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Varying the exposure period and duration of neuroactive pharmaceuticals and their metabolites modulates effects on the visual motor response in zebrafish (*Danio rerio*) larvae

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ABSTRACT

Pharmaceuticals and personal care products are emerging contaminants that are increasingly detected in surface waters around the world. Despite the rise in environmental detections, measured concentrations are still typically low, raising the importance of environmental risk assessments that focus on ecologically relevant sublethal endpoints, such as altered behavior. Neuroactive pharmaceuticals, like mental health medications, pain killers, etc., may be particularly potent in this regard as they are specifically designed to cause behavioral changes without causing physiologic impairment in mammalian systems. We screened 15 different popular neuroactive pharmaceuticals, ranging from antidepressants (including 3 major antidepressant metabolites), anxiety medications, and pain killers, under three different exposure scenarios (repeated, late acute and early transient exposure) to look for behavioral effects in larval zebrafish using the visual motor response (VMR). Drugs were screened at 0, 1, 10, and 100 μg/L in the repeated exposure scenario, and at 0 and 100 μg/L in the late acute and early transient exposure scenarios. Eight of the 15 compounds tested, specifically the antidepressants amitriptyline, fluoxetine, nor-fluoxetine, paroxetine, sertraline, nor-sertraline, venlafaxine, and the antipsychotic drug haloperidol decreased swimming activity by 25% to 40% under repeated exposure conditions. Five of the compounds (amitriptyline, fluoxetine, nor-fluoxetine, paroxetine, and sertraline) also significantly decreased activity by 17% to 31% in the late acute exposure paradigm. Three compounds (fluoxetine, paroxetine and venlafaxine) significantly altered swimming activity with early transient exposure, however creating a hyperactive response and increasing activity from 24% to 28%, while haloperidol significantly decreased activity by 31%. This paper is, to our knowledge, the first to screen so many neuroactive pharmaceuticals, including major metabolites, in parallel under multiple exposure conditions. We show that antidepressants most consistently alter VMR swimming activity. Additionally, we show that major antidepressant metabolites can potentially alter behavior as much as their parent compounds. Furthermore, we show that the magnitude and direction of behavioral effect is dependent on the exposure duration and period, indicating that a more diverse experimental approach might be needed to more accurately assess the risk these compounds pose to the environment.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are widely considered contaminants of emerging concern. Although PPCPs have almost certainly been released into the environment since the beginning of their use, it was only with recent technological advances in analytical methods that we have been able to detect their presence in the environment, with detailed reports on their environmental impact appearing only in the last 20 years (Williams, 2008; Calisto and Esteves, 2009). Among the diverse groups of compounds within PPCPs, neuroactive pharmaceuticals such as antidepressants, anxiety medications, and other psychiatric drugs may represent elevated environmental risk as they are designed to cause biological effect at low doses (Ankley et al., 2007), are sufficiently lipophilic to facilitate transport into the central nervous system and are relatively resistant to degradation (Calisto and Esteves, 2009).

There are multiple potential sources of neuroactive pharmaceuticals to the environment, such as manufacturing waste or disposal of unused medications, but it is widely accepted that excretion of ingested pharmaceuticals into municipal wastewater systems is the predominant

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source of pharmaceuticals to the environment (Williams, 2008). Various studies have shown that typical secondary wastewater treatment processes are ineffective at removing most pharmaceutical compounds (Wu et al., 2015; Metcalfe et al., 2010; Kolpin et al., 2002; Ternes et al., 2004). In contrast, more modern treatment procedures like advanced oxidative processes have demonstrated much greater removal efficiencies for certain neuroactive pharmaceuticals compared to others (Shao et al., 2018; Lajeunesse et al., 2013) Because of the current lack of government regulation of pharmaceutical discharges in the United States, there is little incentive for managers to invest in expensive upgrades to address pharmaceutical contaminants (Halling-Sørensen et al., 1998).

To effectively characterize the environmental risk of an emerging contaminant, researchers must understand the potential exposures and effects on wild organisms (Williams, 2008). Recent studies have detected a wide variety of neuroactive pharmaceuticals in surface waters all over the world, in sediment, and even animal tissues, indicating pervasive environmental contamination. While wastewater effluents are diluted by discharging into larger bodies of water, surface water measurements of some popular neuroactive pharmaceuticals like antidepressants and anxiety medications report detections at high nanogram to low microgram per liter levels (Alonso et al., 2010; Gurke et al., 2015; van der Aa et al., 2013). While not as well studied, sediments in urban estuaries and areas downstream of wastewater treatment plants also accumulate neuroactive pharmaceuticals at parts per billion levels (Lara-Martin et al., 2015; Schultz et al., 2010). Time series experiments have shown that sediment loads can persist despite changes in hydrologic conditions and surface water concentrations, making sediment a likely secondary source of exposure for aquatic organisms (Koba et al., 2018). Both caged fish and fish residing downstream of wastewater treatment plants show considerable bioaccumulation of neuroactive pharmaceuticals from parts per trillion levels in surface waters to parts per billion levels in fish tissues (Arnnok et al., 2017; Metcalfe et al., 2010; Schultz et al., 2010).

Most neuroactive pharmaceuticals range from short-lived to moderately persistent in the environment but continual human use can create a near constant supply of pharmaceuticals (van Nuijs et al., 2015; Calisto and Esteves, 2009) resulting in pseudo-persistence, increasing the risk of chronic or long-term effects to aquatic organisms (Schultz et al., 2011; Daughton and Ternes, 1999). While previous research has established the near ubiquity of neuroactive pharmaceuticals in urban water bodies worldwide (Jones et al., 2004), the activity of these compounds in environmentally exposed organisms is not well established, particularly at the low environmental concentrations observed.

