Aquatic Toxicology 140–141 (2013) 145–156

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Anti-androgens act jointly in suppressing spiggin concentrations in androgen-primed female three-spined sticklebacks – Prediction of combined effects by concentration addition

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ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form 28 May 2013
Accepted 30 May 2013

Keywords:
Anti-androgen
Gasterosteus aculeatus
Mixture effects
Concentration addition
Pesticides
Endocrine disruption

ABSTRACT

Increasing attention is being directed at the role played by anti-androgenic chemicals in endocrine disruption of wildlife within the aquatic environment. The co-occurrence of multiple contaminants with anti-androgenic activity highlights a need for the predictive assessment of combined effects, but information about anti-androgen mixture effects on wildlife is lacking. This study evaluated the suitability of the androgenised female stickleback screen (AFSS), in which inhibition of androgen-induced spiggin production provides a quantitative assessment of anti-androgenic activity, for predicting the effect of a four component mixture of anti-androgens. The anti-androgenic activity of four known anti-androgens (vinclozolin, fenitrothion, flutamide, linuron) was evaluated from individual concentration-response data and used to design a mixture containing each chemical at equipotent concentrations. Across a 100-fold concentration range, a concentration addition approach was used to predict the response of fish to the mixture. Two studies were conducted independently at each of two laboratories. By using a novel method to adjust for differences between nominal and measured concentrations, good agreement was obtained between the actual outcome of the mixture exposure and the predicted outcome. This demonstrated for the first time that androgen receptor antagonists act in concert in an additive fashion in fish and that existing mixture methodology is effective in predicting the outcome, based on concentration-response data for individual chemicals. The sensitivity range of the AFSS assay lies within the range of anti-androgenicity reported in rivers across many locations internationally. The approach taken in our study lays the foundations for understanding how androgen receptor antagonists work together in fish and is essential in informing risk assessment methods for complex anti-androgenic mixtures in the aquatic environment

1. Introduction

Considerable attention and concern has been focused on contaminants in the aquatic environment that interfere with the functioning of the vertebrate reproductive system (endocrine disrupting chemicals: EDCs), the most-documented of which are EDCs that target oestrogen-dependent pathways (Sumpter and Johnson, 2008). However, chemicals that interact with other elements of the reproductive endocrine system are of equal interest. In particular, EDCs with anti-androgenic properties are believed to be ubiquitous within the aquatic environment (Hill et al., 2010; Johnson et al., 2007; Urbatzka et al., 2007) and may be important contributors to reproductive dysfunction in aquatic animals (Jobling et al., 2009). Nonetheless, the biological significance of anti-androgenic contaminants is not yet fully understood. Relatively little is known about the disposition and identity (although see Rostkowski et al., 2011) of anti-androgenic EDCs or the extent of their effects on aquatic wildlife. These knowledge gaps highlight a need for further investigation and assessment.

The aquatic environment is a chemically complex medium in which individual contaminants may be present at low concentrations yet still contribute to joint effects on organisms as part of the overall assemblage of chemicals. In this context, without the ability to extrapolate likely combined effects, reference data derived from single-agent exposure studies are uninformative regarding the overall risk and potential adverse effects for exposed animals

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0166-445X/$ – see front matter.
http://dx.doi.org/10.1016/j.aquatox.2013.05.020

(Kortenkamp, 2007). Thus, there is a need to develop and refine methods that allow the prediction of effects of chemical mixtures on target organisms in the aquatic environment. To date, most studies using fish as an environmentally relevant model target organism to investigate mixture effects of EDCs, have focused on chemicals with estrogenic modes of action (Brian et al., 2005; Correia et al., 2007; Jukosky et al., 2008; Thorpe et al., 2001; Zhang et al., 2010). The purpose of the present study was to extend this approach to investigate the use of single agent concentration-response data to predict the effects on a relevant fish model of a mixture of chemicals with anti-androgenic properties.

In order to retain relevance to real-world exposure scenarios we adopted an in vivo assay system that utilises unique features of the three-spined stickleback (*Gasterosteus aculeatus* L.). The stickleback is ubiquitous in northern latitudes and widely employed in ecological, ecotoxicological and behavioural investigations (Katsiadaki et al., 2007; Pottinger et al., 2002, 2011, 2013; Sanchez et al., 2008). Male sticklebacks synthesise an androgen-dependent glycoprotein (spiggin) which is used to glue together the structural components of the nest (Jakobsson et al., 1999; Jones et al., 2001). Androgen-inducible spiggin is also present in the kidney of females but normally at very low levels and this feature has been exploited to provide a bioassay for EDCs with anti-androgenic activity (Katsiadaki et al., 2002). Priming females by exposure to a standardised concentration of androgen in order to stimulate the synthesis of spiggin provides a sensitive in vivo quantitative assay system for the detection and evaluation of anti-androgenic EDCs (Jolly et al., 2009; Katsiadaki et al., 2006). The use of females, in which spiggin levels are normally low, provides a relatively constant baseline from which consistent androgen-induced spiggin levels can be achieved. This would not be possible using males in which the annual cycle of endogenous androgen causes large inter-individual fluctuations in kidney spiggin content. The use of females also reduces the likelihood that non-receptor mediated mechanisms, for example those acting on steroid synthesis, might affect spiggin levels; in females the synthesis and accumulation of spiggin is primarily a direct consequence of an androgen receptor-mediated process (Olsson et al., 2005). Because of this, the AFSS is an in vivo assay with a sound mechanistic basis that specifically identifies androgen receptor antagonists.

