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ANTI-ANXIETY DRUGS AND FISH BEHAVIOR: ESTABLISHING THE LINK BETWEEN INTERNAL CONCENTRATIONS OF OXAZEPAM AND BEHAVIORAL EFFECTS

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Abstract: Psychoactive drugs are frequently detected in the aquatic environment. The evolutionary conservation of the molecular targets of these drugs in fish suggests that they may elicit mode of action–mediated effects in fish as they do in humans, and the key open question is at what exposure concentrations these effects might occur. In the present study, the authors investigated the uptake and tissue distribution of the benzodiazepine oxazepam in the fathead minnow (*Pimephales promelas*) after 28 d of waterborne exposure to 0.8 $\mu g\,L^{-1}$, 4.7 $\mu g\,L^{-1}$, and 30.6 $\mu g\,L^{-1}$. Successively, they explored the relationship between the internal concentrations of oxazepam and the effects on fish exploratory behavior quantified by performing 2 types of behavioral tests, the novel tank diving test and the shelter-seeking test. The highest internal concentrations of oxazepam were found in brain, followed by plasma and liver, whereas muscle presented the lowest values. Average concentrations measured in the plasma of fish from the 3 exposure groups were, respectively, $8.7 \pm 5.7\ \mu g\,L^{-1}$, $30.3 \pm 16.1\ \mu g\,L^{-1}$, and $98.8 \pm 72.9\ \mu g\,L^{-1}$. Significant correlations between plasma and tissue concentrations of oxazepam were found in all 3 groups. Exposure of fish to 30.6 $\mu g\,L^{-1}$ in water produced plasma concentrations within or just below the human therapeutic plasma concentration (H_TPC) range in many individuals. Statistically significant behavioral effects in the novel tank diving test were observed in fish exposed to 4.7 $\mu g\,L^{-1}$. In this group, plasma concentrations of oxazepam were approximately one-third of the lowest H_TPC value. No significant effects were observed in fish exposed to the lowest and highest concentrations. The significance of these results is discussed in the context of the species-specific behavior of fathead minnow and existing knowledge of oxazepam pharmacology. *Environ Toxicol Chem* 2016;35:2782–2790. © 2016 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Read-across Behavior Bioaccumulation Fish Psychoactive drug

INTRODUCTION

In recent years, interest in the potential effects of psychoactive pharmaceuticals in aquatic organisms has grown rapidly, mainly because of the frequent detection of these compounds in the aquatic environment [1]. Several psychoactive drugs have been detected in surface waters, generally in the nanograms per liter range [1,2], as well as in fish tissues [3,4], fish plasma [5], invertebrates [6], and biofilm [7], usually in the low nanograms per gram range. An increasing number of studies have reported a range of biochemical and behavioral effects of these compounds in aquatic organisms across an extremely wide range of exposure concentrations (picograms per liter to milligrams per liter). Because of the apparent highly variable potency of these compounds in aquatic organisms, the environmental implications of exposure to these drugs are as yet unclear [1].

Among these psychoactive compounds, the benzodiazepine oxazepam has received significant attention after a recent report in which relatively low concentrations (1.8 $\mu g\,L^{-1}$) affected the behavior of wild fish inhabiting Swedish surface waters [8].

Oxazepam is a drug widely used for the treatment of anxiety but is also prescribed to treat insomnia and acute alcohol withdrawal. Oxazepam acts as an agonist of an allosteric binding site on the γ-aminobutyric acid subtype A ($GABA_A$) receptor and increases the binding efficiency of GABA binding, a major inhibitory neurotransmitter and key regulator of anxiety [9], leading to a reduction of the communication between neurons and hence to a calming effect in the brain [10,11]. Importantly, this neurotransmitter system is well conserved in teleost fish: several studies have identified benzodiazepine–GABA receptors in fish and shown that they have similar binding characteristics as those present in mammals [12,13]. Moreover, exposure of zebrafish to GABA–enhancing drugs, such as diazepam (another benzodiazepine drug), has been shown to have an anxiolytic effect, thus confirming that fish are sensitive to GABA modulation and respond to benzodiazepines [14]. The conservation of the drug target in fish supports the hypothesis that exposure to oxazepam may lead to a mode of action–mediated effect in fish, as it does in humans [11,15].

Considering the importance of characterizing accurate effect concentrations to better inform the environmental risk assessment of pharmaceuticals, the aim of the present study was to investigate, firstly, the uptake and tissue distribution of oxazepam in the fathead minnow (*Pimephales promelas*) after chronic waterborne exposure and, secondly, the relationship between internal concentrations of oxazepam and behavioral effects.