The biological effects of low dose neuroactive pharmaceutical exposure are not well understood, requiring toxicity evaluation in a variety of organisms, which can be an overwhelming task. Furthermore, due to economic and resource constraints, it is imperative that we maximize our efficiency in evaluating emerging contaminants for ecological risk (Ankley et al., 2007). As neuroactive pharmaceuticals are designed to affect the central nervous system and its components, animal behavior is an obvious endpoint to focus on. Animal behavior is well suited for chemical risk assessment frameworks as behavioral assays are non-invasive and do not harm the study organism (Huang et al., 2015). Depending on the behavior of interest, behavioral assays can also be relatively easy to conduct and amenable to multiplexing. Behavior can be a sensitive indicator of altered physiological function and indicate toxicant effect before physiologic dysfunction is measurable (Sloman and McNeil, 2012), and in certain cases can pinpoint specific pathways that are impacted by toxicant exposure (Scott and Sloman, 2004). In addition to its practical utility, behavior is also an important ecological parameter. Even small changes in how an organism interacts with conspecifics, predators or prey can have population level effects. Toxicant exposure that causes behavioral impairment, rather than morphological or physiological impacts, may prevent an animal (or an entire population) from fulfilling ecosystem functions, leading to ecological death (Scott and Sloman, 2004).

Previous work established various behavioral anomalies that occur with neuroactive pharmaceutical exposure, however the existing literature represents only a handful of compounds among the hundreds of potential pharmaceutical contaminants. Furthermore, major metabolites of many neuroactive pharmaceuticals are regularly detected in the environment (Alygizakis et al., 2016; Gurke et al., 2015; Metcalfe et al., 2010; Lajeunesse et al., 2013), sometimes at concentrations higher than the parent compounds, and are oftentimes the dominant form of the pharmaceutical found to bioaccumulate in animal tissues (Arnnok et al., 2017). We are unaware of studies that directly expose study organisms to major metabolites of neuroactive pharmaceuticals, which represents a large gap in the literature. Additionally, the temporal patterns of exposure that wild fish experience with these pharmaceuticals is highly variable depending on the environmental context, which limits the ability of experiments testing only a single exposure pattern to predict environmental risk. The research presented here used the spontaneous behavior of an easily manipulated aquatic vertebrate, the zebrafish (*Danio rerio*), to screen 15 different neuroactive pharmaceuticals (including three common antidepressant metabolites), assessing the visual motor response (VMR), a simple but well characterized sequence of locomotory changes in response to a change in light conditions. We ran these experiments under three exposure scenarios – continuous (or repeated), late acute, and early acute exposure – to investigate the impact of exposure period and duration on behavior. We hypothesized that: (1) pharmaceuticals in the same class of drugs would affect behavior similarly, (2) major metabolite compounds would cause similar effects as their parent compounds, and (3) increased exposure through solution renewals would have greater behavioral effects than short term acute or transient exposures. This represents, to our knowledge, the most comprehensive side-by-side toxicity evaluation of these contaminants and their major metabolites to date.

2. Materials and methods

2.1. Exposure solutions

The pharmaceuticals tested include the tricyclic antidepressant amitriptyline, the selective serotonin reuptake inhibitors (SSRIs) fluoxetine, citalopram, sertraline, paroxetine (and the SSRI metabolites nor-fluoxetine and nor-sertraline), the norepinephrine dopamine reuptake inhibitor (NDRI) bupropion, the serotonin norepinephrine reuptake inhibitor (SNRI) venlafaxine (and its metabolite des-venlafaxine), the anticonvulsant carbamazepine, the benzodiazepine oxazepam, the opioid painkiller tramadol, and the antipsychotics haloperidol and risperidone (Table 1). These pharmaceuticals represent a wide range of neuroactive pharmaceuticals in current use. Pure pharmaceutical stocks were dissolved in methanol and stored at 4 °C. Working stocks were made by drying pharmaceutical stock aliquots with pure nitrogen gas and resuspended in an appropriate volume of embryo media (0.3 g/L Instant Ocean, 7.5 mg/L HCO_3^-, 1 ml/L methylene blue) to achieve a working stock concentration of 500 μg/L. Working stock was further diluted using embryo media to obtain final test concentrations of 1, 10, and 100 μg/L. The lowest test concentration roughly corresponds to typical high-end surface water concentrations measured in the published literature (Metcalfe et al., 2010; van der Aa et al., 2013), though occasionally environmental measurements > 100 μg/L have been made (typically outside of industrial plants), which is where we capped our test concentrations (Fick et al., 2009).

2.2. Fish husbandry

Adult wildtype zebrafish (a hybrid of Tubigen Longfin/Brian's wildtype strain) were maintained at 28.5 °C under 13/11 h light/dark cycle and were fed pellet food or newly hatched brine shrimp on alternating

Table 1
Neuroactive pharmaceuticals used in the larval zebrafish behavioral screen. The chosen pharmaceuticals represent a broad range of pharmaceuticals that are popular in current human use.

Drug class	Generic name	Brand name(s)	Commonly prescribed for	Early life stage fish behavior studies
Tricyclic Antidepressant	Amitriptyline	Elavil™	major depressive disorder, anxiety, attention deficit hyperactivity disorder, bipolar disorder, migraines, neuropathic pain, insomnia	
Selective Serotonin Reuptake Inhibitors (SSRI)	Fluoxetine	Prozac™, Sarafem™	major depressive disorder, obsessive-compulsive disorder, bulimia nervosa, panic disorder, premenstrual dysphoric disorder	Airhart et al. (2007); Chiffre et al. (2014); Cunha et al. (2018); Painter et al. (2009); Pelli and Connaughton (2015)
	Nor-Fluoxetine		Fluoxetine metabolite	
	Citalopram	Celexa™, Cipramil™	major depression, panic disorder, obsessive-compulsive disorder	Chiffre et al. (2014); Holmberg et al. (2011)
	Sertraline	Zoloft™	major depressive disorder, obsessive-compulsive disorder, panic disorder, anxiety disorder	Chiffre et al. (2014); Hedgespeth et al. (2014); Painter et al. (2009)
	Nor-Sertraline		Sertraline metabolite	
	Paroxetine	Paxil™, Seroxat™	major depressive disorder, obsessive-compulsive disorder, social anxiety disorder, panic disorder, posttraumatic stress disorder	
Norepinephrine Dopamine Reuptake Inhibitor (NDRI)	Bupropion	Wellbutrin™, Zyban™	depression, smoking cessation, attention deficit hyperactivity disorder, sexual disfunction, obesity	Painter et al. (2009)
Serotonin Norepinephrine Reuptake Inhibitor (SNRI)	Venlafaxine	Effexor™	major depressive disorder, generalized anxiety disorder	Painter et al. (2009); Thompson et al. (2017)
	Des-Venlafaxine	Pristiq™	Venlafaxine metabolite; major depressive disorder, menopause	
Anticonvulsant	Carbamazepine	Tegretol™	epilepsy, neuropathic pain, schizophrenia, bipolar disorder	Qiang et al. (2016); Weichert et al. (2017)
Benzodiazepine	Oxazepam	Serex™	anxiety, insomnia, alcohol withdrawal syndrome, posttraumatic stress disorder, premenstrual syndrome	Brodin et al. (2013); Chiffre et al. (2014); de Boissel et al. (2017)
Opioid	Tramadol	Ultam™	moderate to moderately severe pain (acute and chronic)	
Typical Antipsychotic	Haloperidol	Haldol™	schizophrenia, Tourette syndrome, bipolar disorder, nausea/vomiting, delirium, agitation, acute psychosis, alcohol withdrawal	
Atypical Antipsychotic	Risperidone	Risperdal™	schizophrenia, bipolar disorder, autism irritability	Kalichak et al. (2017)

