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Acute exposure to waterborne psychoactive drugs attract zebrafish

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ABSTRACT

Psychotropic medications are widely used, and their prescription has increased worldwide, consequently increasing their presence in aquatic environments. Therefore, aquatic organisms can be exposed to psychotropic drugs that may be potentially dangerous, raising the question of whether these drugs are attractive or aversive to fish. To answer this question, adult zebrafish were tested in a chamber that allows the fish to escape or seek a lane of contaminated water. These attraction and aversion paradigms were evaluated by exposing the zebrafish to the presence of acute contamination with these compounds. The zebrafish were attracted by certain concentrations of diazepam, fluoxetine, risperidone and buspirone, which were most likely detected by olfaction, because this behavior was absent in anosmic fish. These findings suggest that despite their deleterious effects, certain psychoactive drugs attract fish.

1. Introduction

Psychotropic medications such as antidepressants, antipsychotics and anxiolytics are widely used (Bocquier et al., 2008) and its prescription has increased worldwide in the last 20 years (Carta et al., 2004; Paulose-Ram et al., 2007; Alonso et al., 2004; la Poza et al., 2013). Consequently, increasing its presence in aquatic environments (Santos et al., 2007) which are monitored especially in urban and hospital wastewater, effluent from water and sewage treatment plants, surface and drinking water (Calisto et al., 2011; Al Aukidy et al., 2012). The main concern is that these contaminants may cause toxicity, affecting the health of non-target humans and animals. Also, many of these drugs are resistant to wastewater treatments and are only partially removed (Palmer et al., 2008; Silva et al., 2011).

The most commonly prescribed, consumed, and consequently detected drugs in aquatic environments are benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), buspirone, risperidone, and ethanol. Benzodiazepines, such as diazepam and clonazepam, potentiate $GABA_A$ receptor function by increasing the channel opening frequency, producing hypnotic effects by acting on the α1 subunit (McKernan et al., 2000) and anxiolytic effects by acting on the α2 subunit (Löw et al., 2000). Fluoxetine is a potent and highly selective inhibitor of the transporter for serotonin reuptake at the presynaptic membrane, causing increases in serotonin concentrations at postsynaptic receptor sites (Wong et al., 1995). Buspirone exerts anxiolytic effects by acting as a partial agonist at serotonin 5-HT1A receptors (Ohlsen and Pilowsky, 2005), and it also interacts to a lesser degree with other receptors, such as the dopamine D2 receptor (Dhavalshankh et al., 2007). The antipsychotic drug risperidone belongs to the benzisoxazole chemical class (Kumar et al., 2008; Courchesne et al., 2007) and has been reported to act therapeutically by blocking serotonin and dopamine receptors (Grant, 2007); thus, it is useful for studying increases in serotonin neurotransmission. Ethanol also has acute anxiolytic effects that are most likely mediated by $GABA_A$ receptors (Radcliffe et al., 1999; Kumar et al., 2009), with depressant effects on the central nervous system at higher doses.

Although the concentrations of these drugs in aquatic environments are lower than the lethal concentrations for most of the species present in these ecosystems, studies have shown that their concentrations in organs such as the brain, liver and muscles are higher than those in the water (Brodin et al., 2013; Brooks et al., 2005; Sackerman et al., 2010). Benzodiazepines and SSRIs may trigger a set of morphological, physiological, neuroendocrine, reproductive, motor and behavioral changes (Brodin et al., 2013; Sackerman et al., 2010; Airhart et al., 2007; Gebauer et al., 2011; Park et al., 2012; Prieto et al., 2012; Abreu et al., 2014; Idalencio et al., submitted for publication).

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Since these psychoactive drugs are potentially dangerous to fish, we posed the following question: are these drugs attractive or aversive to fish? To answer this question, adult zebrafish were placed into a chamber that allowed them to avoid or to swim into a lane containing contaminated water. This enabled the evaluation of the attraction and aversion paradigm in zebrafish exposed to acute contamination of these compounds.

2. Methods

2.1. Ethics statement

This study was approved by the Ethics Commission for Animal Use (CEUA) at the Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol 29/2014-CEUA) and met the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Subjects

A mixed-sex stock population of adult wild-type zebrafish (*Danio rerio*) from the short-fin (SF) strain was used. In the experiment 1, ten fish were subjected to each substance treatment, totalizing 210 fish (21 treatments, each with 10 fish). In the 2nd experiment, ten anosmic fish were subjected to the substances that are attractive or aversive in the 1st experiment and also a saline only control, thus, a total of 200 fish were used in this study.