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The experimental design was driven by the "read-across" approach [5,16], according to which the human therapeutic plasma concentrations of a given drug can be used as reference to predict the likelihood of occurrence of relevant mode of action–mediated effects in fish at comparable levels of biological organization. The closer the drug concentration in fish plasma is to the human therapeutic plasma concentration (H_TPC), the higher is the probability to observe the effect (i.e., behavioral alterations) [16]. This approach can be used to estimate the water concentration that will elicit a therapeutic effect in fish and can therefore drive the selection of the exposure concentrations and test specific hypotheses. This approach has been successfully applied in previous studies on antidepressant drugs by Margiotta-Casaluci et al. [5] and Valenti et al. [17]. In the present study, the exposure concentrations were selected to produce drug plasma concentrations in fish within the H_TPC in 1 group of fish and lower than the H_TPC range in another 2 groups. This design was intended to test whether behavioral effects occur only at plasma concentrations equal to the H_TPC, or also at lower concentrations.

MATERIALS AND METHODS

Chemicals

Oxazepam (CAS no. 604-75-1) was purchased from Sigma-Aldrich with purity higher than 98%. Stock solutions were prepared every 7 d in *N,N*-dimethylformamide (Fisher Scientific) in amber bottles to preserve them from light exposure. High-performance liquid chromatography (HPLC)–grade methanol, acetonitrile, water, and formic acid (98%) were supplied by Merck. The Ostro™ 96-well plate used for purification of plasma samples was acquired from Waters. Dechlorinated tap water (5 μm and 10 μm carbon-filtered) was used as dilution water, and general parameters (pH, temperature, and dissolved oxygen) were monitored daily throughout the study.

Test species and ethical statement

Adult fathead minnows (*P. promelas*) were supplied from breeding stocks maintained at Brunel University (London, UK). Two weeks before the beginning of the present study sexually mature males and females were separated to prevent any spawning activity and acclimated to the test conditions. Fish were fed 3 times/d: once with adult brine shrimp (Tropical Marine Centre; Gamma-irradiated) and twice with flake food (King British Tropical flake food). Fish were not fed on the sampling day. The present study was carried out under Project and Personnel Licences granted by the UK Home Office, under the United Kingdom Animals (Scientific Procedures) Act 1986, and European Animal Directive 2010/63/EU.

Experimental design

The 28-d exposure was carried out using a continuous flow-through system comprising 16 20-L glass tanks (455 mm length × 260 mm width × 250 mm depth). The test was run at a water temperature of 25 ± 1 °C, with a 16:8-h light:dark photoperiod, with 20-min dawn/dusk transition periods. During the experiment, dissolved oxygen concentrations were maintained between 6.4 mg L^{-1} and 7.8 mg L^{-1}, and pH was 7.5 (±0.5). Water flowed into the 16 glass mixing chambers at a rate of 222 mL min^{-1}, which supplied 16 tank volumes/d to each test tank. The same mixing chambers also received concentrated stock solutions of oxazepam delivered via peristaltic pumps at a rate of 0.1 μL min^{-1}. In total, there were 4 exposure tanks/treatment (2 for males and 2 for females) for each of 4 treatments (0 μg L^{-1}, 1 μg L^{-1}, 5 μg L^{-1}, and 25 μg L^{-1}). Eight males or females were randomly allocated to the appropriate tanks, giving a total of 32 fish/treatment (16 males and 16 females). Both the allocation of the treatment groups in the experimental room and the allocation of each fish into 1 of the 16 tanks were randomized with the aid of a random number generator.

Exposure concentrations were selected to cover both pharmacologically and environmentally relevant concentrations. The selection of external exposure (water) concentrations expected to result in pharmacologically relevant internal (plasma) concentrations was driven by application of the fish plasma model of Huggett et al. [18] and information on the H_TPC of oxazepam. The latter was obtained from a study in which patients were administered 15 mg and 30 mg of oxazepam on 2 separate occasions with a 7-d interval, which produced therapeutic plasma concentrations in the range 100 ng mL^{-1} to 280 ng mL^{-1} [19].

After the 26-d exposure, 2 behavioral tests, the shelter-seeking test and the novel tank diving test, were performed on all fish as detailed in the next section, *Analysis of oxazepam in water, fish plasma, and other tissues*. On the day 28 of exposure, fish were terminally anesthetized according to UK Home Office regulations using a buffered ethyl 3-aminobenzoate methane sulfonate solution (MS-222; 0.5 g L^{-1}, pH 7.5; Sigma; CAS no. 144-55-8). Standard length and wet weight were measured, and blood was taken from the caudal vein using heparinized capillary tubes. Blood samples were centrifuged at 4 °C at 10 000 *g* for 6 min; plasma was collected and stored at –80 °C until analysis; and brain, muscle, and liver were removed, weighed, snap-frozen in liquid nitrogen, and stored at –80 °C until subsequent analysis.

