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Estrogens counteract the masculinizing effect of tributyltin in zebrafish

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Abstract

Recently, it has been demonstrated that the biocide tributyltin (TBT) can interfere with fish sex differentiation, leading to a bias of sex toward males. On the contrary, it is well known that estrogenic compounds can induce fish feminization. Yet, the combined effects of mixtures of androgenic and estrogenic compounds on fish sex differentiation have never been investigated before, even though in the environment animals are frequently exposed to both groups of xenobiotics. Therefore, in order to investigate whether exposure to estrogenic compounds can block the masculinizing effect of TBT, 5 days post-fertilization zebrafish (*Danio rerio*) larvae were exposed for a four month period to TBT and to the synthetic estrogen–ethinylestradiol (EE2). The fish were fed a diet containing TBT at nominal concentrations of 25 and 100 ng TBT/g, and two groups of animals were also dosed with TBT plus EE2 at nominal water concentration of 3.5 ng/L, using a flow-through design. As expected, fish exposed to TBT showed a bias of sex toward males (62.5% males in control tanks and 86% and 82% in TBT 25 and TBT 100 ng TBT/g, respectively). Co-exposure to EE2 completely blocked the masculinizing effect of TBT, with 7% males in the TBT 25 ng/g+EE2 treatment and 0% in the EE2 alone and in the TBT 100 ng/g+EE2 exposed groups. These results clearly indicate that EE2, at environmentally relevant concentrations, can block the TBT masculinizing effects in zebrafish, which suggests that in the aquatic environment the presence of estrogens may neutralize the fish masculinizing effect of TBT. Our findings highlight the need of testing the combined effects of contaminants, as single exposure studies may not be sufficient to predict the effects of mixtures of xenobiotics with antagonistic properties.

Keywords: Tributyltin; Xenoandrogen; Ethinylestradiol; Xenoestrogen; Mixture; Masculinization; Sex ratio; Endocrine disruption; Fish

1. Introduction

Recently, concerns have increased about chemicals in the environment which alter the normal endocrine function of animals and Humans, commonly termed endocrine disruptors (EDCs). The majority of the studies on EDCs in vertebrates have focused on the effects of estrogenic chemicals (EC) (natural steroid 17-β-estradiol (E2); alkylphenolic compounds; the synthetic estrogen ethinylestradiol (EE2), etc.) because many of the observed effects (reduce testicular development and fertility, increase production of vitellogenin, the presence of male fish feminization) are believed to result from disruption of this axis (Jobling et al., 2003; Kinnberg et al., 2003; Andersen et al., 2003; Solé et al., 2003; Tollefsen et al., 2004; Rasmussen and Korsgaard, 2004; Angus et al., 2005). However, the best documented example of endocrine disruption in wildlife is the masculinization of female neogastropods (imposex) by the antifouling compound tributyltin (TBT) (Matthiessen and Gibbs, 1998; Santos et al., 2005). TBT is a ubiquitous persistent xenobiotic that can be found in freshwater, estuarine and costal ecosystems (Fent, 1996). Although its use has been recently restricted to all ship sizes (Anon., 2001), the levels of TBT in the aquatic environment are still a cause of great concern (Santos et al., 2002).

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In contrast to the extensive literature dealing with the negative impact of TBT in gastropods, only two articles have addressed the effects of TBT in fish sex differentiation (Shimasaki et al., 2003; McAllister and Kime, 2003). Both studies show that TBT can alter the sex ratio towards males at extremely low levels. This may indicate that many natural fish population may be negatively impacted by this organotin compound.

Although estrogenic and androgenic compounds exist in combination in the aquatic environment, there are no available studies focusing on the combined effects of EDCs mixtures with antagonistic effects on fish sex differentiation. Clearly, studying the effects of single EDCs may not be sufficient to understand what is really occurring in the environment, which points to the need for a more robust approach which integrates the fact that animals are simultaneously exposed to both groups of xenobiotics.

Therefore, the aim of this study was to investigate if an environmentally relevant concentration of a model xenoestrogen (EE2) could neutralise the masculinizing effect of TBT. To this end, we have exposed 5 days post-fertilization zebrafish (*Danio rerio*) larvae to environmentally low doses of TBT (25 and 100 ng TBT/g diet) for a period of four months (which includes the sex differentiation period), and concomitantly tested whether co-exposure to an environmentally relevant concentration (3.5 ng/L) of the synthetic estrogen—EE2—could neutralize the masculinising effect of the androgenic compound. The selection of zebrafish as the model species was based on the fact that it reaches sexual maturity in a short period of time (within 3–4 months) and also because TBT had previously been shown to induce masculinization of zebrafish at extremely low levels (nominal concentrations between 0.1–1 ng/L) in a life-cycle test (McAllister and Kime, 2003).