The fish were fed twice per day at 10:00 and 16:00 h with a commercial flake food until satiation (Alcon® Basic, MEP 200 Complex, Brazil). The mean water temperature in the holding tank was maintained at 24 ± 2 °C, and the dissolved oxygen concentrations varied from 5.6 to 7.2 mg/l (both measured using YSI model 550A oxygen meter; Yellow Springs Instruments, USA). The pH values ranged from 6.2 to 7.4 (measured using a Bernauer pH meter). The total ammonia–nitrogen concentration was less than 0.5 mg/l (measured using a colorimetric test).

2.3. Substances

Clonazepam (Rivotril®), diazepam (União Química, Brazil), fluoxetine (Daforin, EMS), risperidone (Risperidona, EMS), buspirone (Ansitec®, LIBBS) and ethanol were purchased from common commercial suppliers. The details of the substances examined in the experiment are listed in Table 1. The food odor positive controls were prepared using two distinct methods. Positive control 1 was prepared by adding flaked food to the water at a rate of 0.5 g/l, followed by the homogenization and the immediate use of the mixture in specific test trials. Positive control 2 differed from positive control 1 only in that the flaked food remained in the water overnight (12 h) before the mixture was homogenized and used in specific test trials.

2.4. Experimental apparatus

The experimental apparatus consisted of a modified, 30-liter acrylic tank (50 × 25 × 25 cm, length × width × height). Metal mesh was added to prevent the fish from escaping the tank. A short segregation panel and a fine mesh baffle were inserted at the other end of the tank to create two chambers leading to two lanes of water with laminar flow run in parallel without mixing. See the schematic drawing of the apparatus in Fig. 1A and the dye (gentian violet) colored confirmation of laminar flux for all substances in Fig. 1B. The use of the dye aimed to verify if the separate flux was maintained in all drug tests, and drugs were not mixed to the dye during the experiments. A flow rate of 2 l/min was used for each track, and the manifold for each mixing chamber had a single door to allow for the introduction of the test substance.

2.5. Experimental protocol

In experiment 1, individual fish were transferred from the holding tank in a small volume of water. After transfer, the fish were allowed to acclimate for 150 s, and a continuous dose of the test compound was subsequently introduced into one of the mixing chambers for 150 s at a predetermined concentration. During the tests, fish were not fed. The position (left or right) of the clean and contaminated water lanes was switched between each of the trials to prevent a possible bias caused by a fish preference for the left or right lane. The horizontal gradient created by the laminar flow within the tank allowed for the untreated lane to remain uncontaminated, thus creating two lanes between which the fish could move freely (Readman et al., 2013). Following each single fish testing, the system was manually flushed to remove any test substance residue. The location and locomotor activity of the fish with access to both the treated and untreated lanes were recorded with a video camera for the entire experimental period. The video camera was positioned directly above the tank. The analysis of the video recordings was conducted using ANY-maze® video tracking system (Stoelting Co., USA) for both the 150-s acclimation period and the 150-s exposure period to show that the fish responded only after substance introduction, and the results for each test substance were analyzed separately.

The experiment 2 reproduces the 1st one but using zebrafish with temporary anosmia by the application of lidocaine gel (50 mg/g) in the nares and olfactory surface as described by Johansen (Johansen,

Table 1
Effects of substances and concentrations.

Substance	Concentration	Effect	Reference
Water (control)	–	–	–
pH 3 (Trichloroacetic acid)	pH 3	Escape behavior	Readman et al. (2013)
Ethanol	1%	Neuroendocrine changes	Oliveira et al. (2013)
Ethanol	0.5%	Neuroendocrine changes	Oliveira et al. (2013)
Ethanol	0.25%	Neuroendocrine changes	Oliveira et al. (2013)
Clonazepam	0.057 μg/l	Ambient concentration	Almeida et al. (2013)
Clonazepam	300 μg/l	Behavior changes	Gebauer et al. (2011)
Diazepam	160 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Diazepam	16 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Diazepam	0.88 μg/l	Ambient concentration	Calisto and Esteves (2009)
Fluoxetine	50 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Fluoxetine	25 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Fluoxetine	1 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Risperidone	0.00034 μg/l	Ambient concentration	Calisto and Esteves (2009)
Risperidone	100 μg/l	Behavior changes	Magno (2012)
Risperidone	170 μg/l	Neuroendocrine changes	Idalencio et al. (submitted for publication)
Buspirone	10 μg/l	Behavior changes at 1% concentration	-
Buspirone	1000 μg/l	Behavior changes	Gebauer et al. (2011)
Buspirone	3000 μg/l	Behavior changes	Gebauer et al. (2011)

