Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles

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ABSTRACT

Whereas biochemical and molecular parameters have been well recognised as important “signposts” of individual disturbance to endocrine disrupting chemicals’ (EDCs) exposure, behavioural endpoints are yet greatly overlooked as a routine tool in environmental risk assessment of EDCs. However, life histories are intimately associated with numerous inter- and intra-specific interactions, which invariably depend on the performance of effective behaviours. Within fish species, one of the most important factors influencing energy turnover earlier in the development is locomotor activity. This essential trait reflects the organism’s ability to generate and coordinate the metabolic energy required for both reproductive and non-reproductive behaviours. Inappropriate movement responses due to toxic effects of contaminants may ultimately impact important ecological variables.

Therefore, in the present study, the swimming bursts of zebrafish juveniles exposed for 40 d to the synthetic estrogen ethinylestradiol (EE2), tested at environmentally relevant concentrations (nominal concentrations of 0.5, 1 and 2 ng L\(^{-1}\)), were investigated in order to address the potential of rapid-behaviour patterns as an effective response indicator of estrogenic endocrine disrupting chemical’s exposure. This synthetic estrogen was selected due to its high prevalence in aquatic ecosystems, ability to mimic natural estrogens and proven record of causing negative effects in fish reproduction. The behavioural responses were compared with established endpoints used in the screening of EE2 effects at adulthood.

Results indicate that zebrafish juveniles’ swimming activity was significantly decreased upon EE2 exposure. Since reduced locomotion of zebrafish may impact foraging, predator avoidance, drift and transport, and even interfere with social and reproductive behaviours, a fitness decline of wild fish populations can ultimately be hypothesized. Furthermore, behavioural endpoints were found to display higher sensitivity to EE2 than either vitellogenin gene induction or reproductive parameters determined at adulthood.

Overall, the findings of this work not only demonstrate the power of high-throughput behavioural responses, able to act as sensitive early warning signals of EDC exposure, but also highlight the potential of behavioural endpoints in providing a more comprehensive and non-invasive measure of EDC’s exposure.

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1. Introduction

The detrimental impact of several groups of chemicals in different dimensions of the environment is now well documented. Given the ubiquity and persistent nature of numerous organic pollutants exhibiting endocrine disrupting activity (endocrine disrupting chemicals – EDCs), a large number of studies have been focusing on whether and how EDCs interfere with the normal endocrine system of animals as well as their impact in fundamental processes such as reproduction (WHO, 2002). Evidence suggests that both invertebrates and vertebrates have been impacted by EDCs exposure, although most reports derive from aquatic animals (Fent et al., 1998; Santos et al., 2002; Thibaut and Porte, 2004; Rodrigues et al., 2006; Hotchkiss et al., 2008; Rempel and Schlenk, 2008).
This is a predictable outcome considering that a large group of chemicals are released to the aquatic ecosystems, both from point and non-point sources (e.g. wastewater plant discharges and silt-laden runoffs, respectively).

Chemicals with the ability to mimic estrogens, estrogenic disrupting chemicals (EDCs), have been shown to be prevalent in most aquatic ecosystems in the vicinity of urban areas. Most of these chemicals act as agonists of the estrogen receptors (ERs), thus eliciting biological responses similar to those of the natural estrogens (Kime, 1998). The most bioactive EDC, at the receptor level, is the 17α-ethinylestradiol (EE2), used in oral contraceptives and hormone replacement therapies (Gutendorf and Westendorf, 2001). EE2, at concentrations below 2 ng L⁻¹, has been reported to disturb several crucial processes in fish such as gonad development, egg production, embryo mortality or courtship behaviour (Lange et al., 2001; Robinson et al., 2003; Balch et al., 2004; Soares et al., 2009) and, at 5 ng L⁻¹ it has been reported to lead to population collapse of fathead minnow, *Pimephales promelas* (Kidd et al., 2007).

