



Effect of antidepressants on circadian rhythms in fish: Insights and implications regarding the design of behavioural toxicity tests.



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ABSTRACT

Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) are widely prescribed for the treatment of depression and anxiety disorders. Consequently, these compounds are frequently identified in global waterways where they may pose a hazard to aquatic biota. Evidence demonstrates these compounds to be capable of influencing the behaviour of fish, but the relevance of many reported behavioural endpoints is unclear and the value of some findings has been questioned. Since these compounds act on neuroendocrine-mediated pathways in vertebrates, the present study explored how exposure to two representative SSRIs (fluoxetine and sertraline) and an SNRI (venlafaxine) affect circadian rhythms in fish. Male mosquitofish (*Gambusia holbrooki*) were exposed to 1, 10 and 100 $\mu\text{g/L}$ concentrations of these compounds individually and when present as a full mixture, for a period of one week. Neither fluoxetine nor sertraline had an impact on diurnal activity patterns when fish were exposed to these compounds alone at any concentration, whereas venlafaxine significantly disrupted normal circadian rhythmicity but only at 100 $\mu\text{g/L}$. When fish were exposed to the full mixture, significantly altered diurnal activity patterns were rapidly observed at nominal concentrations of 1 and 100 $\mu\text{g/L}$, but there was no effect at 10 $\mu\text{g/L}$. This sort of non-monotonic dose relationship is not altogether unusual for fish exposed to antidepressants, but it poses a problem when attempting to evaluate potential risks to the aquatic environment. To evaluate the possibility for misinterpretation when collecting behavioural data over short temporal scales, the data for each day of the experiment was analysed separately. The outcomes demonstrate the importance of longer periods of data collection, which may be necessary to capture the full range of natural behavioural variability that exists both amongst and within individual fish. More importantly, these findings may help reveal why discrepancies are commonly being reported in the literature with regards behavioural effects in fish exposed to antidepressants. It is thus suggested that research be aimed at documenting behavioural variability in fish species used in toxicity testing, to establish guidelines for quality control and where possible inform the development of standardised methodologies so that behavioural analysis can be more appropriately applied to the broad field of aquatic toxicology.

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1. Introduction

Pharmaceuticals have been garnering considerable attention as emerging threats to the aquatic environment and are being identified in global waterways with increasing regularity (Kuster and Adler, 2014). These compounds originate from a variety of sources, including the discharge of treated domestic sewage and hospital wastewater (Frédéric and Yves, 2014), runoff from aquaculture and livestock farming, and in effluents from pharmaceutical man-

ufacturing facilities (Bottoni et al., 2010). Regardless their source, environmental concentrations of most pharmaceuticals normally tend to fall in the mid to low ng/L range (Subedi and Kannan 2015; Fent et al., 2006; Kümmerer, 2010). While such concentrations seem negligible, the fact that pharmaceuticals serve as active chemical ingredients in consumer medications and therapeutic drugs means that many of these compounds have been carefully designed to elicit their intended biological responses at relatively low doses. Consequently, the high biological potency that makes pharmaceuticals desirable for human usage may also make them a hazard for aquatic wildlife, since physiological and biochemical pathways are generally well conserved amongst vertebrate species and thus animals may also be susceptible to low doses of these compounds

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(Fick et al., 2010; Margiotta-Casaluci et al., 2014). Indeed, mounting evidence has revealed many aquatic animals to be susceptible to various pharmaceuticals at environmentally relevant concentrations (Arnold et al., 2014).

Antidepressants represent an extremely important class of pharmaceutical contaminant, with numerous studies demonstrating that these compounds may pose a particularly high level of risk for aquatic wildlife (Brodin et al., 2013). One specific area of interest is the ability for psychoactive pharmaceuticals to alter normal behavioural patterns, an outcome that has been observed in both vertebrate (Barry, 2013; Margiotta-Casaluci et al., 2014; Olsén et al., 2014) and invertebrate (De Lange et al., 2006; Hamilton et al., 2016) species. Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) are currently amongst the most widely prescribed medications for treating conditions associated with depression and anxiety (Cascade et al., 2007). These chemicals act by blocking neurotransmitter reuptake (i.e., serotonin and/or norepinephrine) by pre-synaptic neurons, resulting in greater concentrations in the synaptic cleft and consequently increased binding to receptors at post-synaptic neurons (Lambert and Bourin, 2014; Racagni and Popoli, 2008). In fish, documented responses to SSRI exposures have generally involved reductions in swimming velocity and associated impacts on feeding efficacy (reviewed in Melvin et al., 2016). Recent findings have confirmed linkages between observed behavioural effects and altered concentrations of serotonin in fish exposed to SSRIs (Bisesi et al., 2014, 2015). Importantly, several fundamental biological processes including the regulation of circadian rhythms are controlled via the neuroendocrine system (Urbanski, 2011; Wayne, 2000), and serotonin is known to play a role in patterns of sleep and wakefulness (Portas et al., 2000). Considering the established mechanism of action of these compounds it is therefore hypothesised that fish exposed to SSRI or SNRI antidepressants may exhibit alterations to normal circadian rhythmicity, but there have been no studies exploring such effects.