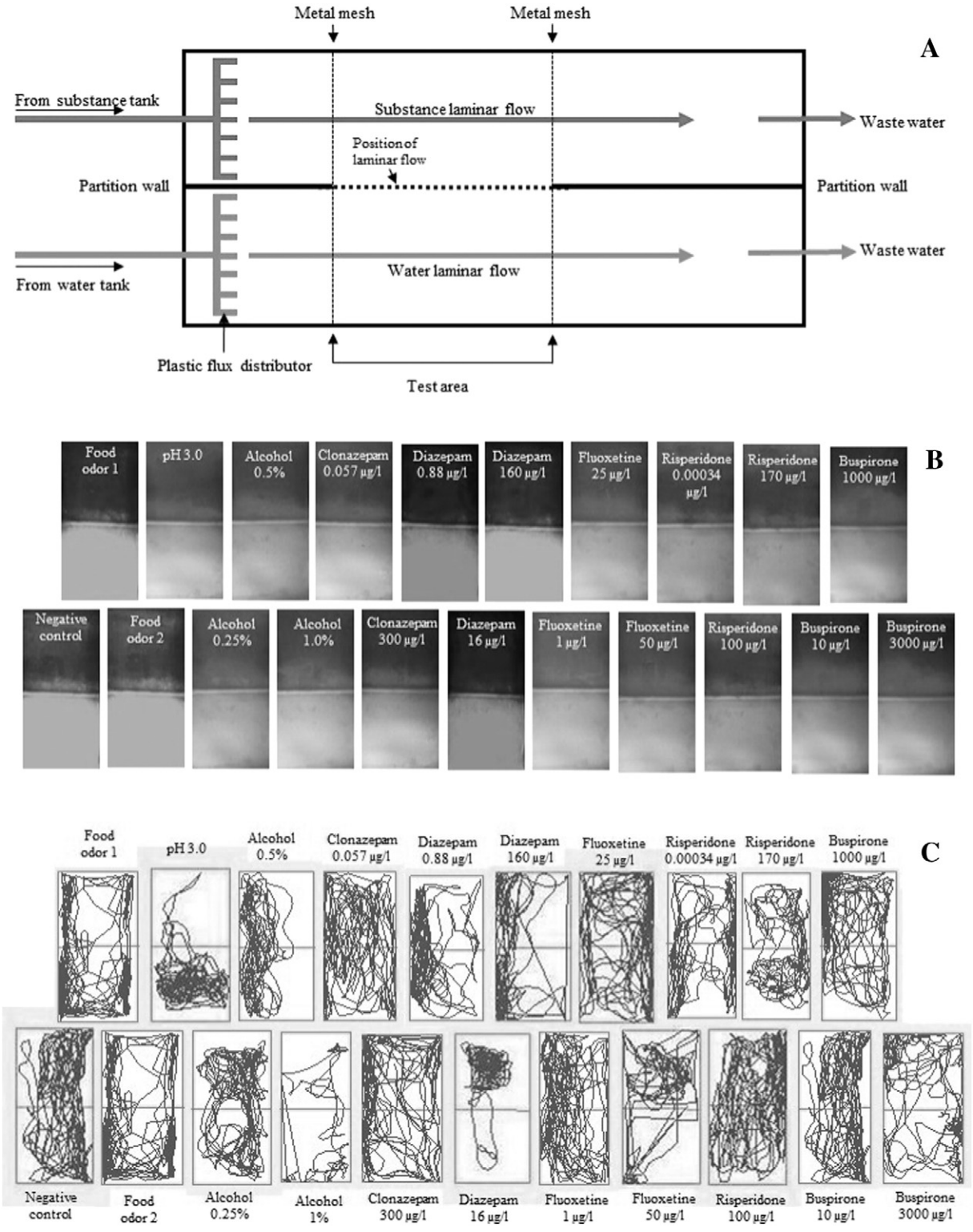

Fig. 1. Schematic representation of the test chamber (A), photographic confirm maintenance of laminar flow during dosage. Images show the stability of laminar flow during dosing. Each compound is dosed with violet as an indicator in order to follow the progression of the compound (B) and representative video tracking the movement of the zebrafish in each treatment (C).

1985). Briefly, each zebrafish was captured and placed on a wet sponge, and the lidocaine gel was gently applied with cotton into the nares. Then, each fish was returned to the aquarium and used immediately in the experiment. To control the influence of the procedure, we repeat the exact temporary anosmic protocol, but with only saline solution in the cotton. The substances tested were those that are attractive to zebrafish in the experiment 1 (diazepam 16 and 160 µg/l, fluoxetine 25 and 50 µg/l, risperidone 100 µg/l and buspirone 1000 µg/l), plus the control situations. As in experiment 1, the time spent in each lane was evaluated. Temporary anosmia is an effective technique to study olfactory participation in odorant detection such as sex pheromones (Souza et al., 1998).