The most common approach to evaluate the exposure to EDCs consists in determining the induction of the egg-yolk protein precursor, vitellogenin (VTG), in adult male fish liver (*Sumpter and Jobling, 1995; Kime et al., 1999*). Although VTG induction in male fish has been largely used both in the field and under laboratory conditions to monitor the exposure to EDCs, it also shows some constraints mostly related with the ecological relevance of the response, species and gender differences in response, life-cycle period of exposure (adult VTG is more inducible than larvae and juvenile) (Tyler et al., 1996, 1999; Navas and Segner, 2006; Shi et al., 2006; Liao et al., 2009). In addition, seasonal-dependent regulation of gene expression reported for some annual cycle species can also induce difficulties in data interpretation (Moussavi et al., 2009).

Within fish species, one of the most important factors influencing energy turnover earlier in the development is locomotor behaviour (Brett and Groves, 1979). The ‘free-swimming’ stage (after yolk depletion and swim-up) is a crucial phase for survival, since it reflects the juveniles’ ability to generate and coordinate the metabolic energy required for both reproductive and non-reproductive behaviours. Therefore, unorthodox movement responses due to the toxic effects of contaminants may ultimately impact population sustainability. Previous studies on vertebrates have demonstrated that natural estrogens can exert neuromodulatory effects, by directly affecting neuron functioning, which influence vertebrate locomotor activity (Mermelstein et al., 1996; Kelly et al., 2003; Dickinson, 2006). Yet, despite the well-conserved basic structure and function of vertebrate endocrine axis, data regarding effects of teleost fish movement patterns under EDC exposure remains scarce, especially when long-term exposures to contaminants are considered (e.g. Sárria et al., 2011a). In fact, whereas endpoints at the cellular, molecular and biochemical level have been well recognised as important “signposts” of individual disturbance to EDCs exposure (Hutchinson et al., 2006), behavioural endpoints are yet greatly overlooked as a routine tool for detecting environmental pollution effects. Importantly, recent studies show a good agreement between selected behavioural endpoints in fish and EDCs and pharmaceutical toxicological action. The observed behavioural effects reported in these non-invasive toxicity tests ranged from marked changes in locomotion patterns and predation escape, to more subtle disorders in courtship and aggression responses (Airhart et al., 2007; Speedie and Gerlai, 2008; McGee et al., 2009; Kokel et al., 2010; Saaristo et al., 2010; Sárria et al., 2011a). The application of rapid behaviour indicators on EDCs risk assessment may potentially contribute in providing very expedite responses, both ecologically relevant and cost-effective, and thus overcome the often less immediate response which is commonly provided by other types of approaches (Clotfelter et al., 2004).

Zebrafish, *D. rerio* Hamilton (1822), has been well recognised as a model vertebrate species for developmental studies, an essential work basis to several scientific areas, such as behavioural neuroscience and ecotoxicology. As an example, zebrafish locomotor activity has already allowed for a specific high-throughput behavioural profiling that ultimately led to the discovery and characterisation of psychotropic drugs (Kokel et al., 2010; Rihel et al., 2010). In contrast, there is virtually no information available on zebrafish swimming behaviour under EDC exposure. Thus, in the present study, taking into account the already anticipated estrogenic potency of EE2, the swimming bursts performed by juvenile zebrafish, exposed to environmental relevant concentrations of this EC, was investigated. Since *D. rerio* locomotor network development and kinematics are well described (Drapeau et al., 2002; Müller and van Leeuwen, 2004), and considering that the motor-sensory endocrine axis is transversal through the generality of the vertebrate species, we discuss not only the potential use of this rapid-behaviour pattern as an effective responsive indicator of sub-lethal EDC toxicity, but also the applicability of this approach in other teleost species.

## 2. Materials and methods

### 2.1. Chemicals

17-α-ethinylestradiol [C₂₀H₂₄O₂] (EE2, purity ≥98%, Sigma – stock solution: 1 mg mL⁻¹); dimethylsulphoxide [(CH₃)₂SO] (DMSO, purity 99.5%, Sigma).

