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Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*

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ABSTRACT

Assessing the risks of emerging contaminants, such as pharmaceuticals in the environment requires an understanding of their exposure regime and their effects at environmentally relevant concentrations across species. *Daphnia magna* represents an excellent invertebrate model species to study the mode of action of emerging pollutants, allowing the assessment of effects at different biological levels. The present study aims to test the hypothesis that different families of neuro-active pharmaceuticals at low environmentally relevant concentrations may lead to similar phenotypic responses in *D. magna*. Phenotypic traits included reproduction and behavioural responses. Selected pharmaceuticals were carbamazepine, diazepam and propranolol, three widely prescribed compounds, already detected at considerable levels in the environment (ng to few μg/L). Fluoxetine was also included in behavioural assays. The three tested neuro-active pharmaceuticals were able to enhance reproduction at 1 ng/L of propranolol, 0.1 μg/L of diazepam and 1 μg/L of carbamazepine. Fluoxetine, carbamazepine and diazepam increased positive phototactic behaviour at concentrations ranging from 1, 10 and 100 ng/L, respectively. Reported responses were nonmonotonic, which means that eco-toxicity testing of pharmaceuticals need to assess effects at the ng/L range.

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1. Introduction

Assessing the risks of long-term exposure to low doses of human prescribed pharmaceuticals is an identified research need (Fent et al., 2006). Treated and untreated wastewater effluents are the main route that brings human pharmaceuticals and/or their metabolites to water. Consequently pharmaceuticals are continuously released into the environment and thus their negative effects are independent from their persistence in the environment (Fent et al., 2006; Petrović et al., 2003). In surface waters concentrations of measured human pharmaceuticals are often in the ng/L range. However, targeted ecotoxicological studies using environmental relevant concentrations and focusing on subtle environmental effects are scarce. Recently several studies have reported that at very low concentrations of antidepressants and anxiolytic drugs alter the behaviour of fish, molluscs and crustaceans (Brodin et al., 2013; Fong and Ford, 2014; Ford and Fong, 2015). Human targets of antidepressants, anxiolytic and neuropathic drugs such as selective serotonin re-uptake inhibitors (SSRI), drugs blocking voltage-gated sodium channels and/or GABA agonists and certain antihypertensive compounds are highly conserved across vertebrates and 61% of them are also found in the invertebrate crustacean *Daphnia* (Gunnarsson et al., 2008). Therefore, neuro-active drugs may also affect aquatic invertebrates. It is important to note, that several neuro-active compounds are designed to affect neurotransmitters (serotonin, dopamine, epinephrine, *gamma*-aminobutyric acid-GABA), which regulate many physiological and behavioural processes (Fong and Ford, 2014; Ford and Fong, 2015). There is also an increased number of studies showing that effects of antidepressants at low concentrations do not follow a monotonic response {Fong, 2014 #176; Ford, 2015 #210}. This behaviour is common among endocrine and neuro-active compounds that at low concentrations act specifically on their target sites, whereas at high concentrations became toxic and hence impair survival, growth and/or reproduction irrespectively of its primary mode of action (Vandenberg et al., 2012). This means that there is an urgent need to measure subtle but consistent effects of human pharmaceuticals at low concentration levels in non-target organisms.

The crustacean and aquatic ecotoxicological model organism *Daphnia magna* share with vertebrates several of the neurotransmitters that are targeted by antidepressant and other neuro-active drugs. These include the presence of serotonin, dopamine, epinephrine and GABA receptor signalling pathways

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(Campbell et al., 2004; Campos et al., 2013b; Ehrenström and Berglind, 1988; McCoole et al., 2012a,b; Weiss et al., 2012). There is also evidence that the SSRI fluoxetine, carbamazepine and propranolol increase offspring production at 10, 1 and 50 μg/L, respectively (Campos et al., 2012a; Lürling et al., 2006; Stanley et al., 2006). In amphipods the SSRIs fluoxetine and sertraline altered phototaxis and swimming behaviour at quite low concentrations ranging from 1 to 100 ng/L (Bossus et al., 2014; Guler and Ford, 2010). In *D. magna*, negative phototactic behavior is directly linked to diel vertical migration along the water column, which prevents *Daphnia* to be preyed upon fish during daylight (Cousyn et al., 2001; De Meester, 1993). Thus, this response is an ecologically relevant trait. The aim of the present study is to determine changes in phototactic behaviour and reproduction in *D. magna* individuals exposed to four widely prescribed neuro-active drugs using low environmental concentrations ranging from high ng/L to low μg/L. The studied compounds included the anti-depressant SSRI fluoxetine, the anxiolytic diazepam, the neuropathic and anti-epilepsy drug carbamazepine and the antihypertensive compound propranolol. Selective serotonin reuptake inhibitors (SSRIs) act by blocking the re-uptake of serotonin in the nerve synapses. This effect is used worldwide to treat clinical depression in humans (Rang et al., 1995), with the consequences that these compounds are nowadays widespread in the environment. Surveys in US have reported levels of 12–540 ng/L of fluoxetine, the active ingredient of Prozac, in surface waters and effluents (Kolpin et al., 2002) and total concentrations of SSRIs in aquatic systems were measured in the range of 840–3.2 μg/L (Metcalfe et al., 2010; Vasskog et al., 2008). Diazepam, first marketed as Valium, is widely used to treat anxiety. Diazepam enhances the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the $GABA_A$ receptor (via the constituent chlorine atom) leading to central nervous system depression (Riss et al., 2008). Concentrations of diazepam ranging from 4 to 40 ng/L have been found in Spanish urban rivers (Valcárcel et al., 2012). Carbamazepine is a medication used primarily in the treatment of epilepsy and neuropathic pain. It stabilizes the inactivated state of voltage-gated sodium channels, making fewer of these channels available to subsequently open. This leaves the affected cells less excitable until the drug dissociates (Ambrósio et al., 2002). Carbamazepine is also a GABA receptor agonist since it potentiates GABA receptors made up of alpha1, beta2, and gamma2 subunits (Ambrósio et al., 2002). Carbamazepine is fairly persistent in water and hence can be found at concentrations ranging from 1 to up to 3000 ng/L in rivers receiving waste water treatment effluents (Muñoz et al., 2009; Tixier et al., 2003). Propranolol is a nonselective beta blocker widely prescribed to treat high blood pressure and a number of heart dysrhythmias. It blocks the action of epinephrine and norepinephrine on both β_1- and β_2-adrenergic receptors (Wisler et al., 2007). Propranolol is also quite persistent in water and can be found at 10–60 ng/L in surface water (Bendz et al., 2005; Muñoz et al., 2009).