2.6. Statistics

The homogeneity of variance was determined using Hartley's test, and normality was assessed using the Kolmogorov–Smirnov test. For the 150-s analysis intervals (pre- and postdrug influx), the times spent in the two lines were dependent on one another. Thus, the time spent in the treated lane was compared with that spent in the control lane by a paired Student's *t*-test or the Wilcoxon matched-pairs signed-ranks test, depending on data normality. The different drugs and concentrations of the same drug were not compared. The frequency of crossings between the two lanes was compared by the unpaired Student's *t*-test or Mann–Whitney *U*-test, depending on data normality. The locomotor parameters distance traveled, mean speed, absolute turn angle and path efficiency were compared against the control values by one-way ANOVA followed by Dunnet's post hoc test. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Experiment 1 — attraction and aversion test

Fig. 2 shows the time spent in the contaminated and clean lanes, and the pre-trial analysis (initial 150 s) before drug influx, showing that attraction or aversion began only at the moment of drug influx. With clean control water in both lanes, there was no preference of the zebrafish for the right or left lane ($p = 0.7214$), whereas in positive control situations, the zebrafish showed a clear aversion to pH 3.0 ($p = 0.0002$) and to two food odor controls ($p = 0.0195$ and 0.0005).

The zebrafish spent more time in the lanes containing diazepam at 16 and 160 μg/l ($p = 0.0413$ and $p = 0.0078$, respectively), suggesting that the fish are attracted by diazepam at these concentrations. Similar attraction was found for fluoxetine (25 and 50 μg/l, $p = 0.0195$ and $p = 0.0222$, respectively), risperidone (100 μg/l, $p = 0.0323$) and buspirone (1000 μg/l, $p = 0.0020$).

No attraction or aversion was detected for ethanol (0.25, 0.50 and 1.0%), clonazepam, or diazepam (0.88 μg/l) or for other concentrations of fluoxetine (1 μg/l), risperidone (0.00034 and 170 μg/l), or buspirone (10 and 3000 μg/l).

Only the fish exposed to pH 3.0, 1% ethanol and 170 μg/l risperidone presented a higher lane crossing frequency than that of the control group exposed to two lanes of clean water (Table 2, $p = 0.0321$, 0.0053 and 0.0311, respectively). Fig. 1C is taken from a representative video and shows the movement of fish tracked during exposure to the substances that elicited significant differences.

No differences were found, except for food odor 1 and 2, in the locomotor parameters (distance traveled, mean speed, absolute turn angle and path efficiency) in all drugs against control values (Table 3).

3.2. Experiment 2 — attraction and aversion test with anosmic zebrafish

The anosmic zebrafish were not attracted by the drugs that were attractive in experiment 1 (diazepam, fluoxetine, risperidone and buspirone). The aversion to food odor was also abolished, whereas the fish maintained the strong aversion to pH 3.0 (Fig. 3A). Fish of control group (identical anosmia protocol but with only saline solution) maintain the attraction verified in the intact ones (Fig. 3B).

Importantly, substances used did not significantly alter pH and DO levels as depicted in Table 4.

4. Discussion

Here, we demonstrated that some psychoactive drugs, such as diazepam, fluoxetine, risperidone and buspirone, were attractive to the fish and that its detection in the water is probably via olfaction. These are very intriguing results if considered from an environmental perspective because the fish did not swim far from the contaminated lanes as expected; in fact, they may have sought these sites. The protocol and apparatus used for this chemotaxic preference test were previously validated in an evaluation of the aversion of fish to anesthetics (Readman et al., 2013), but this is the first study assessing the attraction and aversion paradigm in relation to waterborne psychoactive drugs using a chemosensory chamber test.

