Aquatic Toxicology 128–129 (2013) 34–42

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

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

The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the Fish Sexual Development Test

Lisa Baumann[a,*], Henrik Holbech[b], Susanne Keiter[a], Karin Lund Kinnberg[b], Susanne Knörr[a], Tina Nagel[a], Thomas Braunbeck[a]

[a] Aquatic Ecology and Toxicology Section, Centre for Organismal Studies, University of Heidelberg, Im Neuenheimer Feld 230; D-69120 Heidelberg, Germany
[b] Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

ARTICLE INFO

Article history:
Received 10 October 2012
Received in revised form
16 November 2012
Accepted 20 November 2012

Keywords:
Gonad histology
Gonad staging
Sexual differentiation
Vitellogenin
Zebrafish
Endocrine disruption

ABSTRACT

In July 2011, the Fish Sexual Development Test (FSDT) has officially been adopted as OECD test guideline 234 for the detection of endocrine disrupting chemicals (EDCs). Sex ratio and vitellogenin (VTG) induction are the mandatory endocrine endpoints within this test, whereas gonad staging is only included as an option. In the present study, five FSDTs with zebrafish (*Danio rerio*) were conducted with EDCs with different modes of action (17α-ethinylestradiol, dihydrotestosterone, 17β-trenbolone, prochloraz and 4-*tert*-pentylphenol). Results document that not only sex ratio and VTG production of the exposed fish were massively affected, but also gonad maturation. As a novel approach for the quantification of gonad maturation in zebrafish, the maturity index was developed to allow not only an improved assessment of dose-dependent EDC-related effects on gonad maturation, but also statistical analysis of histological data. VTG induction and maturity index showed an excellent correlation for all five EDCs tested. Most importantly, the maturity index often helped to find appropriate interpretations for results that seemed contradictory at first sight. Results show that histological analyses and their predictive power for population fitness are currently underestimated and should become a standard component in the evaluation of potential EDCs.

1. Introduction

Endocrine disrupting chemicals (EDCs) have become a major research field in ecotoxicology over the last 20 years. Especially aquatic vertebrates are continuously affected by EDCs and have, therefore, frequently been used for EDC research. In the environment, numerous chemicals have been identified as EDCs showing effects like shift of sex ratio or occurrence of intersex gonads, on wildlife populations (Guillette et al., 1996; Jobling and Tyler, 2003; Lye, 2000; Sumpter, 1998). In the future, even more compounds might be identified to show endocrine activity, especially under the EU regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, EC 2007), which enforces the (re-)registration of thousands of new and existing chemicals. As a consequence, considerable efforts have been made to reduce the impact of EDCs on nature and humans, and a suite of standardized test systems were developed at the OECD level (Organization for Economic Co-operation and Development, OECD, 2009a,b,c, 2011a).

The most critical issue of an in vivo test system is its capability to predict endocrine effects in wildlife populations. Full life-cycle or even multi-generation tests are usually regarded as the method of choice for the detection of population-relevant endpoints such as reproductive failure, but they are very time- and cost-intensive (Nash et al., 2004). In July 2011, the Fish Sexual Development Test (FSDT) has officially been adopted as OECD test guideline no. 234 for the detection of EDCs within the OECD conceptual framework at level 4 (OECD, 2011b). Although reproduction is not included as an endpoint, the FSDT has potential as a promising compromise for the gold standards, the full life-cycle or the multi-generation test, since it has been designed for a safe evaluation of potential EDCs within an extended exposure period of 60 days, which is still much shorter than ≥6–7 months for a full life-cycle or even a multi-generation test with, e.g., zebrafish (*Danio rerio*).

As a protogynic fish species, zebrafish represents an interesting test organism for the investigation of effects on reproduction and the hormonal system. Its gonad development covers an all-female state, which means that all individuals first develop ovaries, from which around 50% will be transformed to testis later (Takahashi, 1977). This developmental process is under hormonal control and makes zebrafish particularly sensitive to EDCs (Andersen et al.,

* Corresponding author.
E-mail addresses: lisa.baumann@cos.uni-heidelberg.de (L. Baumann), hol@biology.sdu.dk (H. Holbech), susanne.keiter@cos.uni-heidelberg.de (S. Keiter), kinnberg@biology.sdu.dk (K.L. Kinnberg), susanne.knoerr@gmx.de (S. Knörr), tina.nagel@stud.uni-heidelberg.de (T. Nagel), braunbeck@uni-hd.de (T. Braunbeck).

0166-445X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2012.11.016

Table 1
Chemicals and test concentrations.

Test substance	CAS-no.	Replicates	Test concentrations
4-*Tert*-Pentylphenol	80-46-6	4	32, 100 and 320 μg/L
17α-Ethinylestradiol	57-63-6	2	0.1, 1, 3 and 10 ng/L
17β-Trenbolone	10161-33-8	2	1, 3, 10 and 30 ng/L
Dihydrotestosterone	512-18-6	4	100, 320 and 1000 ng/L
Prochloraz	67747-09-5	2	10, 30, 100 and 300 μg/L

2000; Maack and Segner, 2004). All experiments conducted so far during the validation of the FSDT and several studies before (for reviews, see Holbech et al., 2012 as well as Scholz and Klüver, 2009) demonstrated that exposure of zebrafish to EDCs in this sensitive phase of gonadal transformation resulted in a dose-dependent shift of sex ratio and an up- or down-regulation of vitellogenin (VTG). Histopathological lesions in the gonads have been given less attention, even though a guidance document on the diagnosis of endocrine-related histopathology in fish gonads has been made available (OECD, 2010) and although several chemicals are known to inhibit sexual differentiation and gonad maturation (for review, see Danzo, 1998). Moreover, there are numerous reports showing significant effects in the gonads of fish exposed to EDCs (Dang et al., 2011; Keiter et al., 2012; Van der Ven et al., 2003; Weber et al., 2003).

