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Effects of short-term exposure to the model anti-androgen, flutamide on reproductive function based endpoints in female Murray rainbowfish (*Melanotaenia fluviatilis*)

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ABSTRACT

The aim of the present study was to evaluate the responses of female Murray rainbowfish (*Melanotaenia fluviatilis*) to the model anti-androgen, flutamide in a short-term exposure. Adult female Murray rainbowfish were exposed to nominal (measured) concentrations of 125 (104), 250 (163), 500 (378) and 1000 (769) μg/L of flutamide for seven days in a semi-static set-up. Plasma vitellogenin (VTG), 11-keto testosterone (11-KT) and 17β-estradiol (E2) concentrations, brain aromatase activity and ovarian histology were assessed following the exposure. No treatment-related mortality was found in rainbowfish and there was no effect of flutamide on the developmental stage of the ovaries. Histological investigation revealed absence of mature oocytes in flutamide-treated fish. In addition, a significant reduction in the sizes of the vitellogenic oocytes was found after treatment with 500 and 1000 μg/L flutamide. The circulating levels of VTG and the activity of aromatase in the brain were also significantly reduced in fish treated with 500 and 1000 μg/L flutamide. Treatment with higher concentrations of flutamide reduced the levels of 11-KT and E2 in plasma. The results from this study demonstrate that a short-term exposure to the model anti-androgen, flutamide can adversely affect the reproductive function based on end-points such as plasma VTG, 11-KT and E2; brain aromatase activity and sizes of the oocytes in female Murray rainbowfish. Further, a positive correlation between these experimental variables suggests hormonal imbalance.

1. Introduction

During the past two decades, a large body of research has focused on the potential deleterious effects on the reproductive physiology and behaviour of aquatic animals of the release of pharmaceuticals in the freshwater environment. Anti-androgens (androgen receptor [AR] antagonists) are an emerging class of endocrine disrupting chemicals (EDCs) that can cause phenotypic feminisation similar to environmental estrogens in male fish by blocking or down-regulating the expression of ARs (Sohoni and Sumpter, 1998). Since the discovery of reproductive and developmental abnormalities in wild roach (*Rutilus rutilus*) from the presence of minute amounts of estrogenic EDCs in the discharges of sewage treatment plants (STPs) (Jobling et al., 1998), most research in aquatic ecotoxicology has focused on hormone mimics acting through the estrogen receptors (ERs). However, the role of AR antagonists in causing endocrine disruption in fish is now being speculated. In addition, it has now been established that some natural or anthropogenic chemicals recognised to be estrogenic, can act through an anti-androgenic mode-of-action (MoA) (Sohoni and Sumpter, 1998).

The mammalian anti-androgens, phthalates have been detected in the rivers of South Africa at concentrations ranging between 10 and 80 mg/L (Fatoki et al., 2010; Ogunfowokan et al., 2006). Anti-androgenic activity of 935 μg flutamide equivalent concentration (FEQ)/L has been detected in the surface water in China (Zhao et al., 2011). A recent study reported that more than 1800 mg FEQ/L anti-androgens flow into the graywater every day in Beijing (China) (Ma et al., 2013). *In vitro* anti-androgenic activity (370–4223 μg FEQ/L) has also been determined in the freshwater in Italy (Urbatzka et al., 2007). A 21-day exposure to STP effluent containing 328.56 μg FEQ/L of anti-androgenic and 3.32 ng E2/L estrogenic activities adversely affected the reproductive behaviour of male

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stickleback (*Gasterosteus aculeatus*) (Sebire et al., 2011). However, these reproductive effects are likely due to the presence of estrogens and anti-androgens together in the effluents.

In utero exposures to anti-androgens such as phthalates, vinclozolin, procymidone, iprodione, chlozolinate, ketoconazole, linuron, fenitrothion and dichlorodiphenyldichloroethylene (*p,p′*–DDE) during sensitive stages of sexual development have been reported to cause malformations of the reproductive tract in male mammals (Gray et al., 1999a, 1999b). Some studies have reported reduction in sperm count and decrease in the size of the testes in guppy (*Poecilia reticulata*) after exposures to anti-androgens like flutamide, vinclozolin and *p,p′*–DDE (Baatrup and Junge, 2001; Bayley et al., 2002). Recent studies demonstrated a reduction in aggressiveness and induction of nurturing behaviour in male bluegill sunfish (*Lepomis macrochirus*) and the monogamous cichlid, (*Amatitlania nigrofasciata*) exposed to flutamide (Rodgers et al., 2013; van Breukelen, 2013). Research in aquatic toxicology using AR antagonists has lately been limited to the male fish. However, the female fish thrive in the same freshwater and are equally prone to endocrine disruption by the anti-androgens. In one study, a significant reduction in the gonadosomatic index (GSI) was found in sexually mature female fathead minnow (*Pimephales promelas*) exposed to 700 μg/L of vinclozolin (Makynen et al., 2000). However, the evidence for vinclozolin to act as an anti-androgen in fish is weak since it failed to competitively bind to the testosterone (T) sites in the brain and ovary of fathead minnow (Makynen et al., 2000). The anti-androgenic nature of phthalates is also uncertain in fish because they have been reported to displace 17β-estradiol (E2) from the ERs in rainbow trout (*Oncorhynchus mykiss*) (Matthews et al., 2000), thereby, suggesting an *in vivo* estrogenic MoA. The detection of anti-androgenic activity in water worldwide (Jobling et al., 2009; Jolly et al., 2009; Ma et al., 2013; Urbatzka et al., 2007) and foreseeing the importance of androgens in the process of oocyte maturation in fish such as yellow croaker (*Larimichthys crocea*), European sea bass (*Dicentrarchus labrax*), Japanese eel (*Anguilla japonica*) and Atlantic cod (*Gadus morhua*) (Endo et al., 2011; Garcia-Lopez et al., 2011; Kortner et al., 2009; Pu et al., 2013), have necessitated the evaluation and characterisation of the effects of a reference anti-androgen on the reproductive function based end-points in female fish.

