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Environmentally relevant concentrations of tramadol and citalopram alter behaviour of an aquatic invertebrate

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ABSTRACT

Environmental pollution by pharmaceutically active compounds, used in quantities similar to those of pesticides and other organic micropollutants, is increasingly recognized as a major threat to the aquatic environment. These compounds are only partly removed from wastewaters and, despite their low concentrations, directly and indirectly affect behaviour of freshwater organisms in natural habitats. The aim of this study was to behaviourally assess the effects of an opioid painkiller (tramadol) and antidepressant drug (citalopram) on behaviour patterns of a clonal model species, marbled crayfish. Animals exposed to environmentally relevant concentrations of both tested compounds ($\sim 1\ \mu g\ l^{-1}$) exhibited significantly lower velocity and shorter distance moved than controls. Crayfish exposed to tramadol spent more time in shelters. Results were obtained by a simple and rapid method recommended as suitable for assessment of behaviour in aquatic organisms exposed to single pollutants and combinations.

1. Introduction

Pharmaceutically active compounds (PhAC) are an important group of pollutants that represent a serious threat to aquatic ecosystems worldwide (Boxall et al., 2012). Freshwater ecosystems are exposed to mixtures of PhACs and their residues (Azuma et al., 2017; Li et al., 2011) originating from municipal wastewater from which they are removed only partially or not at all by sewage treatment (Golovko et al., 2014a; Heberer, 2002; Petrović et al., 2003). Pharmaceutically active compounds are designed to be effective at low concentrations and their residues entering environments through wastewaters can therefore affect non-target organisms (Huerta et al., 2012; Hughes et al., 2012; Santos et al., 2010). Toxic effects of PhACs on mammals are well-known, but information of their ecotoxicity and ecological effects is sparse (Boxall et al., 2012). Antibiotics and hypertension drugs, the most common PhACs found in the environment as well as being those at the highest levels, were the focus of most past studies (Lindberg et al., 2014; Padhye et al., 2014; Petrie et al., 2016). Pharmaceutically active compounds without obvious toxic effects, such as antidepressants, psycholeptics, and opioids have received less attention (Brodin et al., 2013; Brodin et al., 2014).

Psychoactive pharmaceuticals are designed to impact signal pathways in the brain, so they may be expected to affect organism behaviour (Fedorova et al., 2014b; Feighner, 1999; Fong and Ford, 2014; Irvine et al., 2011; Thomas et al., 2014). Some antidepressants bioaccumulate in fish tissues (Du et al., 2012; Gelsleichter and Szabo, 2013; Grabicova et al., 2017; Grabicova et al., 2014) and in the benthos community (Grabicova et al., 2015), which increases the possibility of detectable effects on these organisms.

Similar to fish and aquatic and semi-aquatic insects (Barry, 2013; Jonsson et al., 2014), crayfish are susceptible to behavioural changes induced by extraneous substances in water (Cook and Moore, 2008; Lahman et al., 2015). Exposed aquatic animals often exhibit alterations that affect predation, social interactions, reproduction, and migration (Brodin et al., 2013; Corcoran et al., 2010; Valenti et al., 2012). These effects are not lethal but can indirectly influence entire populations, and thus ecosystem functioning, through disrupted prey/predator relationships and altered defence and reproductive behaviour. Several psychoactive PhACs are still labelled as environmentally safe due to their non-lethal effects, despite their known influence on behaviour of aquatic organisms (Boxall et al., 2012; Fong and Ford, 2014).

Assessment of the effects of PhACs requires a method simple enough to be repeated in precisely-defined conditions as well as a suitable model species. Crayfish show a complex morphology, development, and behaviour, including elaborate social interactions (Gherardi, 2002; Vilpoux et al., 2006). They are considered keystone species in freshwater ecosystems and are strong ecosystem engineers (Creed and Reed, 2004; Dorn and Wojdak, 2004). Hence, pollution impact on native

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crayfish stock can result in ecosystem instability (Creed and Reed, 2004; Momot, 1995; Usio and Townsend, 2004).

The marbled crayfish (*Procambarus virginalis* Lyko 2017) is an ideal model organism for many research areas with the potential to generalise results to other members of Crustacea/Decapoda/Reptantia (Vogt, 2011). They are easily cultured, of suitable size, and exhibit individuality, tolerance to handling, high fertility, a relatively short generation time, and adaptability to a wide spectrum of environmental and nutritional conditions (Kawai et al., 2015; Vogt, 2008). In addition, mother, offspring, and all batch-mates of marbled crayfish are genetically identical (due to reproduction by obligatory apomictic parthenogenesis), which can partly eliminate variability related to genotype (Martin et al., 2007; Vogt, 2008). On the other hand, these clones need not be sensitive enough due to their low demands and high adaptability (Kawai et al., 2015).

Behavioural patterns can be detected using specialised software enabling movement tracking in defined, appropriate conditions for testing PhAC effects. The goal of this study was to compare the behaviour of size matched, genetically uniform, marbled crayfish exposed to environmentally relevant concentrations of an opioid painkiller tramadol and the antidepressant drug citalopram with un-exposed controls.

2. Material and methods

2.1. Chemicals

Tramadol hydrochloride and citalopram hydrobromide were obtained from Sigma-Aldrich (USA). Individual stock solutions were prepared in ultra-pure water (aqua-MAX-Ultra system, Younglin, Kyounggi-do, Korea) at a concentration of 10 mg L^1 and stored at 4 °C. The exposure solutions of 1 µg L^{-1} were prepared by dilution of the stock solution with aged tap water.