### 2.2. Experimental fish

Zebrafish, *D. rerio* (Teleostei, Cyprinidae) is a small benthopelagic tropical freshwater fish, native from India and Southeast Asia (*Engeszer et al., 2007*). The species size, robustness, short life cycle, high fecundity and potential to easily breed in captivity are advantages for experimental bioassays. Zebrafish has been widely recognised as a model vertebrate species for developmental biology and gerontology assessment (*Grunwald and Eisen, 2002*), behavioural ecology, genetics research (*Eisen, 1996; Engeszer et al., 2004*), and ecotoxicology (*Hill and Janz, 2003; Micael et al., 2007; Segner, 2009*).

### 2.3. 17-α-ethinylestradiol (EE2) exposure

Wild-type parental zebrafish (F₀) were obtained from local suppliers in Singapore, and used as breeding stock according to Soares et al. (2009). In the afternoon before breeding, two groups of 4–6 males and 10–12 females were housed in cages with a net bottom cover with glass marbles within a 30 L aquarium under the same water and photoperiod conditions as the stock and fed with live brine shrimp. At the following day, breeding fish were removed 1.5 h after the beginning of the light period and the eggs were collected and cleaned. Fertilised eggs were randomly allocated to experimental aquaria. A flow-through system was used to administer EE₂ with slight modifications of Santos et al. (2006). The experimental setup, including details on fish basic monitoring, exposure conditions, physico-chemical parameters are described in detail in Soares et al. (2009). Briefly, after 15 d of aquaria system equilibration, 450 eggs collected ≈2 h post-fertilisation (hpf) were randomly allocated to 5 L aquaria (each placed within 30 L aquarium), and exposed to EE₂ (nominal concentrations: 0.5; 1 and 2 ng L⁻¹). Each treatment was replicated twice, including experimental and solvent controls. DMSO was used as solvent
in all treatments, except control, at a volume of 0.000002%. At 20 d
post-fertilisation (dpf), zebrafish were allocated to 30 L aquaria
and their number adjusted to fit 100 juveniles per aquarium.
Feeding was initiated at day 6 dpf with two meals of Tetramin
supplemented with one brine shrimp meal per day; at day 9 dpf
this was changed to two brine shrimp meals and one Tetramin
meal per day, which was maintained up to the end of exposure.
The feeding regime of larvae was based on a slight modification of
Carvalho et al. (2006), which maximises survival and growth.

2.4. Water chemical analysis

Concentrations of EE2 were found to be below the detection limit
in the reference aquaria and to be 0.19 ± 0.02, 0.24 ± 0.02 and
1.00 ± 0.12 ng L\(^{-1}\), respectively, for the 0.5, 1 and 2 ng L\(^{-1}\) nominal
concentrations. Briefly, the extraction procedures consisted of a pre-
treatment according to the "Sample Pre-treatment Protocol
for Female Steroid Hormones" (Japan Envirochemicals); extraction
in a solid phase C18 column (Sep-Pak Plus C18 cartridges, Waters
Corporation); 80% methanol elution and concentration under nitro-
gen gas flow. EE2 concentration was then determined using the "Jec
ELISA kit" (Japan Envirochemicals) according to the manufacturer’s
protocol.

2.5. Collected data

2.5.1. Swimming behaviour

During the entire exposure period, mortality was assessed daily
and dead individuals were removed. At 40 dpf, 12 juveniles from
each treatment (six per replicate) were randomly selected and the
number of movement bursts was counted during sixty seconds.
Basically, each time a fish actively propelled itself in either direc-
tion, a movement was scored. In order to avoid bias, "blind" obser-
vations were performed by a single person.