days. 6 to 8 pairs of breeding adults were set up to ensure enough embryos were produced for each experiment. We ran three different exposure experiments: repeated exposure, late acute exposure, and early transient exposure. Fig. 1a presents a schematic comparing the three different exposure scenarios. Tests on each pharmaceutical using each exposure paradigm were carried out over two different days, where half the data was acquired using embryos or larvae collected from different pairs of adults spawned on different days to control for potential variation from confounding factors like batch effects from different parents or handling. All animal husbandry and experimental manipulation of larvae were approved by Stony Brook University's Institutional Animal Care and Use Committee (IACUC).

2.2.1. Repeated exposure experiment

We developed the repeated exposure scenario as a 'worst case' environmental exposure, where embryos were continuously exposed to pharmaceuticals, starting from potentially sensitive early developmental periods all the way to behavior screening. Fish embryos naturally have a protective chorion that helps protect the embryos from environmental stressors, including chemical contaminants. However, the precise time that fish emerge from their chorion is variable even within a clutch. Furthermore, it is unknown how efficiently many of the test compounds cross the chorion. For these reasons, we dechorionated embryos to remove a potential confounding factor in drug exposures to facilitate cross comparisons of different pharmaceuticals. Embryos were dechorionated at 3 h post fertilization (hpf) using a dilute protease solution (1 mg/mL Pronase; Sigma Aldrich). Embryos were then transferred to agarose lined 60 mm plastic petri dishes containing 15 mL of exposure solutions (1, 10, 100 μg/L pharmaceutical or control embryo water) in groups of 25. At 24 hpf, normally developing embryos were transferred into individual wells of a 48-well plate containing 1 mL of their respective exposure solution. All pharmaceuticals were run on individual plates including control fish. 80% of exposure solution was renewed daily until 5 dpf. Survival and development were tracked, and dead and severely deformed larvae were removed from the plate. Only batches with > 90% survival at 6dpf were used in the experiment (Supplemental Table 1). Final sample sizes were 22–24 larvae per dose treatment.

2.2.2. Late acute exposure experiment

We developed the late acute exposure experiment to test whether pharmaceutical treatment could alter behavior with limited exposure outside of a sensitive developmental period. This represents a relatively more conservative environmental exposure scenario. Embryos were collected from wild-type adults and divided into groups of 25 in agarose lined 60 mm plastic petri dishes. At 24 hpf, normally developing embryos were transferred to individual wells within a plastic 48-well plate, which contained 1 mL of embryo water. Survival and normal development were tracked until 6dpf. At 6 dpf, concentrated pharmaceutical stocks were added to the treatment wells to give an exposure concentration of 100 μg/L. Equal volumes of clean embryo water were added to control wells. Drugs were run in groups of three per plate with each group sharing control fish. Larvae were exposed for only 1 h at room temperature immediately before behavioral screening. Only batches with > 90% survival were used in the experiment (Supplemental Table 2). Final sample sizes were 23–24 larvae per drug treatment.

2.2.3. Early transient exposure experiment

We developed the early transient exposure experiment to test whether these pharmaceuticals could generate persistent changes in endpoints measured later. Despite exposing the embryos during critical developmental periods, we consider this exposure scenario the most conservative as the exposures washed off after 24 h. Embryos were collected and dechorionated with a dilute Pronase solution as described in section 2.3.1. Embryos were placed in groups of 25 in 60 mm agar lined plastic petri dishes and exposed to 15 mL of exposure solution

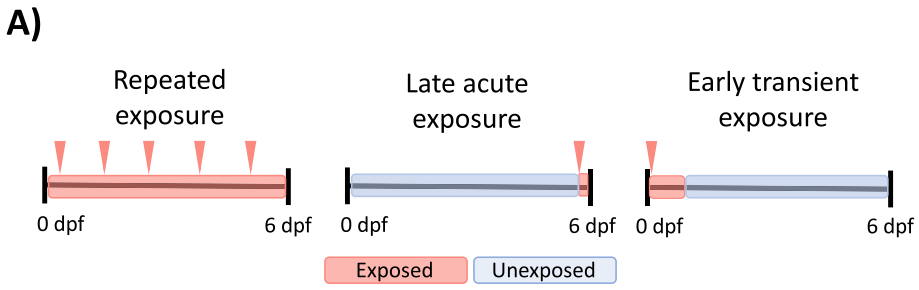

Fig. 1. The experimental design consists of three pharmaceutical exposure paradigms (A), which test different environmentally plausible exposure scenarios, including a repeated exposure (worst case scenario), late acute exposure (more conservative) and a transient early exposure (most conservative) design (B) The behavioral paradigm for our visual motor response (VMR), which consists of a 20 min acclimation period, 5 min of spontaneous swimming in light, and 5 min of swimming in dark.