Analysis of oxazepam in water, fish plasma, and other tissues

Water samples (40 mL) for the chemical analysis were collected in Falcon tubes on days 0, 7, 14, 21, and 28 from all tanks and kept at –20 °C until analysis. For analysis, 1 mL of each sample was centrifuged at 8000 rpm for 10 min, after which 10 μL of each sample was directly injected in the detector without further extraction. For plasma samples, acetonitrile (400 μL) was added to 50 μL of plasma and vortexed for 30 s for protein precipitation. Samples were then transferred to an Ostro 96-well plate connected to a vacuum system for the removal of phospholipids. An aliquot of 300 μL was collected from each extract and placed under an N_2 current to dry completely. Finally, extracts were dissolved in 100 μL of a 1:1 mixture of methanol to water. Five microliters of diazepam-d_5, prepared at 1 μg mL^{-1} in methanol, was added as an internal standard to each extract to account for matrix effects during subsequent analysis.

Brain, liver, and muscle samples were extracted using a method adapted from that of Valdes et al. [20]. Samples (ranging 20–200 mg) were sonicated in 1 mL of methanol for 15 min and then centrifuged at 10 000 rpm for 10 min. After this procedure was repeated 3 times, the supernatant was collected, pooled, and evaporated to dryness. Extracts were subsequently dissolved in 1 mL of HPLC-grade water and then purified in a solid-phase extraction Oasis HLB 96-well plate. Solid-phase extraction sorbent in the wells was previously conditioned with 1 mL of methanol and 1 mL of HPLC-grade water at a flow of 0.5 mL min^{-1}, with extracts being loaded at the same flow rate. After drying the well for 5 min, 1 mL of methanol was loaded in the plate to elute the samples, which were collected and placed

under an N_2 current to dry completely. Finally, extracts were dissolved in 750 μL of a mixture of methanol and water (1:1) for analysis. Ten microliters of diazepam-d_5, prepared at 1 μg mL^{-1} in methanol, was added to each extract to account for matrix effects during analysis.

All extracts were analyzed by ultra-HPLC (Waters) coupled to a hybrid quadrupole linear ion trap mass spectrometer (Qtrap 5500; Applied Biosystems), equipped with an electrospray ionization source in positive mode. Ten microliters was injected in an Acquity HSS T_3 column, with 10 mM formic acid/ammonium formate (pH 3.2) and methanol as the mobile phase set at a flow rate of 0.5 mL min^{-1}.

Compound-dependent mass spectrometric parameters (declustering potential, collision energy, and collision cell exit potential) as well as compound-selected reaction monitoring transitions were optimized by direct infusion of individual standard solution of each analyte at 10 ng mL^{-1}. A summary of these parameters is presented in Supplemental Data, Table S1. All transitions were recorded in a scheduled multiple reaction monitoring algorithm with a 30-s detection window. Source-dependent parameters were determined by flow injection analysis: curtain gas, 30 V; nitrogen collision gas, medium; source temperature, 500 °C; ion spray voltage, 5500; ion spray gases 1 = 60 V and 2 = 50 V. Instrument control data acquisition and data analysis were carried out using Analyst software (Applied Biosystems).

Prediction of oxazepam plasma concentration using the fish plasma model

Measured plasma concentrations were compared with the concentrations predicted by the fish plasma model [18]. The model aims to predict the fish steady-state plasma concentration of a drug starting from a certain water concentration and is based on the following equations:

$$\log P_{\text{Blood:Water}} = 0.73 \times \log D_{7.4} - 0.88$$

$$\text{FssPC} = [\text{water concentration}, \mu g/L] \times P_{\text{Blood:Water}}$$

In these equations $P_{\text{Blood:Water}}$ represents the partitioning between blood and the aqueous phase, log $D_{7.4}$ is the logarithm of the distribution coefficient (D) at pH 7.4 for the drug of interest, and FssPC is the fish steady-state plasma concentration. Average water concentrations throughout the 28 d were used to compare measured versus predicted plasma concentrations.

