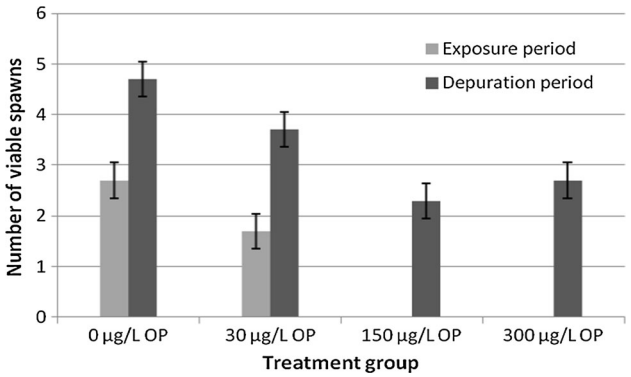

Fig. 4 Reproductive capacity of male *C. dimerus* during exposure to sublethal concentrations of OP and during the subsequent depuration period. The number of successful spawning events, as evidenced by egg fertilization and hatching was recorded for each treatment. Data are expressed as means ± SE. Viable spawns in the 150 and 300 µg/L OP treatments were recorded from day 28 after transfer to clean water

pairs from the treatments with high OP concentrations resumed the expected reproductive rate and start producing viable F1 embryos, similarly to control and 30 µg/L OP-exposed fish (Fig. 4). Previous spawnings had been recorded after a week in OP-free water, however males failed to fertilize the eggs and those unfertilized spawnings were lost. Then, the impairment of testis functionality during the first weeks of the depuration period could be linked to the alterations of gonadal morphology, evidenced until day 14 or 21, depending on OP concentration.

The analysis of reversibility of the effects of xenobiotic exposure at the reproductive level is of particular interest for risk assessment at the population level. While in some fish species a depuration period in clean water allows reversion of sexual disruption, in others the reproductive impairment persists. Secondary sex characteristics of *Oryzias latipes* reverted from female to male when fish were returned to clean water after being continuously exposed to OP or 4-nonylphenol (NP) from fertilized eggs to 60 days post-hatch. However, gonadal histology showed that intersex gonads still existed, even after the fish were transferred to clean water for 2 months (Seki et al. 2009). On the other hand, Liney et al. (2005) reported that the disruption in the development of the reproductive ducts of *Rutilus rutilus* exposed during early life stages to effluents from waste water treatment plants containing alkylphenolic chemicals were not reverted after exposure cessation. Jespersen et al. (2010) showed that OP exert estrogenic effects during early development of *Z. viviparus* embryos, and that depuration has a positive effect on the motherfish and her embryos, although they were inconclusive for the changes observed in the development of male gonads.

Our results show that both morphological and functional impairment induced in male *C. dimerus* by OP treatments are reversible upon exposure interruption and fish recover their fertilizing capacity, yielding viable F1 embryos, after 28 days in OP-free water. These results are consistent with those of Genovese et al. (2012) who reported that for biochemical and molecular biomarkers, such as induction of female proteins in male fish exposed to OP, complete recovery is achieved after a 28-days depuration period.

The drop of actual chemical concentrations in exposure chambers tends to be especially noticeable in static renewal systems as the one employed in our study. Various factors, including glass adsorption, uptake by fish, microbial degradation and photodegradation can explain this tendency (Ekelund et al. 1993; Ahel et al. 1994; Lewis and Lech 1996). Our results show that the initial nominal and actual OP levels were in good agreement, while measured levels of OP declined to 20 %–25 % of the initial concentrations over the 72 h water renewal interval (Table 1). Similar results were obtained by Balch and Metcalfe (2006) who recorded a comparable drop of the actual

Table 1 Actual concentrations of OP in water of the experimental tanks

Nominal OP concentration	150 μg/L OP		300 μg/L OP	
Time (h)	Actual concentration (μg/L)	%	Actual concentration (μg/L)	%
0	152.0 ± 0.7	101.3 ± 0.5	298.2 ± 3.8	99.4 ± 1.3
24	60.3 ± 1.6	40.2 ± 1.1	191.7 ± 4.1	63.9 ± 1.4
48	51.5 ± 0.9	34.3 ± 0.6	114.9 ± 3.1	38.3 ± 1.0
72	32.3 ± 1.2	21.5 ± 0.8	73.7 ± 2.8	24.6 ± 0.9

Measurements were made 0, 24, 48 and 72 h after addition of OP. Values are means ± SDs of the concentrations recorded in two samples. % are values for the measured concentrations expressed as percentage of the nominal concentration. OP was not detected in samples from the control treatment

concentration of NP in the same time period. Therefore, it can be argued that the effects registered in this study are being produced by exposure to concentrations considerably lower than the nominal values, according the characteristic of OP in the assay water. Semi-static experiments as the one used in our study emulate what happens when environmental contamination is caused by alkylphenolic compounds arising mainly as a result of discharges from point sources to surface waters.

As two of the main breakdown products of alkylphenol ethoxylates, both NP and OP are ubiquitous in the aquatic environment. NP is the preponderant AP, constituting 80 % of APs found in surface waters and sediments (Ying et al. 2002). In comparison, OP accounts for about 15 % of the commercial AP input (Bennett and Metcalfe 1998). Both APs are estrogenic and exert their effects through a common mechanism involving estrogen receptors (White et al. 1994; Céspedes et al. 2004). The OP concentrations that induced gonadal alterations in *C. dimerus* are higher than the levels of alkylphenolic compounds reported in most aquatic systems. Generally, environmental levels are below the estimated threshold for induction of histological damage in fish. Concentrations of APs rarely exceed 20 μg/L (Blackburn and Waldock 1995; Bennie 1999), although in rivers receiving significant amounts of industrial and/or domestic effluents, point source contaminant levels may reach over 600 μg/L (Ying et al. 2002). According to the results of our study, it could be considered that the adverse effects induced by pulse-exposure to OP over a limited time would be temporary and do not predict an impact at the population level at least in terms of reproductive detriment. However, it should be noticed that in the natural environment, exposure to different xenoestrogens may result in additive effects (Jobling and Sumpter 1993). Then, extrapolation of these results to the field must be undertaken carefully considering the concentrations present in the environment and in the context of investigations that also contemplate duration of exposure, bioaccumulation and the presence of other chemicals.

In summary, exposure to relatively high concentrations of OP produced histopathological changes in the testes of *C. dimerus*, generating infertile males. These effects proved to be reversible after transfer to clean water. Taking into account the concentrations of APs usually recorded in the aquatic environment, these xenobiotics would not represent a major threat for adult fish of this species, at least under the tested conditions. However, further assessment of the effects of more prolonged exposure to low, environmentally relevant levels of APs would be necessary.

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