2. Methods

2.1. Chemicals

Fluoxetine hydrochloride (CAS-No. 56296-78-7; analytical standard, purity 100%), diazepam (CAS-No. 439-14-5; analytical standard, purity 99%), carbamazepine (CAS-No. 298-46-4; analytical standard, purity 99%) and propranolol hydrochloride (CAS-No. 318-98-9; analytical standard, purity 99%) were purchased from Sigma–Aldrich (USA/the Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

2.2. Experimental animals

A single *D. magna* clone F, extensively characterized in previous studies (Barata and Baird, 2000) was used for all assays. Individual or bulk cultures of 10 animals/L were maintained in ASTM hard synthetic water (ASTM, 1994) as it has been describes previously (Barata and Baird, 2000). Individual or bulk cultures were fed daily with *Chorella vulgaris* Beijerinck (5×10^5 cells/mL), corresponding to 1.8 μg C/mL; (Barata and Baird, 2000). The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14 h light: 10 h dark cycle and temperature at $20 \pm 1\,^{\circ}C$.

2.3. Reproduction tests

Reproduction tests followed established OECD guidelines with only minor modifications (Barata and Baird, 2000). Effects of fluoxetine on reproduction responses of *D. magna* have been already studied in previous studies (Campos et al., 2013b, 2012b), thus, reproductive responses of this compound were not tested. Two independent experiments were performed. In the first one neonates (<24 h old) were exposed until their fourth brood (approx. 21–23 days at 20 °C) to 0.01, 0.1, 1, 10 and 100 μg/L of diazepam, carbamazepine and propranolol. The previous concentration range allowed to define lowest effect concentrations for all compounds but propranolol since this compound already affected measured responses at 0.01 μg/L. Therefore, a second experiment was conducted to test lower concentrations of propranolol: 0.1, 1, 10, 100 and 1000 ng/L. Animals were exposed individually to the tested chemicals in 100 mL of ASTM hard water at the food ration of 5×10^5 cells/mL of *C. vulgaris*. The same concentration of ethanol (50 μL/L) was used in all treatments as a carrier solvent and a solvent treatment was also included. Each treatment was replicated 10 times. The test medium was changed every other day. For each individual its survival, age at first reproduction and brood size were monitored. The intrinsic rate of population growth (r) was computed iteratively from the Lotka (Lotka, 1922) Eq. (1) using the measured age, specific survival and fecundity rates:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1 \quad (1)$$

where l_x is the proportion of the females surviving to age x (days) and m_x is the number of juveniles produced *per* surviving female between the ages x and $x+1$. The age at birth was set to 0 days and survival probability (l) to 1 since mortality was absent in most treatments (in 16 out of 23) and when occurred it was low (10%) and related to handling rather than to toxic effects.

2.4. Phototactic behaviour

Changes in phototactic behaviour were quantified by determining the mean phototactic response of 5 individuals in the presence and absence of the tested chemical concentration. Tested concentrations were selected from previously conducted reproduction assays. Behavioural assays were replicated four times. Three different type of behavioural experiments were conducted: with 8 day old adults exposed during their entire life (experiment 1); with 8 day old adults exposed for 48 h, from 6 until day 8 (experiment 2); with 48 h old juveniles exposed during their entire life (experiment 3). In experiments 2 and 3 performed with carbamazepine, diazepam and propranolol exposure concentrations were limited to those that in experiment 1 had the highest effects. Exposures were performed in groups of 5 individuals in 500 mL of test media for adults or 100 mL of test media for juveniles. Ethanol (<50 μL/L) was used as a carrier solvent and a solvent treatment was also included.

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