2.5.2. Additional endpoints measured 8 mpf

In order to allow for a comparison between behavioural
parameters determined at 40 dpf, and more established endpoints
used in the screening of EE2 impact in fish, the exposure was
extended up to 8 months (mpf), when zebrafish females reach
their maximum fecundity. A vast array of endpoints were deter-
mined at 8 mpf, such as fecundity and fertility rates, male and
female gonad histology coupled with a stereological analyses,
sperm quality parameters, VTG 1 induction in male liver, as well
as embryonic development of the F2 generation. A detailed description of these endpoints has been reported in Soares et al. (2009).

2.6. Statistical analysis

In order to assess any hypothetical bias in mortality patterns,
a Wilcoxon matched pair test was applied to detect if the replic-
cates were indeed homogenous. Then, using the average mortal-
ity of the control as the expected value, a chi-square test was
conducted where the observed values derived from the average
mortalities observed in the solvent control and EE2 treatments.
Furthermore, in order to investigate the influence of EE2 on zeb-
rafish juveniles swimming behaviour (number of movement
bursts) a one-way ANOVA was conducted, with five levels:
experimental control, solvent control (DMSO), lower, interme-
diate and higher EE2 concentrations. All analyses were conducted
in STATISTICA (V7).

3. Results

3.1. Mortality

The registered mortality, which occurred solely up to 20 dpf,
varied between 36% and 55%. These values are well within the
ranges reported by several other zebrafish partial life-cycle studies
(Hill and Janz, 2003; Santos et al., 2006). No differences among rep-
licates were observed (Wilcoxon matched pairs test: N = 5;
Z = 0.944; P = 0.345). Also, there were no significant differences in
mortality between controls and EE2-exposed groups (\(\chi^2 = 3.043;
DF = 3; P = 0.385\)).

3.2. Swimming behaviour

EE2 concentration directly affected zebrafish juveniles swim-
m originated activity [one way ANOVA: \(F(4,55) = 7.985, P < 0.01\) causing
a significant reduction in the number of juvenile fish swimming
bursts. No differences were recorded between the controls (SNK,
data not shown), which were significantly different from the EE2 ex-
posed groups. The increase in EE2 concentration, although not signif-
icant, was indeed translated into a decrease in the number of
movement bursts (Fig. 1): Experimental control = 102.33 ± 1.80
(average number of bursts ± S.E.); Solvent control = 98.25 ± 1.83;
[EE2]0.5 ng L\(^{-1}\) = 91.42 ± 2.82; [EE2]1 ng L\(^{-1}\) = 89.00 ± 2.36; [EE2]2 ng L\(^{-1}\) = 87.92 ± 2.08.

3.3. Additional endpoints measured at 8 mpf

With the exception of VTG 1 gene expression in males that was
induced at the highest EE2 exposure level (2 ng EE2 L\(^{-1}\), nominal
concentration), none of the other monitored parameters was al-
tered at adulthood at the selected environmentally low EE2 levels
(i.e. fecundity, fertility, male sperm quality parameters and male
and female gonad stereological analysis; Soares et al., 2009). In
addition to the effects of EE2 in the F1 generation, the impact in
the embryonic development of the F2 generation was also tested,
with significant changes observed in zebrafish larvae for all tested
EE2 concentrations (Soares et al., 2009).

4. Discussion

In contrast to biochemical and molecular endpoints, the use of
fish behavioural parameters in environmental risk assessment is
still not a common approach. However, recent ecotoxicological
and drug discovery studies support the sensitivity of fish behaviour
endpoints in the hazard assessment of chemicals and in the char-

![Fig. 1. The effects of EE2 exposure on zebrafish (Danio rerio) juveniles swimming behaviour (bars depict standard errors and different letters indicate differences among treatments).](image-url)
acterisation of pharmaceuticals action mechanisms (e.g. Kokel et al., 2010; Rihel et al., 2010). Therefore, if one wishes to integrate this approach in environmental risk assessment of EDCs, validation under laboratory control conditions is still required. In the present study, exposure of zebrafish larvae to the ubiquitous EDC, EE2, led to a significant alteration in juvenile zebrafish behaviour patterns, with a decrease in swimming activity even at the lowest nominal concentration of 0.19 ng L⁻¹. Even though it is out of the scope of the present study to determine the mechanisms of EE2 disruption, it could be hypothesised that the observed increase in lethargy of the EE2-exposed zebrafish juveniles might be caused estrogenic neurotoxicity. In fact, not only the motor-sensory endocrine axis of vertebrates tends to be well conserved, but also estrogens have already been shown to be neurotoxic in mammals, causing changes on locomotor activity (Mermelstein et al., 1996; Kelly et al., 2003).

