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A study of temporal effects of the model anti-androgen flutamide on components of the hypothalamic-pituitary-gonadal axis in adult fathead minnows

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ABSTRACT

The aim of this study was to investigate temporal changes in the hypothalamic-pituitary-gonadal (HPG) axis of fathead minnows (*Pimephales promelas*) treated with the model androgen receptor (AR) antagonist flutamide. Reproductively-mature fish were exposed in a flow-through test to analytically-confirmed concentrations of either 50 or 500 μg flutamide/L for 8 d, followed by an 8-d recovery period in clean water. Fish were sampled at 1, 2, 4 and 8 days during each phase of the experiment. Flutamide (500 μg/L) caused significant reductions in relative gonad size of the females on day 8 of the exposure and day 1 of the recovery, and reduced expression of secondary sex characteristics in males during the exposure phase of the experiment. *Ex vivo* gonadal synthesis of testosterone in both sexes (and 17β-estradiol in females) was reduced in the 500 μg/L treatment within 2 d of exposure; however, steroid synthesis returned to levels comparable to controls by the end of the exposure portion of the test. *Ex vivo* testosterone synthesis in males exposed to 50 μg flutamide/L was greater than in controls on days 4 and 8 of the exposure. Both the enhanced steroid production in the low treatment males, and return to control levels in the high treatment males and females during chemical exposure are indicative of a compensatory HPG response. One contributor to this response could be increased expression of genes responsible for enzymes involved in steroid synthesis; for example, transcripts for both cytochrome P450 side- chain cleavage and 11β-hydroxysteroid dehydrogenase were significantly elevated in flutamide-exposed males. Overall, responses of the HPG axis in adult male and female fathead minnows exposed to flutamide were both dynamic and comparatively rapid during exposure and recovery. These observations have ramifications both for the development of short-term fish assays to detect endocrine-active chemicals, and the derivation of robust adverse outcome pathways for AR antagonists in fish.

1. Introduction

Regulatory bodies throughout the world are developing and implementing approaches to identify hazards and risks of endocrine-active chemicals (EACs) to humans and the environment (OECD, 2010a). In the US, the Environmental Protection Agency (USEPA) is responsible for an endocrine disruptor screening program (EDSP) which aims to evaluate on the order of 10,000 chemicals for possible effects on function of the vertebrate hypothalamic-pituitary-gonadal and thyroidal (HPG/T) axes (http://www.epa.gov/endo/). To support this effort, a variety of *in vitro* and *in vivo* assays have been identified and/or developed to prioritize, screen, and test chemicals for their endocrine-disrupting

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properties. An important test protocol for the EDSP is the fish short-term reproduction assay (FSTRA), which examines a diverse range of endpoints, including plasma sex steroid and vitellogenin (VTG; egg yolk protein precursor) concentrations, gonad histology, secondary sex characteristics, fecundity and fertility in 21-d chemical exposures with the fathead minnow (*Pimephales promelas*). The FSTRA is effective in identifying HPG axis perturbation caused by agonists and antagonists of the estrogen and androgen receptor (ER, AR) and inhibitors of sex steroid synthesis (Ankley and Jensen, 2014). In addition to use by the EDSP, the FSTRA has been standardized and validated for international screening/testing programs for EACs (OECD, 2010b).

Given the large number of chemicals that need to be assessed through the EDSP, USEPA scientists associated with the program have investigated different approaches that could streamline the identification of EACs. For example, Ankley et al. (2009) describe an effort, based on the adverse outcome pathway (AOP) framework (Ankley et al., 2010), that employed a combination of the FSTRA and shorter-term, time-course assays to identify a range of mechanistic endpoints for rapidly identifying perturbation of HPG function in the context of possible apical effects. One goal of this work was the development of a fathead minnow EAC screening assay that would be shorter and more focused than the FSTRA. Model EACs assessed in that effort affect several relevant HPG pathways, including: inhibition of different enzymes involved in sex steroid synthesis (fadrozole, prochloraz, trilostane, ketoconazole); activation of the ER (17α-ethinylestradiol); activation of the AR (17β-trenbolone); and, antagonism of the AR (flutamide, vinclozolin). The FSTRA and time-course data for the steroid synthesis inhibitors and the two receptor agonists have been previously described in the open literature (see Ankley and Villeneuve, 2015; and references therein), while the flutamide time-course data for the overall effort is described in the current paper. The various time-course studies have contributed significantly to the development of a number of AOPs for the reproductive effects of different types of EACs in fish (https://aopwiki.org/wiki/index.php/Main_Page; AOP 23, AOP 25, AOP 30). In addition, a meta-analysis of the entire dataset has provided important insights as to the consequences of compensatory HPG responses and system recovery relative to screening and testing of EACs (Ankley and Villeneuve, 2015).