(control or 100 µg/L of pharmaceutical or embryo water control) for 24 h. After 24 hours of exposure, embryos were transferred in minimal exposure solution (< 1 mL total) to 15 mL of clean embryo water and gently agitated to dilute exposure solutions. Embryos were transferred to another 15 mL of clean embryo water to further dilute any remaining exposure solution. Embryos were then transferred to individual wells in a plastic 48-well plate. Wells from each plate were equally divided between control fish and three other drug treatments. Survival and normal development were tracked until 6 dpf. Only batches with > 90% survival were used in the experiment (Supplemental Table 3). Final sample sizes ranged from 22 to 24 larvae per drug treatment.

2.3. Behavior paradigm

Behavioral screening was conducted in a Zebrabox imaging system (Viewpoint Life Sciences) at 6 dpf, always between 12 and 4 pm to reduce potential swimming behavior variability resulting from circadian rhythm. We monitored locomotor activity in the visual motor response (VMR) (Burton et al., 2017), which is a swimming response evoked by a sudden light change and is characterized by a sharp increase in activity after the light change that is sustained for a couple of minutes. Larvae were allowed 20 min to acclimate to the behavior system and then 5 min of spontaneous swimming in light conditions (pre-stimulus) were recorded. Lights were immediately turned off and 5 min of spontaneous swimming in the dark (post-stimulus) were recorded.

2.4. Data analysis

All data analyses were conducted in RStudio (CRAN). Total number of movements were recorded for each individual fish with data binned in 60 s increments. Data from individual pharmaceuticals were analyzed using a two-way repeated measures ANOVA, using drug treatment and light condition (pre/post-stimulus) as fixed factors. We analyzed the repeated measures of each individual fish, which gives us a measure of the baseline variability within an individual, as a random factor to control for pseudoreplication. Drug doses for the repeated exposure experiment were run as a continuous variable. A p-value of 0.05 was used as a threshold for statistical significance and a pharmaceutical was considered to significantly alter behavior if either drug or drug and light condition interaction terms were statistically significant.

3. Results

3.1. Repeated exposure experiment

Data from the repeated exposure experiment are shown in Fig. 2 and a summary of the results from the repeated measures ANOVA are presented in Supplemental Table 1. Seven of the 15 compounds tested significantly altered spontaneous swimming activity during the VMR assay. The antidepressants amitriptyline (Fig. 2a), fluoxetine (Fig. 2e), paroxetine (Fig. 2i), sertraline (Fig. 2k), and venlafaxine (Fig. 2n), as well as two of the antidepressant metabolites, nor-fluoxetine (Fig. 2f) and nor-sertraline (Fig. 2l), significantly affected spontaneous swimming by causing hypoactivity, reducing the number of movements anywhere from 25% to 40%.

Additionally, amitriptyline, nor-fluoxetine, sertraline, nor-sertraline, and the antipsychotic drug haloperidol (Fig. 2g) all had a significant interaction between drug exposure and light change. Amitriptyline had only modest effects on behavior in light conditions but had a much larger effect after the light change, showing a dose dependent decrease in the number of movements. In contrast, haloperidol treatment resulted in hypoactivity in light conditions, which appears to be nearly abolished after the light change. Behavior of nor-fluoxetine, sertraline, and nor-sertraline exposed larvae showed greater changes between the different doses in light conditions, but after the light change only the highest treatment was notably different from the control and lower dose treatments. While not statistically significant, bupropion (Fig. 2b), citalopram (Fig. 2d), and des-venlafaxine (Fig. 2o) increased activity on average from 10% to 30%, while tramadol (Fig. 2m) on average decreased activity up to 11% and had drug/light condition interaction terms that were nearly significant, suggesting potential effects of these drugs in affecting swimming behavior under certain conditions.

3.2. Late acute exposure experiment

In the late acute exposure experiment, 5 antidepressants significantly reduced swimming behavior (Fig. 3, Supplemental Table 2). Amitriptyline (Fig. 3a), fluoxetine (Fig. 3e), nor-fluoxetine (Fig. 3f), paroxetine (Fig. 3i), and sertraline (Fig. 3k) exposure led to a significant hypoactive response, reducing swimming activity anywhere from 17% to 30% from controls. In contrast to the results in 3.1, only amitriptyline and fluoxetine had significant drug/light change stimulus interaction. While amitriptyline and fluoxetine treatment alone did not alter swimming behavior, drug treatment coupled with the evoked light change stimulus caused significant hypoactivity, greatly magnifying the difference between treatment and control fish. While not statistically significant, the NDRI drug bupropion (Fig. 3b) caused a consistent hyperactive response in both light and dark conditions, increasing the average number of movements by over 20% from controls.