One of the important classes of anti-androgens is synthetic, non-steroidal drugs such as flutamide, enzalutamide, bicalutamide, cyanolutamide and nilutamide that were designed to competitively bind to ARs *in vivo*. Flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide) was one of the first cancer drugs synthesised and is used extensively to treat prostate cancer in men and polycystic ovarian syndrome in women. Treatment with flutamide has been reported to cause impairment in sperm and Leydig cell development and malformation of the penis in mammals (Kotula-Balak et al., 2012; Lydka et al., 2012; Simon et al., 2012). Flutamide has no environmental relevance but based on mammalian studies it is regarded as a model chemical to elucidate the effects and MoAs of anti-androgens. Flutamide and its metabolite, 2-hydroxyflutamide were reported to competitively bind to ARs in fathead minnow (Ankley et al., 2004; Makynen et al., 2000).

Administration of flutamide has been reported to cause reduction in the proportion of sperms and spiggin levels in male fish (Baatrup and Junge, 2001; Kinnberg and Toft, 2003; Sebire et al., 2008). In female fish, exposure to flutamide can cause oocyte atresia and induction in the levels of vitellogenin (VTG) (Chikae et al., 2004; Jensen et al., 2004). Most of the ecotoxicological research using flutamide has been conducted on fathead minnow, medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), guppy, catfish (*Clarias batrachus*) or carp (*Cyprinus carpio*) (Andersen et al., 2003; Baatrup and Junge, 2001; Bottero et al., 2005; Jensen et al., 2004; Leon et al., 2007; Rajakumar et al., 2012) (Supplementary Table 1), which are not native and hence, not accustomed to Australian freshwater environment.

Murray rainbowfish (*Melanotaenia fluviatilis*) (family: Melanotaeniidae) is a native Australian freshwater fish species residing in Murray-Darling river basin. The fish spawn throughout the year in laboratory conditions. The larvae (3–5 mm at the time of hatching) grow into sexually dimorphic adults in 6–8 months (Crowley et al., 1986). Murray rainbowfish has earlier been used as a model test species to develop and validate biomarkers for estrogenic exposure in the Australian environment (Bhatia et al., 2013; Woods and Kumar, 2011). We proposed to test if a short-term exposure to biologically active concentrations of flutamide would affect the reproductive function based on end-points such as circulating levels of VTG and sex steroid hormones – E2 and 11-keto testosterone (11-KT); and brain aromatase activity in female rainbowfish. Based on the concentrations of flutamide used in overseas *in vivo* fish studies (Kang et al., 2006; Martinovic-Weigelt et al., 2011) and the values of the anti-androgenic activity detected in freshwater by *in vitro* screens (Ma et al., 2013; Urbatzka et al., 2007; Zhao et al., 2011), we used the concentrations of 125–1000 μg/L. These concentrations were not lethal to cause mortality but were biologically active to cause reproductive changes.

Vitellogenin is a phosphoprotein produced in the liver and is incorporated into the maturing oocytes of female oviparous animals. Vitellogenin has been used as the biomarker for estrogenic exposure in male fish (Bhatia et al., 2014a; Woods and Kumar, 2011). However, changes in the VTG levels can occur in female fish after exposure to EDCs which also affect the activity of aromatase, the key enzyme catalysing the conversion of testosterone to E2 (Ankley et al., 2005, 2002). In female fish, 11-KT is crucial for the growth of oocytes (Matsubara et al., 2003). We hypothesised that exposure to flutamide could result in endocrinological changes and that these changes would be reflected in the ovarian histopathology and brain aromatase activity in Murray rainbowfish. Histopathological changes in the gonads can predict the reproductive fitness of the animal and could be extrapolated to population level effects (Leino et al., 2005).

2. Materials and methods

2.1. Test species

Sexually mature adult female Murray rainbowfish (10-months old) were acquired from Aquarium Industries, Victoria, Australia. Fish were maintained in 200 L glass tanks (loading 1 g/L) containing artificial freshwater (7.5 g MgSO4, 9.6 g NaHCO3, 1.5 g CaCl2, 40 g NaCl, 3 g KCl and 6 g CaSO4 in 100 L Milli-Q water at pH 6.8–7.5; conductivity 1200–1500 μS/cm; dissolved oxygen $>$60 percent) and fed four percent (w/w) adult brine shrimp once daily and acclimatised for 14 days to the laboratory environment at 21 $\pm$ 0.1 °C under 16:8 light:dark photoperiod with a gradual sunrise/sunset and 60 min transition period.

2.2. Test chemical

Flutamide (4′-nitro-3′-trifluoromethylisobutyranilide) (CAS number 13311-84-7) was purchased from Sigma Aldrich, Australia. Stock solution (100 g/L) was prepared in methanol (99.9 percent purity) and stored at −20 °C. The nominal concentrations of exposure were 125, 250, 500 and 1000 μg/L flutamide. The concentration of methanol was consistent across all treatment tanks. Water and solvent controls (0.001 percent) were also included.

2.3. Test conditions

Exposures of female Murray rainbowfish to flutamide were conducted in glass tanks (35 cm × 18 cm × 24 cm) containing 5 L water (fish loading 1 g/L) according to the method of (Bhatia et al., 2013). Two replicate tanks containing four fish in each were used. Fish (eight per treatment) were exposed to nominal concentrations of flutamide for seven days. The temperature in the