Acetonitrile (LC/MS grade purity) was obtained from Merck (Germany), formic acid (for acidification of mobile phases of LC) from Sigma-Aldrich (Germany). Isotopically labelled tramadol (D3) and citalopram (D6) were purchased from Toronto Research Chemicals (Canada) and Lipomed (USA), respectively, and were used as internal standards for LC–MS/MS analyses of water samples.

2.2. Experimental animals

Marbled crayfish were cultured in our laboratory at the Research Institute of Fish Culture and Hydrobiology in Vodňany, FFPW USB, Czech Republic, where all experimental work was conducted. Young mature marbled crayfish specimens were randomly selected from the culture tanks. Carapace length to the nearest 0.1 mm was measured using Vernier callipers and weight to the nearest 0.1 g was obtained with an electronic balance (Kern & Sohn GmbH, Balingen, Germany) after video-recordings. The mean length and weight (Table 1) did not differ between control and exposed groups.

Table 2
Concentration of tramadol and citalopram in marbled crayfish exposure solution and control water at 0 and at 48 h (α = 0.05). Data are presented as mean ± standard deviation.

Tested compound	Group	n	Time 0 (µg L^{-1})	Time 48 (µg L^{-1})	Paired *t*-test	*P*
Tramadol	exposed	3	0.98 ± 0.03	0.81 ± 0.11	1.417	0.391
	control	3	< 0.02	< 0.02	—	—
Citalopram	exposed	4	0.87 ± 0.06	0.89 ± 0.09	−0.457	0.678
	control	4	< 0.02	< 0.02	—	—

2.3. Experimental design

Crayfish were exposed to either tramadol (7 d) or citalopram (21 d) at the concentration of ~1 µg L^{-1} of pure compound (Table 2), a level considered environmentally relevant (Fedorova et al., 2014b; Grabic et al., 2012; Grabicova et al., 2015; Rúa-Gómez and Püttmann, 2012; Thomas et al., 2014). The exposure times were based on the mechanisms of action of the selected compounds. Tramadol acts immediately (peak plasma concentration in humans is observed 1.5 h post-ingestion (www.medscape.com, 8 September 2017), in contrast to citalopram in which a steady-state plasma concentration is achieved in one to two weeks (www.fass.se, 8 September 2017). Animals maintained in pharmaceutical-free aged tap water were used as control and handled in the same way as exposed animals. Animals were stocked individually in clear plastic boxes with capacity of 1.25 L (190 × 140 x 75 mm) containing 0.5 L of exposure solution or aged tap water alone. Water quality parameters were ammonium (NH_4^+) < 0.01 mg L^{-1}, nitrite (NO_2-) < 0.01 mg L^{-1}, nitrate (NO_3-) 4.2 mg L^{-1}, fluoride (F-) 0.6 mg L^{-1}, chloride (Cl-) 8.9 mg L^{-1}, iron (Fe) 0.093 mg L^{-1}, calcium (Ca) 32.0 mg L^{-1}, magnesium (Mg) 8.5 mg L^{-1}, manganese (Mn) < 0.02 mg L^{-1}, conductivity 24.1 mS m^{-1}, hardness 1.16 mmol L^{-1}, and turbidity 0.65 FNU.

The animals were fed ad libitum on commercial feed (Sera Granugreen, Sera, Heinsberg, Germany). Boxes were cleaned during the solution/water change every second day. The control group was cleaned first in order to avoid contamination with tested compounds. Animals that moulted or spawned during the exposure period were removed from the experiment. The water temperature (alcohol thermometer accurate to 0.1 °C) did not differ among exposed and control groups in tramadol and citalopram exposure and reached values of (mean ± standard deviation) 20.0 ± 0.5 °C and 20.0 ± 0.2 °C, respectively.

The real concentration of pharmaceuticals in exposure solutions and water were checked by liquid chromatography tandem mass spectrometry (LC–MS/MS) twice over the exposure period for tramadol and four times for citalopram, before (at time 0, i.e. fresh water or freshly prepared exposure solution) and after changing the water (at time 48 h, i.e. water or exposure solution removed after 48 h). Samples were filtered through a syringe filter of regenerated cellulose, 0.45 µm pores (Labicom, Olomouc, Czech Republic) and stored at −20 °C until the

Table 1
Carapace length (CL) and weight (W) of marbled crayfish specimens used in experimental groups. The number of individuals in each experimental group was n = 20. Data are presented as mean ± standard deviation.

Tested compound	Group	Shelter available	CL (mm)	*t*-test	*P*	W (g)	*t*-test	*P*
Tramadol	exposed	no	21.2 ± 1.9	−1.07	0.290	2.8 ± 0.8	−1.39	0.173
	control	no	20.5 ± 2.4			2.4 ± 0.7		
	exposed	yes	20.7 ± 1.6	−0.47	0.643	2.6 ± 0.6	−0.58	0.567
	control	yes	20.5 ± 1.7			2.4 ± 0.7		
Citalopram	exposed	no	21.3 ± 1.8	0.15	0.884	2.6 ± 0.6	0.52	0.608
	control	no	21.4 ± 2.1			2.7 ± 0.9		
	exposed	yes	21.2 ± 1.8	−0.09	0.925	2.5 ± 0.7	0.16	0.873
	control	yes	21.2 ± 1.5			2.6 ± 0.6		