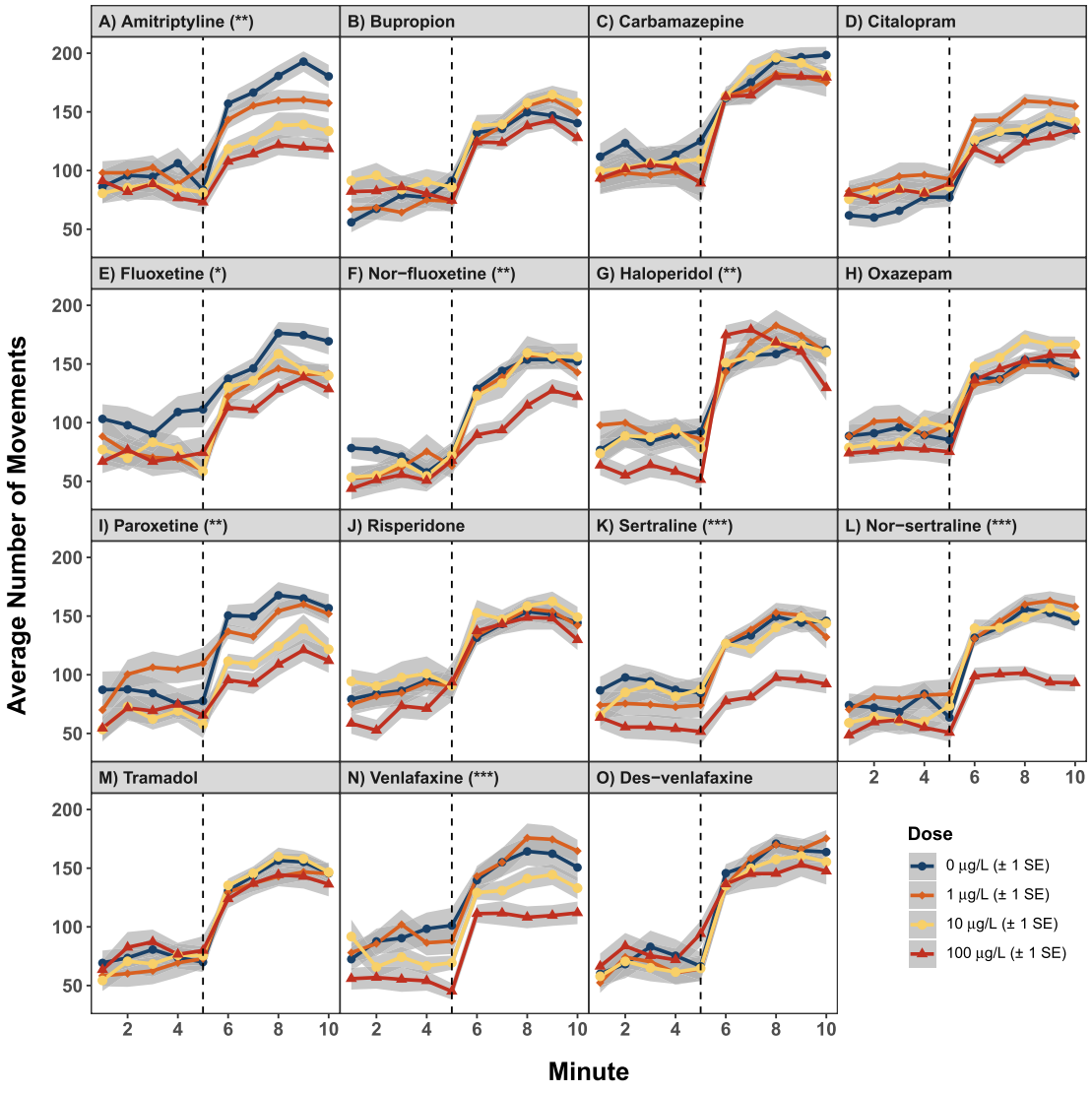

Fig. 2. Average number of movements made per minute in the visual motor response (VMR) with repeated exposure to pharmaceuticals. The dotted vertical line indicates the moment of light change: points to the left of the line are in full light conditions, points to right of the line are in darkness. Significance notation is as follows: < 0.1 (.), < 0.05 (*), < 0.01 (**), < 0.001 (***). There was a significant effect of drug exposure on VMR in fluoxetine, paroxetine, and venlafaxine treated larvae. There was a significant interaction between drug exposure and the light change stimulus on VMR in amitriptyline, nor-fluoxetine, sertraline, and nor-sertraline exposed larvae. The full two-way ANOVA results are summarized in Supplemental Table 1. N = 22–24 larvae.

3.3. Early transient exposure experiment

Four of the 15 drugs significantly altered swimming behavior in the early transient exposure experiment: fluoxetine, haloperidol, paroxetine, and venlafaxine (Fig. 4 and Supplemental Table 3). In contrast to the repeated and late acute exposure experiments, venlafaxine (Fig. 4n) and fluoxetine (Fig. 4e) induced a significant hyperactive response in both light and dark conditions, increasing the average number of movements from controls by roughly 25%. In contrast, haloperidol (Fig. 4g) caused hypoactivity in light conditions, decreasing the number of movements by about 31% before returning to control levels after the light change. Carbamazepine (Fig. 4c) exposure similarly decreased swimming activity before the light change but was not statistically significant ($p = 0.055$). Early exposure to paroxetine interestingly caused a hyperactive response in light conditions, increasing swimming activity up to 24%, but led to a hypoactive response after the light change, decreasing the number of movements by up to 13%.

4. Discussion

4.1. Antidepressants most consistently affected VMR

Our first hypothesis, that drugs from the same class would have similar effects on swimming behavior, was not supported by our data. While our data shows that antidepressants most consistently altered spontaneous locomotion in our three exposure scenarios, the different antidepressant classes (e.g. tricyclics, SSRIs, NDRIs, SNRIs, etc.) had considerable variation in behavioral effects, such as whether effects were readily observed without an environmental stimulus or if effects were only noticeable or more pronounced (or abolished) when coupled with the light change. Even within the same class of antidepressants, such as SSRIs, we see discrepancies in behavioral effects. Despite supposedly having the same physiological mechanism, the SSRI compounds show different behavioral effects in our study. Fluoxetine (Fig. 2e) and paroxetine (Fig. 2i) show decreasing activity with increasing dose, particularly after the light change where fish exposed to even the lowest dose treatment show notable decreases in activity as compared to control fish. In contrast, only fish exposed to the highest