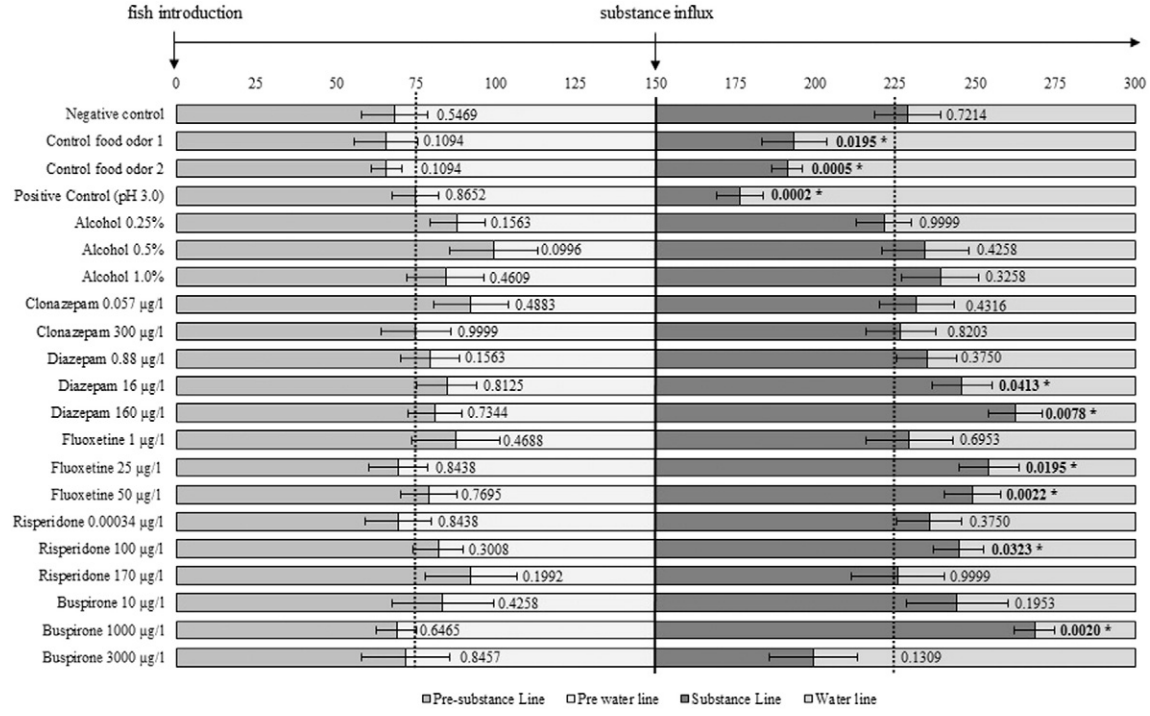

Fig. 2. Time spent (s) in the substance or water lane during the 150-s pre-drug influx and during the 150 s of drug exposure test. The data are expressed as the mean ± SEM for each lane. The means were compared by the paired Student's t test or Wilcoxon matched-pairs signed-ranks test. *P* values depicted following each bar, with 18 degrees of freedom in each comparison.

Table 2
Crossing frequency between contaminated and clean lanes in pre-trial and during drug flux.

Substance	Crossing frequency	
	0–150 s (before drug flux)	151–300 s (during drug flux)
Water (control)	12.25 ± 2.91	14.65 ± 4.99
Food odor 1	22.13 ± 2.67	17.90 ± 4.23
Food odor 2	22.13 ± 2.67	15.33 ± 2.89
pH 3	15.90 ± 3.79	7.57 ± 2.86 *
Ethanol 0.25%	11.63 ± 3.19	17.50 ± 8.45
Ethanol 0.5%	7.10 ± 1.33	8.83 ± 3.39
Ethanol 1%	10.88 ± 3.19	6.29 ± 1.71 *
Clonazepam 0.057 μg/l	4.70 ± 2.16	12.75 ± 6.52
Clonazepam 300 μg/l	15.10 ± 3.55	13.72 ± 3.71
Diazepam 0.88 μg/l	17.67 ± 3.23	11.50 ± 2.72
Diazepam 16 μg/l	8.71 ± 1.30	12.20 ± 5.93
Diazepam 160 μg/l	12.11 ± 2.73	10.33 ± 6.19
Fluoxetine 1 μg/l	5.56 ± 1.82	10.10 ± 5.45
Fluoxetine 25 μg/l	15.20 ± 2.16	11.40 ± 3.43
Fluoxetine 50 μg/l	10.89 ± 2.52	10.85 ± 5.47
Risperidone 0.00034 μg/l	12.25 ± 2.49	10.25 ± 3.84
Risperidone 100 μg/l	17.22 ± 2.05	16.50 ± 3.90
Risperidone 170 μg/l	7.40 ± 2.02	7.15 ± 3.11 *
Buspirone 10 μg/l	6.44 ± 3.49	12.56 ± 6.98
Buspirone 1000 μg/l	7.30 ± 2.35	13.50 ± 5.42
Buspirone 3000 μg/l	8.70 ± 2.07	11.45 ± 1.96

If the fish were truly seeking the drug-contaminated sites, the question that is raised is what were they truly seeking? Our main general hypothesis is that the drugs tested at these specific concentrations were attractive to the fish because they evoked a state of well-being. The premise for the formulation of the title of this study was based on the dangerous and/or disruptive effects of these drugs (Brodin et al., 2013; Gebauer et al., 2011; Park et al., 2012; Abreu et al., 2014; Idalencio et al., submitted for publication) and the notion that despite these effects, they are still attractive for fish.