Analysis of fish behavior

Fish exploratory behavior was used as proxy to assess potential oxazepam-induced behavioral effects [21]. On day 26, 2 behavioral tests, the novel tank diving test and the shelter-seeking test, were performed on all fish ($n = 128$) as described by Margiotta-Casaluci et al. [5] and Valenti et al. [17]. The novel tank diving test is based on the instinctive behavior of fathead minnow to seek protection in a novel environment by diving to the bottom of the tank until the environmental conditions are perceived as safe enough to initiate exploration. Immediately before the initiation of the tests, each individual fish was transferred from the exposure tank to a 9-L observation tank (290 mm length × 345 mm width × 140 mm depth), and the exploratory behavior of each fish individually was recorded for 15 min, after 30-s acclimation, using a Fujifilm digital camera (FinePix JV300, 14.0 Mpix) positioned in front of the tank. VideoTrack analysis software (ViewPoint) was used for the offline analysis of fish exploratory behavior. The observation tank was visually divided in 3 areas of equal size (bottom, middle, top), and the following endpoints were quantified: number of entries into the top and middle areas; percentage of time spent at the bottom, middle, and top areas; and total distance traveled. All fish were tested and returned to their tanks. After 24 h, fish were transferred to a 12-L observation tank (measuring 390 mm length × 200 mm width × 200 mm depth) for the shelter-seeking test. The shelter-seeking test is aimed at quantifying the shelter-seeking behavior of fish when placed in a novel environment (i.e., the observation tank). In this test, individual fish were transferred to a 12-L observation tank (measuring 390 mm length × 200 mm width × 200 mm depth). Each tank contained a tile (i.e., representing the shelter for the fish) placed equidistantly from the tank walls. The exploratory behavior of each fish was recorded for 15 min, after 30-s acclimation, using a Fujifilm digital camera positioned on the top of the tank. Also in this case, VideoTrack analysis software was used for the offline analysis of fish exploratory behavior, which included quantification of the time spent under or outside the shelter and the total distance traveled. Four observational tanks were filmed at the same time in both tests.

Data analysis

Bioaccumulation data were analyzed by the multiple comparison Holm-Sidak test, with analysis of variance assumptions of normality and identical standard deviation (SD) confirmed by D'Agostino-Pearson normality test and Bartlett's test, respectively. All behavioral endpoints were analyzed for treatment-related differences in the medians by the nonparametric Dunn's multiple comparison test. Statistical significance was set at $p < 0.05$ in all cases. A more in-depth analysis by marginal logistic regression was performed. The influence of plasma levels (categorized by their tertiles) and gender on selected behavioral endpoints was examined, the information was reduced to dichotomous levels, and the probability of the behavioral occurrence was estimated in relation to the controls. The analysis accounted for a potential tank correlation effect. Results are reported as odds ratios with 95% confidence intervals, corrected for multiple comparisons (Dunnett). All statistical analyses were conducted using the software SAS (SAS Institute).

RESULTS AND DISCUSSION

Oxazepam concentrations in water, plasma, brain, and muscle

Recoveries, method detection and quantification limits, precision, and accuracy for the analysis of oxazepam in water, fish plasma brain, liver, and muscle are summarized in Table 1 (see also Supplemental Data). Average water concentrations measured throughout the experiment for the lowest, medium, and highest treatment concentrations were 0.8 ± 0.2 μg L^{-1}, 4.7 ± 0.5 μg L^{-1}, and 30.6 ± 2.9 μg L^{-1} (mean ± SD, $n = 20$), respectively. During the 5 sampling events, measured concentrations were within 20% of the nominal concentration in all tanks.

Measured concentrations of oxazepam in plasma of fathead minnows are shown in Table 2 and Supplemental Data, Table S2. All samples in the control group had concentrations below the method detection limits, whereas an increasingly high concentration of oxazepam in plasma was observed at higher exposure concentrations. Average concentrations of oxazepam measured in plasma were 8.7 ± 5.7 μg L^{-1}, 30.3 ± 16.1 μg L^{-1}, and 98.8 ± 72.9 μg L^{-1} (mean ± SD, $n = 32$) for the 0.8 μg L^{-1}, 4.7 μg L^{-1}, and 30.6 μg L^{-1} treatment groups, respectively.

Table 1. Method validation parameters for the analysis of oxazepam in water and fish tissues

	Recoveries (%) (± relative SD)	Method detection limit (ng mL^{-1})[a]	Method quantitation limit (ng mL^{-1})[a]	Precision (% relative SD)		Matrix effect (% ± relative SD)
				Intraday	Interday	
Water	—	0.02	0.06	1.2	2.5	103 (±3)
Plasma	78 (13)[b]	0.04	0.14	7.1	8.7	104 (±9)
Brain	106 (14)[c]	0.54	1.80	0.9	5.3	102 (±8)
Liver	102 (11)[c]	0.12	0.39	0.5	0.3	115 (±1)
Muscle	92 (14)[c]	0.15	0.51	6.1	7.1	99 (±3)

[a]Nanograms per gram for tissues.
[b]Plasma was spiked at 100 ng mL^{-1}.
[c]Tissues were spiked at 50 ng g^{-1}.
SD = standard deviation.

Observed interindividual variability in oxazepam plasma concentrations for fish from the same tank was within the range of 3-fold to 6-fold for 9 out of 12 tanks (Supplemental Data, Table S3). In the 3 remaining tanks, the higher variation was caused by a few samples in which very low concentrations of oxazepam were quantified. If the 5th and 95th percentile values are used, the ratio maximum to minimum in those 3 tanks reduces to the expected range (2.5-fold to 4.2-fold). This degree of intratank variability was similar to that observed for another psychoactive drug, fluoxetine, by Margiotta-Casaluci et al. [5]. Determining the distribution of the drug in the body and potential bioaccumulation dynamics is critical to inform the future development of uptake and pharmacokinetics models able to predict those processes in conditions of chronic exposure [5]. The development of those models is in fact often constrained by the availability of empirical data. Characterization of distribution is particularly important for drugs like oxazepam which act on a specific system (i.e., central nervous system).