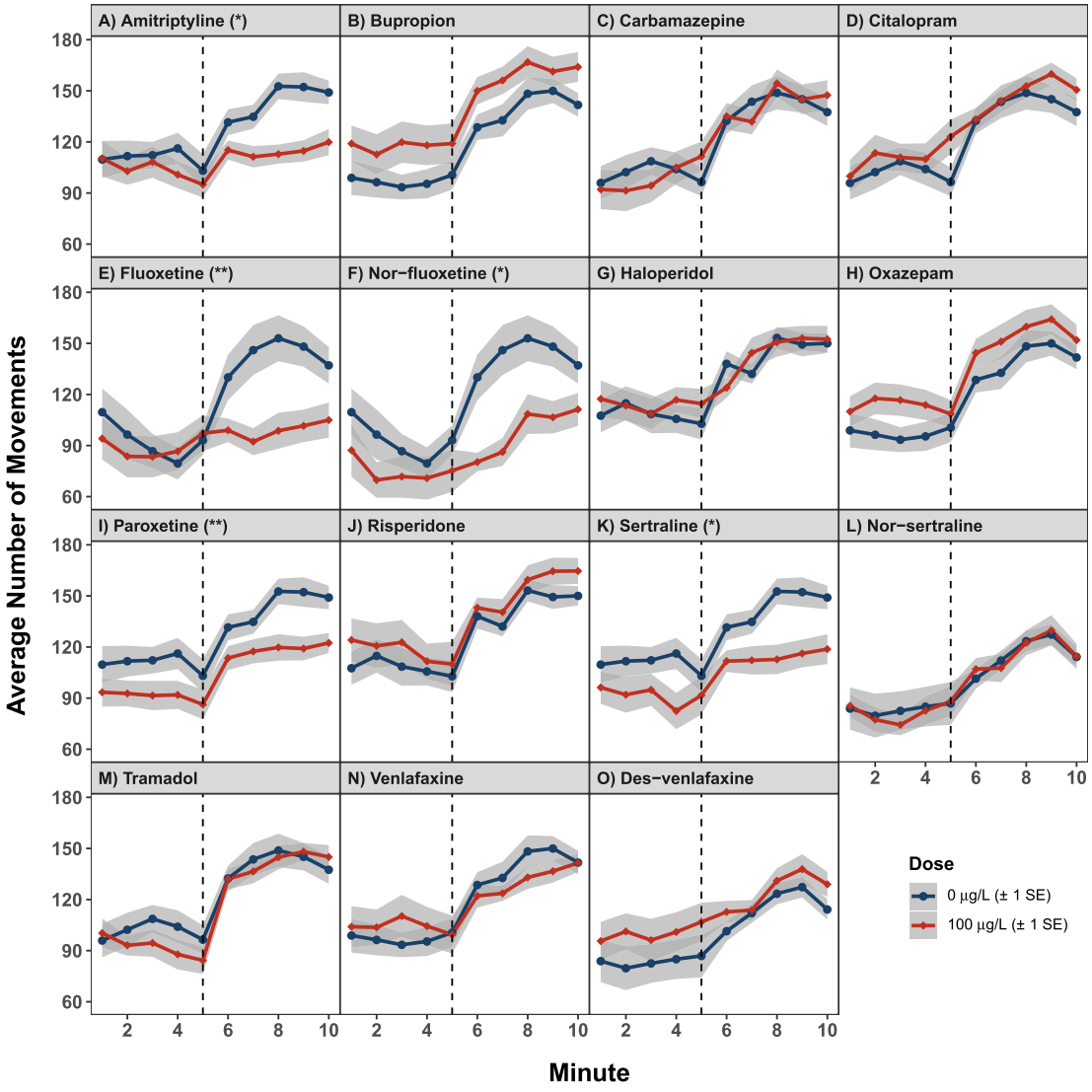

Fig. 3. Average number of movements made per minute in the visual motor response (VMR) with late acute exposure to pharmaceuticals. Dotted vertical line indicates the moment of light change: points to the left of the line are in full light conditions, points to right of the line are in darkness. Significance notation is as follows: < 0.1 (.), < 0.05 (*), < 0.01 (**), < 0.001 (***). There was a significant effect of drug exposure on VMR in amitriptyline, nor-fluoxetine, paroxetine, and sertraline treated larvae. There was a significant interaction between drug exposure and the light change stimulus on VMR in fluoxetine exposed larvae. The full two-way ANOVA results are in Supplemental Table 2. N = 23–24 larvae.

dose of sertraline (Fig. 2k) show hypoactivity after the light change, while fish exposed to citalopram (Fig. 2d) do not show any behavioral effects at all. This illustrates a potential complication for environmental risk assessment as we may not be able to assume that drugs which supposedly have the same chemical mechanism, or drugs that are used to treat the same or similar medical conditions, will have the same biological effect in non-target organisms.

The observed hypoactive effects of antidepressant exposure on larval fish behavior in our repeated exposure experiment are consistent with other recent studies that exposed early life stage fish for prolonged periods. Zebrafish larvae exposed to fluoxetine from early development to 80 hpf showed decreased tail reflexes in response to touch stimuli (Cunha et al., 2018). Similarly, prolonged embryonic exposures to fluoxetine and venlafaxine decreased predator escape responses of fathead minnows at 12 days post hatch (Painter et al., 2009), and 72-h exposures of citalopram, sertraline, and fluoxetine altered the spontaneous swimming of larval Japanese medaka (*Oryzias latipes*) (Chiffre et al., 2014).

The results of our late acute exposure experiment are also generally supported by recent published literature. Adult zebrafish exposed to fluoxetine and paroxetine for 5 to 15 min showed significant increases in latency to move, decreases in the number of grid crossings, and decreases in social behavior (Magno et al., 2015; Giacomini et al., 2016). Our study expands on these findings and shows several other antidepressant pharmaceuticals can potentially alter fish behavior with very short exposure times.

The results from our early acute experiment are particularly of interest as these types of experiments can provide evidence of delayed or persistent effects of brief exposures as well as key developmental periods that are sensitive to pharmaceutical exposure, all of which could be of great importance to environmental managers. However, experiments studying transient exposures during early development are not well represented in the literature. Airhart et al. (2007) found that 24-h fluoxetine exposures in zebrafish larvae occurring before 4dpf led to significant but transient hypoactivity, showing decreased activity up to 7dpf before swimming behavior became indistinguishable from that of control fish.