2. Material and methods

2.1. Chemicals

Ethinylestradiol (EE2) (98% purity) was obtained from Sigma and tributyltin chloride (TBTCl) (96% purity) was obtained from Aldrich. All other chemicals were obtained from Sigma.

2.2. Experimental animals

Adult zebrafish (*D. rerio*) kept in our laboratory for two generations were used as breeding stocks. Fish were initially kept at 27 °C with a 12 h light : 12 h dark photoperiod. Aeration and filtration were provided using sponge filters, and ammonia maintained below detection limit. Two days prior to breeding, fish were fed *Artemia* nauplii and a casein based diet 3 times a day (Carvalho et al., 2004). The day before breeding, four males and four females were housed in a 5 L breeding chambers. At 1 h past the start of the light phase the following day, viable eggs were collected and allowed to hatch in running water. At 5 days post-fertilization (pf), larvae started being fed with a casein based diet (Carvalho et al., 2004). On day 16 pf, a 3 times a week *Artemia* nauplii supplement was introduced (prepared in artificial sea water); starting on day 60 pf, the *Artemia* nauplii supplement was given on a daily base, together with the casein diet.

2.3. Exposure studies

Larvae (5 days pf) were assigned to 5 L aquaria provided with dechlorinated tap water (carbon activated filtration) at a flow rate of 50 L/ day, using a flow-through design. An initial density of 150 larvae per aquarium was used. At day 21, larvae were transferred to 30 L aquaria and at day 50 the density was reduced to 90 animals; the density was further reduced to 70 animals at day 65. Water was maintained at a temperature of 27 °C, and photoperiod at 14 h light : 10 h dark. The pH (7.9±0.5), the dissolved oxygen (>6 mg O_2/L) and ammonium (<0.5 mg NH_4/L) were weekly checked. Waste feed and faeces were daily siphoned from the aquaria. Larvae were exposed to TBT incorporated in the diet at nominal concentrations of 25 and 100 ng TBTCl/g wet mass. The selection of TBT doses was based on two previous studies which reported TBT masculinization of zebrafish and genetically female Japanese flounder at 1 ng TBT/L and 100 ng TBT/g wet mass in the diet, respectively (McAllister and Kime, 2003; Shimasaki et al., 2003). These levels of TBT incorporated in zebrafish diet are environmentally relevant, as they are found in animals from low to moderate TBT-contaminated areas (Albalat et al., 2002). EE2 was dissolved in DMSO and delivered to a mixing chamber by a peristaltic pump (ISMATEC IP-N 16) where it was further diluted in dechlorinated tap water to a final nominal aquarium concentration of 3.5 ng/L. Two treatments with the mixture of both compounds (TBT 25 ng/g+EE2 3.5 ng/L; TBT 100 ng/g+EE2 3.5 ng/L) were also performed, together with solvent control. The solvent (DMSO) concentration in the aquaria did not exceed 20 ng/L. Each treatment was run in duplicate for a four month period. At the end of the experiment, 30 animals per replicate (60 per treatment) were sacrificed, and the sex of animals annotated, after inspection of the gonads under a stereo microscope; animals were classified as males when a white gonad was present, as females when it was possible to observe the presence of oocytes and as undeveloped gonads when a hyaline tissue was present but no oocytes could be detected under the stereo microscope. Animals were then preserved for the study of other parameters. At the end of the experiment, one replicate only (n=30) could be used to determine sex ratio in the TBT 100 treatment. As a routine analysis to confirm male sex identification, a simple methodological procedure was use to observe the presence of sperm in the gonads of those animals displaying white gonad (classified as males): after sacrificing the animal, one of the testis was collected and mixed in 0.5 mL of an extender solution (NaCl 5.8 g/L, KCl 0.2 g/L, $CaCl_2$ 0.22 g/L, $MgCl_2{\cdot}6H_2O$ 0.04 g/L, $NaHCO_3$ 2.10 g/L, $NaH_2PO_4{\cdot}2H_2O$ 0.04 g/L, glycine 3.75 g/L in distilled water, pH 8.6) and 5 μL of 1% of Rose Bengal dye