Exposure to EDCs, including EE2, has been demonstrated to induce a set of negative effects in fish, including decreased reproductive capacity, male feminization, decreased embryonic survival, altered reproductive hierarchies, disrupted sexual selection in group-spawning fish, alterations in parentage outcomes and population collapse (e.g. kidd et al., 2007; Coe et al., 2009; Colman et al., 2009; Saaristo et al., 2009; Soares et al., 2009; Sozano et al., 2010). However, most studies have focused on reproductive-related parameters either at adulthood or during embryonic development. Nevertheless, from an ecological standpoint, juvenile development is also a crucial phase since any alteration in parameters such as feeding and predator avoidance may result in lower survival rate, with clear implications in the number of individuals that reach maturity and reproduce. The findings of the present study indicate that EE2, even at remarkably low levels, decrease juvenile swimming activity which may impact foraging, predator avoidance, drift and transport, and even interfere with social behaviours (e.g. aggression). Interestingly, our recent findings with a pipefish species indicate also that EE2 exposure alters larvae behaviour when facing a potential predator (see Sárria et al., 2011a) while altering the natural patterns of vertical distribution (Sárria et al., 2011b). Taken together, these data indicate that EE2 may impact fish populations through subtle changes in the behavioural patterns expressed during the juvenile stage.

In comparison with established parameters used in the screening of EDC effects in fish, behavioural endpoints were found to display higher sensitivity than the measured endpoints at adulthood (VTG expression, fecundity, fertility, male sperm quality parameters and male and female gonad stereological analysis). Furthermore, the behavioural endpoints displayed the same response pattern as embryonic development in the F2 generation, thus demonstrating the promising role of this behaviour-based approach in anticipating the exposure effects of EE2, and perhaps those of other EDCs. Hence, our results on zebrafish swimming behaviour after EE2 exposure further demonstrate the power of high-throughput rapid behavioural responses as sensitive early warning signals of endocrine disruption.

The ecological level effects of EDCs are difficult to detect in the absence of a massive populational decline over a few generations (Sumpter, 2005), and therefore subtle changes as the one reported in the present study can easily be overlooked. In most field studies addressing the impact of EDCs, only gonad histology and VTG induction are evaluated, and no data is available on behavioural changes. Given the results of the present study, it can be argued that behavioural endpoints could be of great value in the risk assessment of EDCs in wild fish populations. Nevertheless, we have to take into account that field animals are exposed to mixtures of chemicals, which may lead to behavioural responses that differ from those of single exposures. In fact, this seems to be true for mixtures of EE2 and the androgenic chemical tributyrin (Sárria et al., 2011a). Therefore, additional studies under controlled laboratory conditions are required to address the emergent issue of mixture effects of chemicals, as well as their impact in behavioural endpoints.

5. Conclusion

The present study highlights the promising role of cost-effective rapid fish behavioural analyses in EDCs environmental risk assessment, which eases the detection of EE2 effects, a process usually pursued with the use of more time-consuming and methodologically demanding approaches. Locomotion is indeed a relevant ecologically parameter, hence, behavioural endpoints not only contribute as an early warning sign of EE2 effects, as they are also informative from an ecological point of view, a limitation of many biochemical and molecular approaches. Given the findings presented in the present study, additional behavioural studies with other EDCs should be performed to allow the integration of behavioural endpoints in the hazard assessment of EDCs.

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