Several the pharmaceuticals that did not significantly alter behavior in our study have been shown to alter fish locomotion in other recent studies. Calcagno et al. (2016) and Nassef et al. (2010) reported

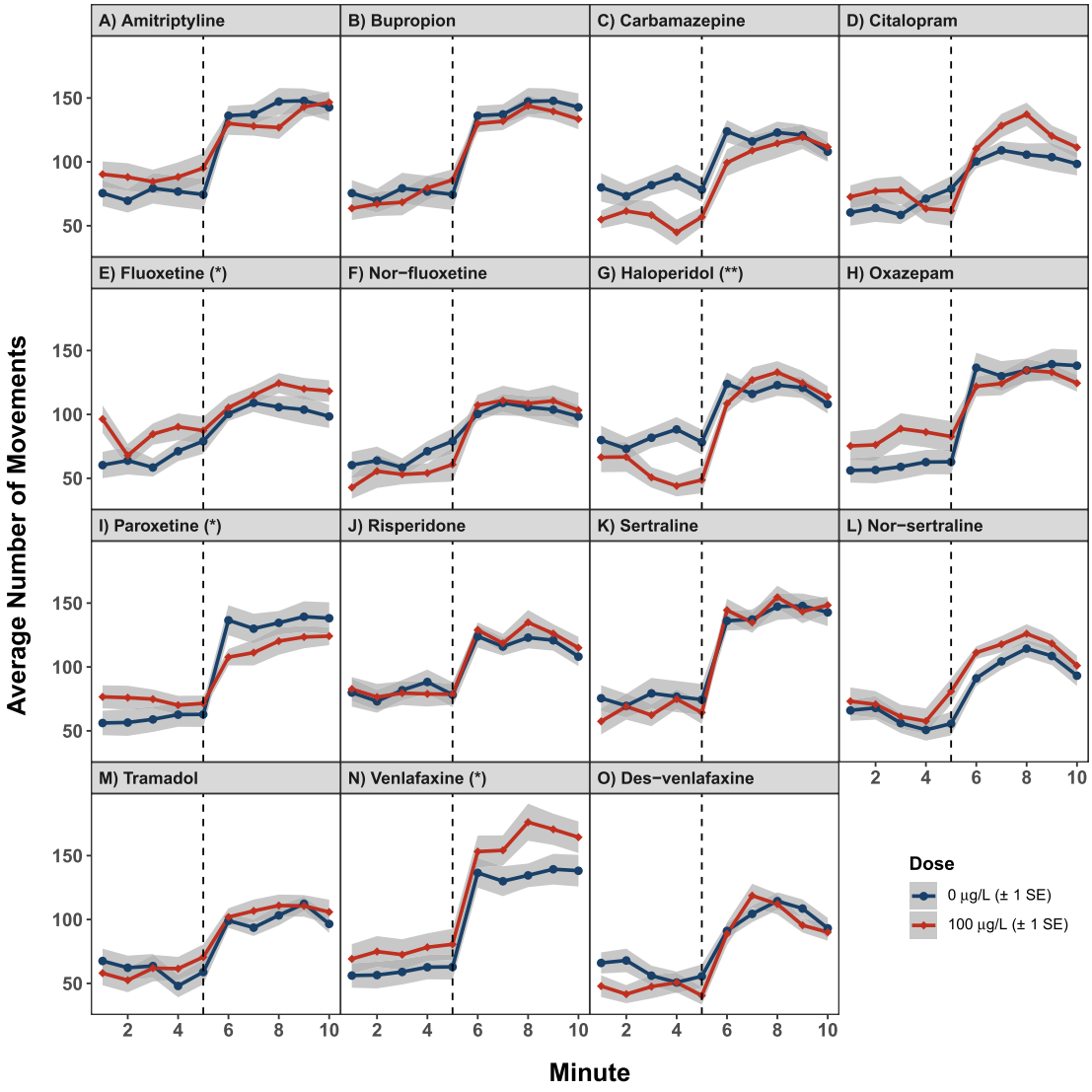

Fig. 4. Average number of movements made per minute in the visual motor response (VMR) with early transient exposure to pharmaceuticals. Dotted vertical line indicates the moment of light change: points to the left of the line are in full light conditions, points to right of the line are in darkness. Significance notation is as follows: < 0.1 (.), < 0.05 (*), < 0.01 (**), < 0.001 (***). There was a significant effect of drug exposure on VMR in fluoxetine and venlafaxine treated larvae. There was a significant interaction between drug exposure and the light change stimulus on VMR in haloperidol and paroxetine exposed larvae. The full two-way ANOVA results are in Supplemental Table 3. N = 22–24 larvae.

anxiolytic effects of carbamazepine using higher doses and longer exposure times in adult *Jenynsia multidentata* and Japanese medaka, respectively. In our study, we found that while not significant, citalopram and oxazepam showed a trend toward altered swimming behavior in conjunction with the light change stimulus, while other studies report significant effects of citalopram on locomotion and novel environment tests (Chiffre et al., 2014; Kellner et al., 2016; Olsen et al., 2014) as well as oxazepam in locomotion and exploratory behaviors (Brodin et al., 2013; de Boissel et al., 2017; Huerta et al., 2016). Additionally, while haloperidol caused significant hypoactivity in our repeated exposure experiment, late acute exposure did not, which contrasts Giacomini et al. (2006)'s findings where 7dpf zebrafish larvae exposed to haloperidol for 2 h caused a significant decrease in swimming speed. However, these studies differ from ours in various key ways, including using higher exposure concentrations, different exposure durations, different behavioral endpoints of interest, and different life stages (which impacts nervous system maturation), all of which may contribute to the varying results observed. These responses in diverse study systems focusing on different behaviors illustrate the complexity of the neurobehavioral system and how different assays capture different aspects of neurobehavior, making it difficult to predict ecologically meaningful impacts of pharmaceuticals from studies with limited or narrow focus.

4.2. Antidepressant metabolites can affect the swimming behavior of larval zebrafish

Our study is unique (to our knowledge) in that we evaluated the behavioral effects of major metabolites of antidepressant drugs, including nor-fluoxetine, nor-sertraline, and des-venlafaxine side by side against their parent compounds. Our results generally support our second hypothesis that major metabolites of neuroactive pharmaceuticals can significantly alter fish behavior as much as their parent compounds (Figs. 2f, l, 3f). However, responses were not always the same between parent and metabolite, indicating we can not necessarily predict the impact of one from the other. Regardless, our data clearly supports the need for environmental managers to conduct environmental risk assessments on major metabolites, as well as their parent compounds.