The concentrations of oxazepam in the liver, brain, and muscle of exposed fish are summarized in Table 2 and Supplemental Data, Table S2. The highest concentrations were found in brain, followed by liver and plasma, whereas muscle presented the lowest values. Oxazepam was detected in all exposed fish except in 3 muscle samples. The tissue-specific bioconcentration factors (BCFs) were calculated according to the measured water concentration and the concentrations in each tissue, and these are summarized in Table 2. In brain BCFs were higher when compared to the other tissues, especially at the lowest exposure concentration, mirroring what has been observed in previous studies [20,22]. The tissue to plasma ratios were also calculated and are summarized in Table 2 and Figure 1A. For all tissues, the ratio between the solid tissue and plasma concentrations in the lowest concentration, calculated as the logarithmic concentration ($\log_{10}$), was significantly different from those of the other 2 concentrations. A linear regression of log-transformed ($\log_{10}$) solid tissue versus plasma concentrations was undertaken and is represented in Figure 1B, to provide an indication of oxazepam organ distribution, as in Tanoue et al. [23]. Significant positive correlations between plasma and tissue concentrations of oxazepam were found in all cases ($0.47 < R^2 < 0.64$, $p < 0.05$, $n = 96$, Pearson correlation coefficient). The distribution of oxazepam in fish supports the current knowledge of the pharmacokinetic and distribution of this drug in mammals [24,25].

Benzodiazepines, including oxazepam, are substances able to cross the blood–brain barrier relatively rapidly [9]. Once in the brain, these compounds equilibrate with brain tissue and, after equilibrium is attained, a constant brain to plasma ratio is maintained so that plasma concentrations are proportionately related to concentrations in the brain [26]. The present study did not have multiple time sampling, so it is not possible to ascertain if that equilibrium was maintained during the exposure period employed. Nevertheless, the brain to plasma ratio observed in the present study (2–6:1) is similar to that observed in rats (3–5:1) [24] and cats (1–3:1) [25], supporting the read-across approach.

Fish plasma concentrations versus H_TPC

Measured plasma concentrations were also compared with the concentrations predicted by the fish plasma model, according to the above equations (see *Prediction of oxazepam plasma concentration using the fish plasma model* and Figure 2). The H_TPC range used as reference [19] in the present study is 100 ng mL^{-1} to 280 ng mL^{-1}. Exposure of fish to 0.8 μg L^{-1} and 4.7 μg L^{-1} resulted in plasma concentrations of oxazepam below the H_TPC, whereas exposure to 30.6 μg L^{-1} produced

Table 2. Measured concentration of oxazepam in plasma, brain, muscle, and liver at the 3 exposure levels[a]

Water	Plasma		Brain			Muscle			Liver		
Concentration (μg L^{-1})	Concentration (ng mL^{-1})	BCF	Concentration (ng g^{-1})	BCF	Brain to plasma ratio	Concentration (ng g^{-1})	BCF	Muscle to plasma ratio	Concentration (ng g^{-1})	BCF	Liver to plasma ratio
0.8	9 (3–18)	10.0	40 (17–73)	51	6.6	7 (0–47)	9	1.1	16 (6–50)	20	2.7
4.7	30 (8–80)	6.4	50 (23–91)	10.6	2.1	22 (0–96)	4.8	0.9	39 (10–96)	8.2	1.5
30.6	99 (10–313)	3.3	147 (57–323)	4.8	2.6	82 (7–192)	2.7	1.5	171 (20–439)	5.6	2.5

[a]Concentration in tissues expressed as mean (minimum–maximum) values.
All values are expressed as average.
DBCF = bioconcentration factor.