Moreover, each of the tested drugs acts on several neurotransmitter systems at different levels, modulating neurotransmitters such as GABA, serotonin, and dopamine. The reason that these drugs attracted the fish may be related to their activities in the limbic and hypothalamic areas and the brainstem, in which they enhance the reward system (Tan et al., 2011; Abler et al., 2012; Kronenberg et al., 2012; Hsu et al., 2014). Because buspirone does not have sedative effects (Seidel et al., 1985; Bencan et al., 2009), sedation is most likely not the cause of the attractiveness of these drugs. In addition, all of the tested drugs provoked changes in the number of crossings between the clean and contaminated lanes (Table 2). Reinforcing this hypothesis, that discard the sedation as attractiveness cause, all the drugs did not change any locomotor parameter (Table 3). These unchanged locomotor parameters also discard possible neuromuscular effects of the drugs tested.

Regarding buspirone, the intermediary concentration showed a clear attractive effect, whereas the lower and higher concentrations did not attract the fish. Similarly, the intermediary risperidone concentration showed attraction, whereas the lower and higher ones did not. A possible explanation for this pattern is that buspirone and risperidone may provoke a U-shaped dose-response curve similar to that found for diazepam (Abreu et al., 2014) and for the proper risperidone (Idalencio et al., submitted for publication) effects on the stress axis of zebrafish.

The zebrafish displayed a strong avoidance behavior in the positive control situations. This response showed that the zebrafish were able to detect the acidic pH and odors and demonstrated that the test was able to elicit responses to various substances.

The strong aversion for the food odor controls (food odor 1 and 2, Fig. 2), was clearly abolished in anosmic animals (Fig. 3A). First, the food odor was an effective positive odorant control. However, the behavior triggered was the complete opposite of that expected from attraction by food. A possible explanation is that the food used was based on fish flour as the protein source. Perhaps this fish odor was interpreted as the "death odor" that fish consistently avoid as an anti-risk behavior. In fact, dead fish odor triggers a clear stress reaction (Oliveira et al., 2014). Another possible explanation is the absence of feeding motivation, since fish, in stock tank were maintained satiated. In the context of test (exploring the apparatus), fish might be misinterpreting the food odor as death odor as postulated above.

In anosmic zebrafish, the attraction verified in the experiment 1 was abolished, suggesting that the drug detection may have been a result of the stimulation of a chemoreceptor associated with olfaction. Considering the chemosensorial nature of test used the aversion to pH 3.0 is probably associated with touch or taste (Chang et al., 2010). In fact, previous studies show that acidic pH is detected by taste (Chang et al., 2010) and/or olfaction (Hidaka and Tatsukawa, 1989).

Table 3
Locomotor activity of zebrafish exposed do different psychoactive substances.

Substance	Distance (mm)	Mean speed (mm/s)	Absolute turn angle	Path efficiency
Water (control)	6968 ± 573	46.52 ± 3.81	27,087 ± 2159	0.01396 ± 0.00245
Food odor 1	11,341 ± 1149*	75.69 ± 7.66*	33,632 ± 3833	0.00941 ± 0.00186
Food odor 2	12,180 ± 1437*	81.6 ± 9.62*	32,719 ± 2997	0.0067 ± 0.00157
pH 3	7398 ± 795	49.45 ± 5.29	28,206 ± 1714	0.0129 ± 0.00234
Ethanol 0.25%	7771 ± 560	51.77 ± 3.70	33,423 ± 2065	0.0074 ± 0.00154
Ethanol 0.5%	4666 ± 580	31.1 ± 3.91	33,563 ± 2558	0.0202 ± 0.0092
Ethanol 1%	4491 ± 390	29.93 ± 2.59	34,632 ± 1403	0.0174 ± 0.0038
Clonazepam 0.057 μg/l	6072 ± 652	40.5 ± 4.35	33,450 ± 1350	0.01575 ± 0.0016
Clonazepam 300 μg/l	8143 ± 754	54.3 ± 5.03	32,083 ± 2665	0.0083 ± 0.0014
Diazepam 0.88 μg/l	8109 ± 524	54 ± 3.5	31,261 ± 665	0.0086 ± 0.0013
Diazepam 16 μg/l	6205 ± 527	41.64 ± 3.48	29,070 ± 1824	0.01392 ± 0.0021
Diazepam 160 μg/l	6544 ± 753	43.58 ± 5.02	24,468 ± 2115	0.0215 ± 0.007
Fluoxetine 1 μg/l	6995 ± 866	46.6 ± 5.77	30,735 ± 2246	0.0147 ± 0.0034
Fluoxetine 25 μg/l	6988 ± 708	46.7 ± 4.74	30,269 ± 1498	0.0112 ± 0.002
Fluoxetine 50 μg/l	7270 ± 407	48.55 ± 2.74	27,874 ± 1886	0.0144 ± 0.001
Risperidone 0.00034 μg/l	6134 ± 445	40.94 ± 2.98	25,442 ± 1937	0.02 ± 0.004
Risperidone 100 μg/l	8468 ± 584	56.52 ± 3.88	24,744 ± 1664	0.0149 ± 0.001
Risperidone 170 μg/l	5826 ± 557	39 ± 3.69	30,524 ± 2516	0.01327 ± 0.002
Buspirone 10 μg/l	6115 ± 986	40.6 ± 6.55	29,872 ± 2429	0.0158 ± 0.0038
Buspirone 1000 μg/l	7109 ± 808	47.31 ± 5.4	24,031 ± 2155	0.018 ± 0.005
Buspirone 3000 μg/l	5667 ± 685	37.8 ± 4.52	26,487 ± 1351	0.0159 ± 0.003