4.3. Exposure period and duration influence the effects of pharmaceuticals on swimming behavior

The repeated exposure experiment had the greatest effect on swimming behavior as over half of the pharmaceuticals screened (eight out of 15) significantly altered swimming behavior as opposed the five and four in the late acute and early transient exposure, respectively. However, we found that the relationship between exposure and behavioral effect was more complicated than anticipated, and that the timing of exposure, particularly the onset and duration of exposure, modulates the potential behavioral effects of these pharmaceuticals. Significant effects in one exposure scenario do not necessarily carry over to other exposure scenarios. For instance, in our study fluoxetine (Figs. 2e, 3e), paroxetine (Figs. 2i, 3i), and venlafaxine (Figs. 2n, 3n) exposure consistently caused hypoactivity in the repeated exposure experiment, but all three compounds had an opposite effect in the early transient exposure experiment, causing hyperactivity (Fig. 4e, i, n). Given the differences in the maturity of the zebrafish nervous system at 1 dpf and 5 dpf it is not surprising that the effects of pharmaceuticals would differ so much. Other studies have also shown how pharmaceutical effect varies with exposure period or duration. Fluoxetine exposure before 4 dpf led to transient changes in locomotor behavior, but exposures on 4 dpf and older larvae led to robust and longer lasting locomotor behavior changes (Airhart et al., 2007). Similarly, fluoxetine exposure in fathead minnow larvae (*Pimephales promelas*) only decreased predator escape response when exposed at embryonic stages and had no effect when exposed as larvae, whereas bupropion had the opposite and only decreased escape response when exposed as larvae, not as embryos (Painter et al., 2009). Furthermore, studies of other chemical contaminants, such as methylmercury, show that variation in effects from varying exposure period and duration are not limited to neuroactive pharmaceuticals (Weis and Weis, 1995), suggesting this might be a sensitive feature of fish development and behavior in general.

Potential causes of the change from hypoactivity observed in the repeated and late acute exposure experiments to hyperactivity observed in the early acute exposure experiment for some of the pharmaceuticals could potentially be explained by the complex relationships between specific neurotransmitters (like serotonin) and the behaviors being studied as well as the timing of exposure. This change from depressive-like behaviors (decreased locomotion with repeated exposure) to anxiety-like behaviors (increased locomotion with early acute exposure) has been observed in other studies in fish, where acute or short-term exposure to antidepressants have shown to cause increased anxious or stressed behavior characterized by increased thigmotaxis (Chiffre et al., 2014), increased preference for and time spent in dark refuges (Magno et al., 2015), decreased boldness and exploration (Hedgespeth et al., 2016), and increased aggression and territoriality (Villeneuve et al., 2010; Sebire et al., 2015). This changing effect is not uncommon with antidepressant drugs as mammalian studies and clinical data have shown that antidepressant treatment can often increase or exacerbate anxious behaviors, particularly during the early or acute phase of treatment (Zienowicz et al., 2006). This is hypothesized to be related to the dual nature of serotonin (the main neurotransmitter targeted by most antidepressant drugs), where increased serotonin levels can have differing effects in different behavior tests, such as light/dark preference tests or novel tank tests (Herculano and Maximino, 2014). This contradiction between anxiolytic and anxiogenic effect is not limited to antidepressants and is also seen in other classes of neuroactive pharmaceuticals such as antipsychotics like haloperidol (Magno et al., 2015; Villeneuve et al., 2010), which in our study generally caused hypoactivity.

4.4. VMR behavior in a broader context

While behavior is an important endpoint to consider, particularly for studies focusing on sublethal and chronic exposure to contaminants, it is nevertheless important to consider the reliability of the assay and the ecological validity of the behaviors measured (Parker, 2016). Relating changes in simple locomotory behaviors to more complex behaviors and population level parameters is not straight forward, and this lack of environmental applicability is an important limitation of the VMR assay. Weis and Weis (1995) showed that while methylmercury exposure to killifish (*Fundulus heteroclitus*) created hypo- or hyper- active larvae depending on the exposure period, both effects increased risk of predation, illustrating how difficult it can be to extend results of laboratory behaviors to more ecologically relevant systems.

However, that is not to say behavior assays like the VMR assay do not have their merit. The VMR assay is relatively easy to conduct and analyze, allowing researchers to answer broad questions regarding toxic potential or relative potency, like the work presented here. Many studies have shown through quantitative models that contaminant exposure can potentially alter population persistence through subtle changes in individual behavior (Murphy et al., 2008). Furthermore, like all other behaviors, the VMR integrates signals from multiple physiological systems and can be an indicator of adverse physiological effects (Kramer et al., 2011). By connecting locomotory responses to molecular responses or other more complex behaviors through mechanistic links and statistical relationships, we can further extend the utility of simple behaviors such as the VMR. In addition to carrying out different assays focusing on more complex and ecologically relevant behaviors, future work should also focus on elucidating the neural and molecular pathways that determine basic motor behaviors like VMR, which could greatly expand the ecological relevance of simple behavior assays.

5. Conclusions

Our study demonstrated a variety of antidepressants, including some of their major metabolites, as well as an antipsychotic drug can induce changes in larval zebrafish behavior in a variety of exposure scenarios. Although hypoactivity was the predominant response observed as expected, large variations in response was observed between individual, even those thought to share a common mechanism of action. Metabolites are potentially as potent as parent compounds in inducing behavioral changes and thus are an important component that needs to be included in environmental assessments. We've shown that the period and duration of exposure is also an important factor in determining the behavioral effect, which highlights the importance of understanding and properly characterizing exposure scenarios to best predict toxic responses. Future studies should continue screening the vast array of pharmaceuticals that are being released into the environment for easy cross comparison as well as test a wider range of animal behaviors to best assess potential effects of pharmaceutical contamination. Effort should be placed on tracking physiological endpoints to better understand how pharmaceutical exposure might initiate changes in behavior while more ecologically relevant behaviors like feeding and predator avoidance should be included to better translate laboratory studies to the environment.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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