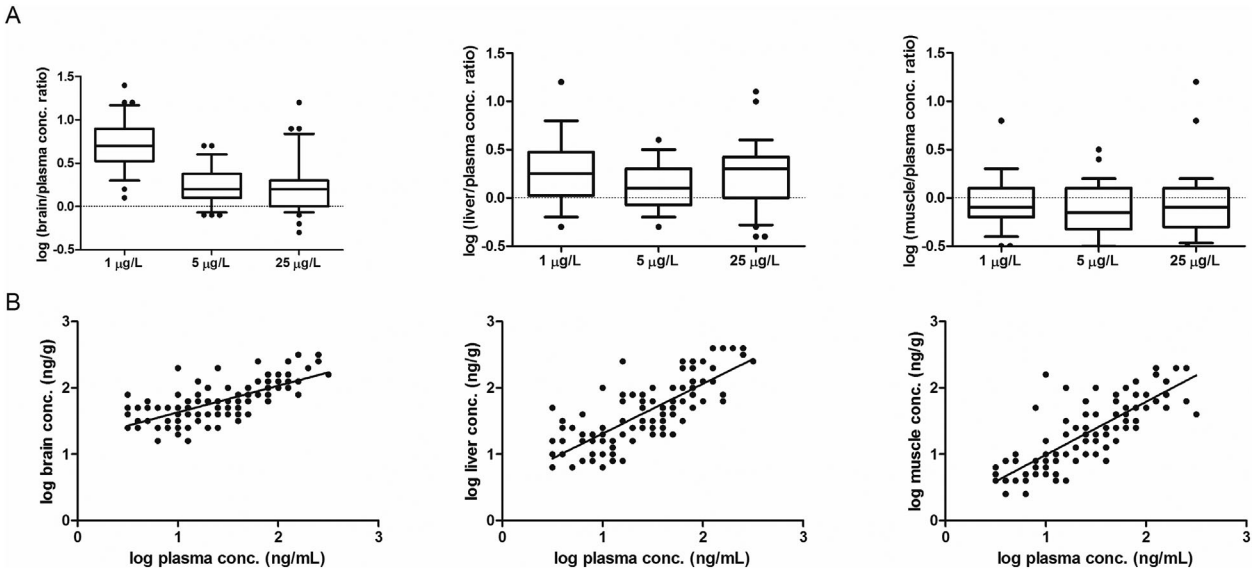

Figure 1. (**A**) Comparative accumulation of oxazepam (log_{10} tissue/plasma concentration ratio) at the 3 nominal exposure concentrations. (**B**) Log-linear correlation between plasma and tissue concentrations of oxazepam in exposed fish. Pearson correlation coefficients for brain, liver, and muscle were 0.336, 0.5228, and 0.4260, respectively. Boxes represent medians, with 10th and 90th percentiles.

plasma concentrations within the H_TPC range in several fish, particularly in males (9 out of 16 males and 3 out of 16 females).

The fish plasma model demonstrated a good degree of accuracy for the prediction of oxazepam uptake in fish plasma in general, despite the fact that it does not consider interspecies or intraspecies variability and that it was developed for very hydrophobic compounds [27]. However, when male and female fathead minnows were assessed separately, the model overestimated the measured concentrations in female fish: the measured concentration was on average 2.7 times lower than the predicted concentration for females exposed at the highest concentration. Two hypotheses arise to explain these differences, although no data are available to test their validity: 1) gender-specific metabolism of oxazepam led to different steady-state plasma concentrations, and 2) smaller gill surface area per unit of body weight in females led to lower uptake rates [28]. Previous studies with a different psychoactive drug, fluoxetine, have demonstrated the occurrence of dose-dependent modulation (e.g., induction, inhibition, saturation) of metabolic enzymes, which can lead to discrepancies between measured and modeled concentrations [10]. These dynamics are anticipated to occur only at high concentrations and are likely not to be relevant at environmental concentrations. In the present study, no metabolites of oxazepam were measured, so the possibility of this effect occurring in the high concentration group remains only theoretical.

In humans, sex-specific differences in pharmacokinetics have been identified for many drugs, including benzodiazepines [29]. For instance, a study about the kinetics of oxazepam in men and women determined that the elimination half-life was 25% longer in females and the clearance of total as well as unbound oxazepam was significantly greater in men than in women [30]. These differences have been attributed to variations in size, composition, and hormonal effect, which