This series of studies was designed to evaluate whether the joint effects of a mixture of anti-androgens on spiggin synthesis in female sticklebacks could be predicted accurately from knowledge of the individual potencies of each component of the mixture. The concept of concentration addition (CA), which is applicable to mixtures of chemicals with a common mode of action (Drescher and Boedeker, 1995), was favoured as the prediction model. In the first instance our intention was to validate the usefulness of CA, rather than to study environmentally relevant mixtures. Accordingly, the following androgen receptor antagonists (Kang et al., 2004; Lambright et al., 2000; Sebire et al., 2009; Tamura et al., 2001; Wong et al., 1995) were selected: fenitrothion [0,0-dimethyl 0-(4-nitro-m-tolyl)phosphorothioate] an organophosphate insecticide; vinclozolin [(RS)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] a non-systemic dicarboximide fungicide, linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] a substituted urea herbicide, and flutamide [2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl] propanamide], a non-steroidal anti-androgenic therapeutant. The potency of each anti-androgen in countering androgen-induced spiggin synthesis in female sticklebacks was evaluated singly and these data were used to predict the outcome of a series of combined exposures in which all four anti-androgens were present in a mixture at ratios proportional to their expected individual potencies. Using this fixed-ratio mixture design, the predictive power of CA was assessed by comparing the predicted anti-androgenicity of the four compounds with that observed. Because differences between nominal and measured concentrations of the anti-androgens in the test mixtures changed the original mixture composition, in each mixture concentration the assumption of a common mixture ratio between the compounds and test concentrations was unavoidably violated. This would have resulted in restricting the comparative mixture assessment to only the analytically determined mixture concentrations, thereby discarding one of the biggest advantages of fixed-ratio mixture designs – the capacity to assess concentration ranges of the mixture that were not directly tested. We overcame these limitations in this study by estimating varying mixture ratios that allowed us to expand the traditional concentration-response analysis established for fixed-ratio mixtures to more complex mixture compositions.

2. Materials and methods

2.1. Chemicals

Analytical grade flutamide (FL), fenitrothion (FN), vinclozolin (VZ) and dihydrotestosterone (DHT) were obtained from Sigma–Aldrich (Gillingham, UK) and linuron (LN) was purchased from QMX Laboratories (Thaxted, UK). All chemicals used in the study were matched across laboratories by batch number and were of high purity (≥99%). All other chemicals were obtained from Sigma–Aldrich unless otherwise stated.

2.2. Fish

Sticklebacks were obtained from a supplier (Moore & Moore Carp, Reading, UK; CEH Lancaster) or captured by beach seine in Oslo fjord (Drøbak Research Station; University of Bergen). At both Lancaster and Bergen, the fish were subsequently kept in glass aquaria supplied with a constant flow-through of water and fed five times weekly with frozen bloodworm. Because of the requirement that the test fish exhibit low levels of endogenous spiggin, only female fish were selected for these studies. Males were identified by inspection of iris and oesophageal colour (immature males exhibit traces of blue and red, respectively) and separated from the females. For a period of at least 1 month prior to the exposure studies the sticklebacks were acclimated to the temperature (Lancaster: 15 ± 2 °C; Bergen: 16 ± 2 °C) and photoperiod (12 h light:12 h dark) under which the studies were conducted.

2.3. Experimental design

Single agent and mixture studies were performed in parallel at two laboratories (Centre for Ecology & Hydrology, Lancaster, UK, and Department of Biology, University of Bergen, Norway) over a period of three years. The in vivo exposures closely followed procedures outlined in the OECD Guidance Document 148 (OECD, 2011). The exposure system comprised the required number of 30 L (working volume) glass aquaria each supplied with a constant inflow of untreated raw water (100 mL/min; PVC tubing, Portex; 5 mm i.d.; Lancaster: lake water; Bergen: seawater) via peristaltic pumps (Watson Marlow 505S; Marprene tubing, 6.4 mm i.d.) with twin head cassettes. The performance of each of the pumps was checked twice weekly by timing the delivery of 100 mL of water into a volumetric flask. Each tank was aerated throughout the study period via a single airstone. Working solutions of the test compounds were formulated in methanol and held in 1.0 L glass bottles. A multichannel peristaltic pump (Watson Marlow 205 U; 0.76 mm i.d. PVC manifold tubing) delivered the test compound solution from the stock bottle to the aquaria via silicone tubing through a three-way connector inserted immediately downstream of the raw water