Flutamide is a drug used to treat prostate cancer (Brogden and Clissold, 1989; Goldspiel and Kohler, 1990) which has limited direct environmental relevance, but nonetheless is a very useful "probe" chemical from an experimental perspective (Ankley et al., 2009). There have been a number of previous studies with fish in which flutamide has been used as a model compound to examine antagonism of the AR (e.g., Bayley et al., 2002; Ankley et al., 2004; Katsiadaki et al., 2006; Rajakumar et al., 2012; Nakamura et al., 2014; Schiller et al., 2014; Bhatia and Kumar 2016). However, none of these studies examined temporal patterns of the effects of flutamide, and few considered interactions among endpoints at multiple biological levels of organization. The 16-d study described herein employed adult male and female fathead minnows exposed to two concentrations of the AR antagonist with four sampling points (1, 2, 4 and 8 d) during 8-d exposure and recovery phases. A variety of endpoints were evaluated, including gonadal expression of several genes known to be related to HPG function, *ex vivo* testicular and ovarian synthesis of testosterone (T) and 17β-estradiol (E2), plasma concentrations of T, E2 and VTG and apical responses reflective of reproductive status (secondary sex characteristics, relative gonad size). In-depth analysis of temporal effects of flutamide on multiple components of signaling and function of the HPG axis could provide mechanistic insights as to the toxicity of AR antagonists in fish, as well as assist in the selection of suitable endpoints and exposure durations for more efficient testing or monitoring of EACs.

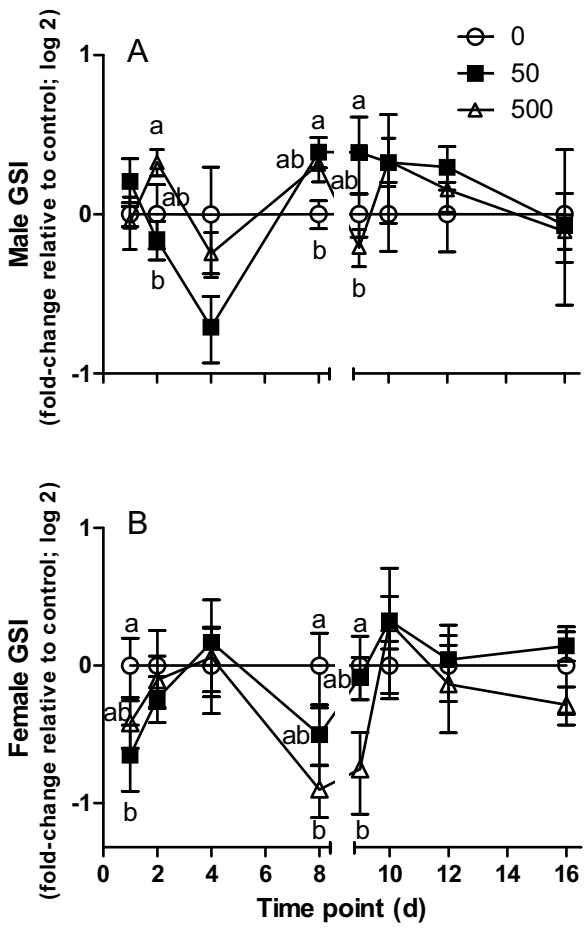

Fig. 1. Effects of an 8/8 d flutamide exposure/recovery on the gonadosomatic index (GSI) in male (A) and female (B) fathead minnows. Data points represent the mean ± standard error (n = 4–12, typically 8) of the fold-change relative to controls from the same time point, expressed as log 2 transformed units. Different letters indicate statistically significant differences among treatments within a given day.

2. Materials and methods

2.1. Experimental design

Flutamide (99% purity) was purchased from Sigma (St. Louis, MO). Solvent-free stock solutions were prepared as described previously (Jensen et al., 2004). The stock was diluted with filtered (control) Lake Superior water to achieve target test concentrations of flutamide. Exposures were conducted in 20 L glass aquaria containing 10 L of Lake Superior control water or flutamide solution (50 or 500 μg/L, nominal) delivered at a flow-rate of approximately 45 mL/min. Test concentrations were chosen based on observed reproductive effect concentrations in a FSTRA (Jensen et al., 2004). Specifically, 500 μg flutamide/L would be expected to reduce egg production in a 21-d test, while 50 μg/L would not.

The basic experimental design for this study consisted of 16 replicate tanks per treatment (48 tanks in total), each containing four sexually mature male and female fathead minnows from an on-site culture unit. The fish ranged from 5 to 7 months in age, and their average (standard deviation, n) size was 1.2 (0.32, 190) and 2.52 (0.71, 192) g for females and males, respectively. All of the fish from two of the replicate tanks were sampled at 1, 2, 4, or 8 days after initiation of the exposure (n = 8 for each sex per treatment at each time point, except the 500 μg/L treatment on day 4, when n = 12). After the 8 day sampling, flutamide delivery to the system was stopped and remaining fish were held in a constant flow of