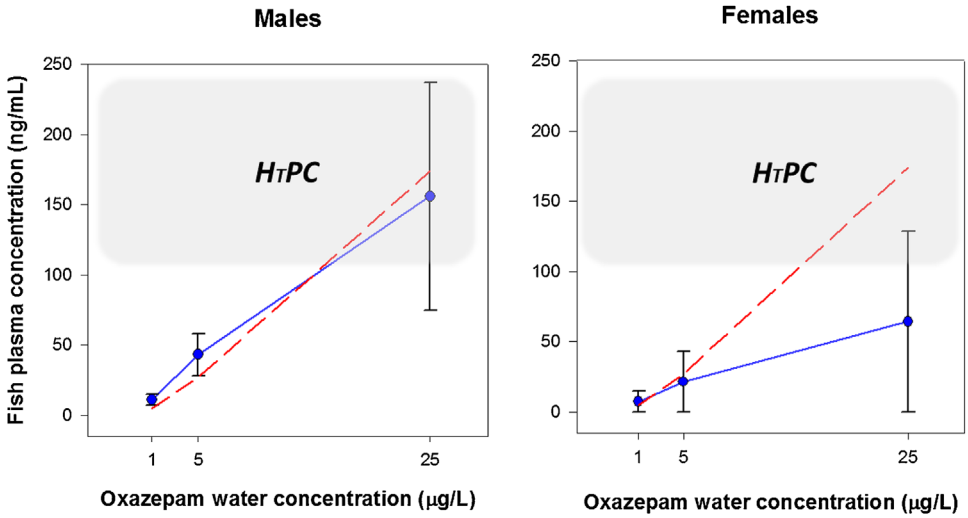

Figure 2. Measured (blue line, mean ± standard deviation, $n = 16$/treatment) and predicted (red dashed line) plasma concentrations of oxazepam after 28-d exposure. Gray area indicates the human therapeutic plasma concentration range. H_TPC = human therapeutic plasma concentration.

ultimately can affect the drug absorption, distribution, metabolism, and excretion profile, although other studies on benzodiazepines, including oxazepam, have not found sex differences in distribution or elimination rates [31,32].

Behavioral effects

Novel tank diving test. In the novel tank diving test, decreased exploratory activity ($p < 0.05$) in both the middle and top areas was observed only for the fish exposed to 4.7 $\mu g\,L^{-1}$ (Figure 3 and Table 3). The same group exhibited an increase in the percentage of time spent in the bottom area. In this group, plasma concentrations of oxazepam were approximately one-third of the lowest H_TPC value. In contrast, no significant effects were observed in fish exposed to the lowest concentration (0.8 $\mu g\,L^{-1}$) and highest concentration (30.6 $\mu g\,L^{-1}$). No indications for significant differences between males and females in behavioral activity were found at any concentration.

Data for individual fish showed a relatively high interindividual and intertank variability across treatments, including the control group (Supplemental Data, Figures S1 and S2). The latter included fish with high as well as poor activity, with fish from tank 1 demonstrating a higher activity than the other control fish. The same control group showed also a behavioral pattern different from the other controls in the shelter-seeking test, indicating that such behavior was tank-specific rather than a random phenomenon. The consequent increase of variability in the behavior of the control group influenced the expected sensitivity of the 2 behavioral tests used in the present study.

To reinforce the results of the nonparametric statistical analysis, which compares only the median behavioral outcomes from the control fish to the treated fish, and as a response to the difficult statistical distribution pattern observed for these endpoints, a marginal logistic regression analysis on the time fish spent on the bottom was conducted (Table 4). The data on the behavioral endpoints were reduced to dichotomous levels, selecting either the median response as the criterion for grouping (balanced approach) or a response considered more relevant (in the present study: all fish that spent at least 90.0% of their time at the bottom). It should be noted that the latter leads to an unbalanced grouping and therefore reduces the statistical power to identify possible treatment-related differences in their group occurrences. The model was set such that it estimates the probability that a treated fish is more (or less) likely to occur in the "upper location" group compared to the controls. Treated

Table 3. Nonparametric statistical analysis (Kruskal-Wallis tests followed by Dunn's post hoc tests)

	Oxazepam concentration ($\mu g\,L^{-1}$)		
Endpoint	0.8	4.7	30.6
Novel tank diving test			
% Time spent at the bottom	— $p = 0.38$	↑ $p < 0.01$	— $p = 0.99$
% Time spent at the middle	— $p = 0.37$	↓ $p < 0.01$	— $p = 0.98$
% Time spent at the top	— $p = 0.12$	↓ $p < 0.01$	— $p = 0.99$
Transition to the middle	— $p = 0.17$	↓ $p < 0.01$	— $p = 0.57$
Transition to the top	— $p = 0.11$	↓ $p < 0.01$	— $p = 0.99$
Distance traveled	— $p = 0.99$	↓ $p < 0.01$	— $p = 0.99$
Shelter-seeking test			
% Time spent under the shelter	— $p = 0.16$	— $p = 0.10$	— $p = 0.99$
% Time spent outside the shelter	— $p = 0.16$	— $p = 0.10$	— $p = 0.99$
Distance traveled	— $p = 0.10$	— $p = 0.11$	— $p = 0.99$