VTG induction is usually regarded as an essential biomarker for the evaluation of EDCs affecting the sexual hormone system (Holbech et al., 2001; Matozzo et al., 2008; Örn et al., 2003; Sumpter and Jobling, 1996). However, an appropriate interpretation of VTG data is frequently only possible with information about the individual gender (Holbech et al., 2006). Since the genetic sex of zebrafish can neither be detected genetically (Tong et al., 2010), nor be unequivocally assessed by phenotype at 60 days post-hatch (dph), especially after exposure to EDCs, gonad histology as the only method for safe and definite sex determination is an indispensable component in the FSDT with zebrafish. However, while sex ratio and VTG induction are mandatory endocrine endpoints in the FSDT, gonad staging is only included as an option in OECD TG 234 (OECD, 2011b), thus giving rise to potential misinterpretations, since differentiation to the female or male gender does not necessarily coincide with reproductive capability. Since EDCs may not only affect differentiation itself, but also the time-scale of gonadal maturation, staging of gonad maturity seems most helpful for the interpretation of EDC-related effects, a fact that should be considered when it comes to the evaluation of an unknown substance, e.g. with respect to decisions at the regulatory level.

In the present study, data from five FSDTs with different EDCs covering diverse modes of action are compared with particular focus on the correlation between the mandatory endocrine endpoints of the FSDT (VTG induction and sex ratio) and the stage of gonadal maturity. The maturity index was developed as a novel approach for the quantification of gonad maturation in young zebrafish, which should allow not only improved visualization of dose-dependent EDC-related effects on gonad maturation, but also the statistical analysis of histological data.

2. Materials and methods

2.1. Test substances

4-*Tert*-pentylphenol, prochloraz, 17α-ethinylestradiol, dihydrotestosterone and 17β-trenbolone and all other substances, unless stated otherwise, were obtained from Sigma–Aldrich (Deisenhofen, Germany). Table 1 gives an overview of the test substances and concentrations used for zebrafish exposure in the FSDTs. The steroids 17α-ethinylestradiol, dihydrotestosterone and 17β-trenbolone were dissolved in dimethylsulfoxide (DMSO) at maximum final solvent concentrations of 0.01%. All treatments were run in at least two replicates (Table 1). A water- and a solvent-control were run in duplicates.

2.2. Exposure

The Fish Sexual Development Test (FSDT) has been performed as described in the OECD Test Guideline no. 234 (OECD, 2011b). Exposure of zebrafish (*Danio rerio*) to the test chemicals started at latest 1 h post-fertilization (hpf) and ended at 60 days post-hatch (dph). At minimum, 40 eggs were used for each replicate. Fish were held in aerated 8–12 L flow-through glass tanks at 26–27 °C and a dark:light cycle of 10:14 h. The exposure water was mixed from tap water and demineralized water to reduce the total hardness. Constant aeration in the reservoirs ensured high oxygen saturation. The effluent was purified by passing over a charcoal filter, before it was released to the municipal sewage treatment plant. Water temperature and flow-through rates (complete water exchange every 8 h) were controlled twice daily. Water hardness (200–280 mg/L), conductivity (600–750 μS), pH (8.0–8.2) and oxygen saturation (90–95%) were checked once weekly. Feces and food remains in the tanks were removed daily. From day 4 to 14, larvae were fed with powdered dry food (Sera Micron, Heinsberg, German)y or Tetra AZ 100™ starter food (Tetra-Werke, Melle, Germany), followed by feeding with granular flake food (TetraMin™, Tetra-Werke, Melle, Germany) and newly hatched nauplii of *Artemia* spec. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Ogden, USA).

2.3. Sampling

Fish were euthanized in a saturated solution of benzocaine (ethyl-4-aminobenzoate) or buffered MS-222 (100 mg/L). Length and wet weight of each individual were documented. Head and tail were cut off with a razor blade behind the operculum and behind the anal fin, weighed together and frozen immediately in liquid nitrogen for subsequent quantification of VTG via enzyme-linked immunosorbent assay (ELISA). Remaining trunks were placed in embedding cassettes (Histosette, Neolab, Heidelberg, Germany) and fixed in modified Davidsons's fixative (Romeis and Böck, 2001) for subsequent histological analyses.

2.4. ELISA

The measurement of the VTG concentration in head and tail homogenate of zebrafish was performed as described by Holbech et al. (2006). In short, the frozen tissues were homogenized with a plastic pistil in 1.5 mL centrifuge tubes and mixed with 10 times the weight of homogenization buffer (50 mM Tris–HCl, pH 7.4: 1% protease inhibitor cocktail (P 8340 (Sigma–Aldrich, MO, USA)). The homogenate was centrifuged for 30 min at minimum 25,000 × *g* at 4 °C where after the supernatant was collected and stored at −80 °C. The VTG concentration in the supernatant was measured by a direct non-competitive sandwich ELISA based on polyclonal affinity purified antibodies against zebrafish lipovitellin developed by Holbech et al. (2001).