Data expressed as mean ± SEM. One-way ANOVA followed by Dunnet's post hoc test. (Distance traveled, $F_{20,290} = 5.351$, $p < 0.0001$ and absolute turn angle $F_{20,290} = 2.453$, $p < 0.0001$).

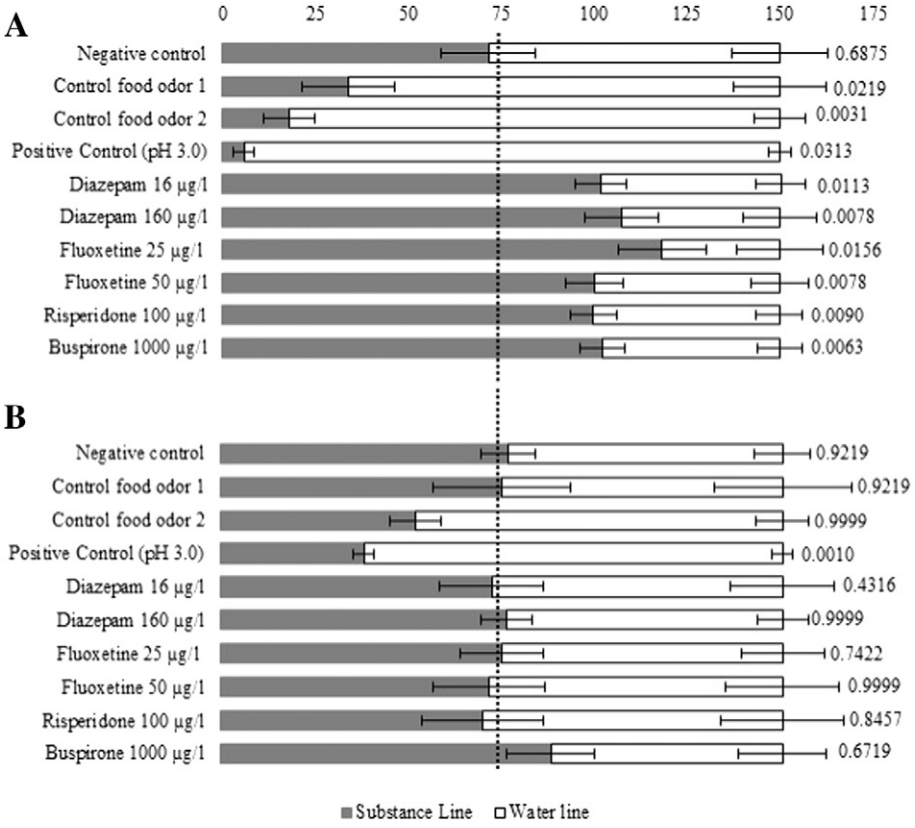

Fig. 3. Time spent (s) in the substance or water lane during the 150-s test in control saline (A) and anosmic zebrafish (B). The data are expressed as the mean ± SEM for each lane. The means were compared by the Wilcoxon matched-pairs signed-ranks test. *P* values in the figure, with 18 degrees of freedom in each comparison.