The potential for antidepressants to influence aquatic wildlife has recently come under scrutiny, due to uncertainty surrounding the validity of non-standard behavioural endpoints and concerns that existing studies may not be reproducible (Sumpter et al., 2014). Considering that in many cases conclusions have been based on observations of fish behaviour over extremely short temporal scales, for example ranging from 5–6 min (Dziewieczynski and Hebert, 2012; Egan et al., 2009; Wong et al., 2013), these concerns seem well founded. Exploring how antidepressants influence fundamental patterns tied to known neuroendocrine-mediated pathways is therefore significant, since this will yield insight about sub-lethal consequences of exposure for an established behavioural trait. A test was recently developed to quantify diurnal activity patterns in fish, providing a mechanism to explore well characterised neuroendocrine-mediated behavioural patterns (Melvin et al., 2016). The approach acquires behavioural data multiple times per hour and is capable of doing so over extended timeframes (e.g., several consecutive days), thereby providing increased confidence in study outcomes due to the incorporation of natural temporal behavioural variability in the subsequent statistical analysis (Melvin et al., 2016). In the context of the current scrutiny surrounding behavioural toxicity testing, this also provides a way to explore how different study timeframes might influence outcomes.

As a final consideration, due to the complex sources of pharmaceuticals it is now widely recognised that exposure of wildlife to these compounds can reasonably be expected to occur as mixtures (Backhaus et al., 2008; Galus et al., 2013). Chemical mixtures comprised of compounds with similar mechanisms-of-action, like SSRIs and SNRIs, are of particular interest because they are predicted to result in increased toxicity due to the inherent expectation of additive or synergistic effects (Bisesi et al., 2015). However, while SSRIs

and SNRIs are quite prevalent in the environment few studies have examined mixture toxicity with these compounds, and this further contributes to uncertainty surrounding the potential hazard that antidepressants might pose to aquatic wildlife. The present study aims to address some of these issues, by investigating alterations to circadian rhythms in mosquitofish (*Gambusia holbrooki*) exposed to the common antidepressants fluoxetine, sertraline and venlafaxine, both individually and when all compounds are present in a full tertiary mixture.

2. Methods

2.1. Chemical standards and stock preparation

Technical grade standards of fluoxetine (FLX), sertraline (SER) and venlafaxine (VEN) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Stock solutions were prepared just prior to use by dissolving 5 mg of each compound in either 1 mL of deionised water (FLX and VEN), or methanol (SER).

2.1.1. Experimental Fish

Adult mosquitofish (*Gambusia holbrooki*) were used for experiments since this species has a wide global distribution, and is known to exhibit diurnal activity patterns characterised by foraging during the daytime and inactivity at night (Pyke, 2005, 2008). Fish were collected from a small woodland pond located near Griffith University's Gold Coast Campus using a fine mesh dip-net, and were transported to the laboratory in buckets filled with water from the collection site. In the lab, fish were gradually acclimated to laboratory conditions, separated by sex and held for at least two weeks prior to experimentation. Water for holding tanks and exposure experiments was prepared according to the USEPA guidelines for moderately hard testing water (USEPA, 1994), and temperature and photoperiod were maintained at $22.9 \pm 0.9^\circ\text{C}$ and 12: 12-h light: dark, respectively. The Animal Ethics Committee at Griffith University approved the study, and all work was performed in accordance with the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (Protocol No. ENV/03/16/AEC).

2.1.2. Exposure protocol for quantifying circadian rhythms

Four separate experiments were performed to examine circadian rhythmicity in fish exposed to each of the three antidepressants individually, and when combined in a full tertiary mixture. Each experiment was carried out by placing 16 male mosquitofish into individual 12 L glass aquaria filled with 5 L of moderately hard test water. Male fish were used for the experiments based on previous findings indicating greater consistency in diurnal rhythms using male compared to female fish (Melvin et al., 2016). Experimental fish had mean weights and lengths of 15.2 ± 0.5 mg and 21.0 ± 2.2 mm, respectively. Each aquarium housed an individual test chamber connected to a 16-channel Multispecies Freshwater Biomonitor® (MFB; LimCo International GmbH, Konstanz, Germany). The MFB is a non-visual technology that measures disturbances to a weak electrical signal, thereby allowing behavioural assessment during both daytime and at night. For each exposure, baseline activity was recorded for a period of 24 hrs to ensure consistent and comparable diurnal rhythms in all fish.

Experiments were performed according to a previously described approach for quantifying diurnal activity patterns in fish (Melvin et al., 2016). Briefly, following baseline recordings the aquaria were randomly assigned to control, 1, 10 or 100 µg/L treatment groups (4 replicates of each treatment). Appropriate stock solutions for FLX, SER, or VEN were spiked at volumes of 1, 10 or 100 µL, with controls receiving 100 µL of deionised water (FLX and

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