The absence in the anosmic fish of attraction to the drugs is a very intriguing result. If our hypothesis that attraction was related to a state of fish well-being caused by a drug action on the reward system is true, these drugs need to be absorbed and act in the central nervous system (CNS). However, is the absorption and action related to the olfactory perception of drugs? A possible explanation is a combined sequential effect wherein a previous olfactory perception is necessary to trigger a hedonic effect in the CNS. Another possibility is that olfaction is fundamental to the drug lane choice by fish, and this choice determines that fish spend more time in the presence of the drug and, consequently, absorb more of it. In the absence of olfaction, fish spend less time in the presence of the drug. In fact, a combined action of senses is common, and the most common cases involve a summation of taste with either olfaction or vision (Delwiche, 2012). In addition, the activation of memories and CNS areas related to smell or taste (Shepherd, 2006), including those related to behavioral expression (Chapuis et al., 2007) is also a common phenomenon. Despite these plausible explanations, the mechanism for the involvement of olfaction with attraction to drugs remains to be elucidated.

Considering the reported deleterious and disruptive effects of psychoactive drugs (Brodin et al., 2013; Park et al., 2012; Abreu et al., 2014) in an environmental perspective, we suggest that fish may seek (or at least, not avoid) drug-contaminated places. This can be very dangerous because the fish did not swim far from the contaminated sites as logically expected; in fact, they may have sought these sites. Since the uptake and bioaccumulation of several drugs in fish seems to be time and dose dependent (Lau et al., 2006; Sackerman et al., 2010; Oxendine et al., 2006; Paterson and Metcalfe, 2008; Brodin et al., 2013), a fish that spends more time in the presence of these drugs (attractive or not perceived drugs) tend to absorb higher concentrations than ones that escape from contaminated sites (aversive drugs). Thus, it is difficult to predict the environmental impact of pharmaceutical residues on fish and aquatic environments.

Table 4
pH and DO (mg/l) levels in clean and contaminated water.

	pH		Dissolved oxygen	
Substance	Water	Substance	Water	Substance
Water (control)	6.8 ± 0.1	6.75 ± 0.07	6.2 ± 0.05	6.15 ± 0.1
pH 3 (Trichloroacetic acid)	6.9 ± 0.1	3 ± 0.1	5.7 ± 0.1	5.8 ± 0.05
Ethanol 1%	6.7 ± 0.15	7 ± 0.06	5.9 ± 0.1	5.75 ± 0.15
Ethanol 0.5%	6.2 ± 0.1	6.5 ± 0.08	5.7 ± 0.05	5.65 ± 0.1
Ethanol 0.25%	6.7 ± 0.15	7 ± 0.05	5.6 ± 0.1	5.7 ± 0.07
Clonazepam 0.057 µg/l	7.4 ± 0.03	7.2 ± 0.08	6 ± 0.09	6.2 ± 0.14
Clonazepam 300 µg/l	7 ± 0.04	6.9 ± 0.05	6.2 ± 0.2	6 ± 0.08
Diazepam 160 µg/l	6.7 ± 0.12	6.9 ± 0.2	5.9 ± 0.18	6 ± 0.13
Diazepam 16 µg/l	7.2 ± 0.03	6.9 ± 0.17	6.2 ± 0.17	6.3 ± 0.12
Diazepam 0.88 µg/l	6.9 ± 0.13	6.75 ± 0.14	6.2 ± 0.07	6.1 ± 0.14
Fluoxetine 50 µg/l	6.7 ± 0.14	6.65 ± 0.08	5.9 ± 0.12	6 ± 0.13
Fluoxetine 25 µg/l	6.8 ± 0.09	6.95 ± 0.12	6.2 ± 0.07	6.1 ± 0.14
Fluoxetine 1 µg/l	6.9 ± 0.13	6.75 ± 0.14	7.2 ± 0.03	7 ± 0.17
Risperidone 0.00034 µg/l	7.3 ± 0.06	7.15 ± 0.18	7 ± 0.08	6.8 ± 0.19
Risperidone 100 µg/l	6.7 ± 0.12	6.6 ± 0.18	6.7 ± 0.14	6.5 ± 0.13
Risperidone 170 µg/l	6.4 ± 0.14	6.6 ± 0.08	5.9 ± 0.2	6.2 ± 0.07
Buspirone 10 µg/l	7 ± 0.1	6.8 ± 0.05	5.9 ± 0.14	6.1 ± 0.1
Buspirone 1000 µg/l	6.8 ± 0.2	6.9 ± 0.13	6.7 ± 0.12	6.9 ± 0.04
Buspirone 3000 µg/l	7.2 ± 0.04	7 ± 0.17	6.5 ± 0.16	6.4 ± 0.06

Authors' contributions

L.J.G.B., M.S.A. and A.C.V.G. conceptualized the study and wrote the paper. L.J.G.B., M.S.A., A.C.V.G. and C.D.B. interpreted the data. M.S.A., A.C.V.G., J.G.S.R., G.K., F.K., R.I., T.A.O., D.G. and H.H.A.B. collected and analyzed the data.

Author information

The authors declare no competing financial interests.

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