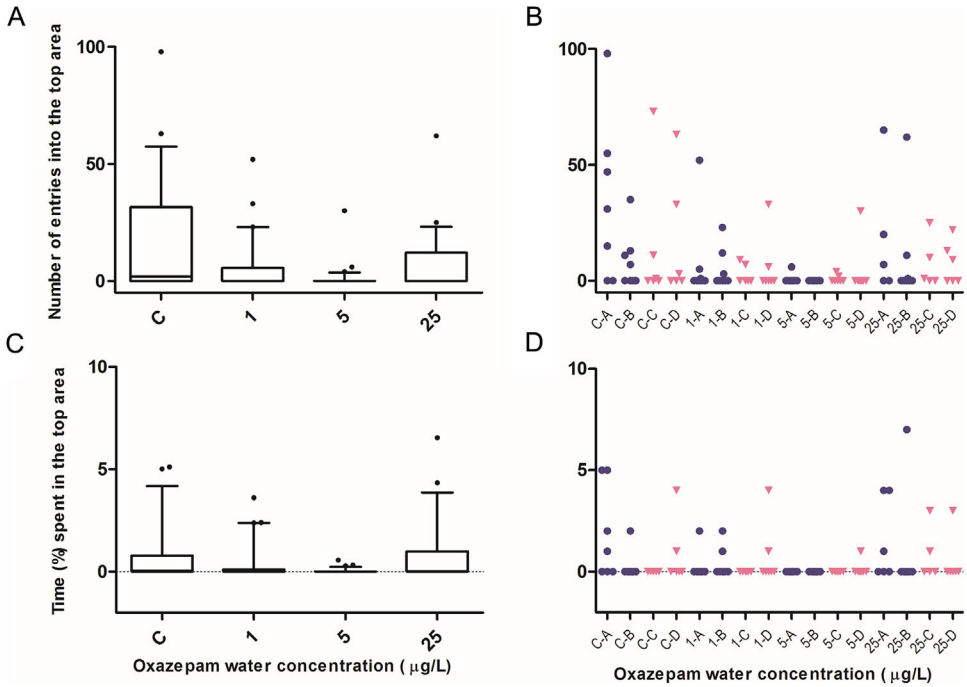

Figure 3. Effect of oxazepam on fish exploratory behavior quantified during a novel tank diving test performed after 28 d of exposure. Number of entries into the top area (**A**) per treatment ($n = 32$) and (**B**) in each individual tank ($n = 8$). Time spent in the top area (**C**) per treatment ($n = 32$) and (**D**) in each individual tank ($n = 8$). Males are represented as blue circles and females as pink inverted triangles. Boxes represent medians, with 10th and 90th percentiles.

Table 4. Association between oxazepam levels measured in fish plasma after 28-d exposure and a dichotomized behavior parameter[a]

	Bottom reference		Upper location			
	n	%	*n*	%	Odds ratio	95% Confidence interval
Novel tank diving test (N = 112)						
Fish spent at least 99.7% of their time at the bottom (balanced approach)						
Control	7	25.9	20	74.1	1.00	—
0–13.3	12	42.9	16	57.1	0.45*	0.21–0.99
13.3–40.1	22	75.9	7	24.1	0.11*	0.03–0.36
>40.1	15	53.6	13	46.4	0.31	0.09–1.04
Fish spent at least 95.0% of their time at the bottom						
Control	16	59.3	11	40.7	1.00	—
0–13.3	22	78.6	6	21.4	0.38	0.09–1.60
13.3–40.1	25	86.2	4	13.8	0.24	0.04–1.39
>40.1	21	75.0	7	25.0	0.49	0.09–2.62
Fish spent at least 90.0% of their time at the bottom						
Control	20	74.1	7	25.9	1.00	—
0–13.3	25	89.3	3	10.7	0.39	0.12–1.29
13.3–40.1	26	89.7	4	10.3	0.31	0.04–2.29
>40.1	24	85.7	4	14.3	0.44	0.07–2.65
Shelter-Seeking test (N=112)						
Fish spent at least 16.8% of their time at the bottom (balanced approach)						
Control	18	66.7	9	33.3	1.00	—
0-13.3	11	40.7	16	59.3	3.86	0.91–16.30
13.3-40.1	16	55.2	13	44.8	1.89	0.80–4.46
>40.1	11	37.9	18	62.1	2.96*	1.12–7.79
Fish spent at least 1/3rd of their time at the bottom						
Control	12	44.4	15	55.6	1.00	—
0–13.3	9	33.3	18	66.6	1.74	0.33–9.19
13.3–40.1	11	37.9	18	62.1	1.37	0.50–3.77
>40.1	9	31.0	20	69.0	1.70	051–5.65
Fish spent at least half of their time at the bottom						
Control	3	11.1	24	88.9	1.00	—
0–13.3	3	11.1	24	88.9	0.83	0.07–10.13
13.3–40.1	3	10.3	26	89.7	0.97	0.09–10.89
>40.1	5	17.2	24	82.8	0.63	0.06–7.11

[a]Odds ratios were estimated by marginal logistic regression, controlled by tank effect, adjusted for gender, and corrected by multiple comparisons (Dunnett). Plasma levels were grouped into 3 even classes according to their tertiles; behavior parameter was dichotomized by its median into 2 even groups (balanced approach) or uneven groups (unbalanced approach).
* Indicates statistical significance ($p < 0.05$).

fish were either used as a single group or grouped according to the tertiles of the measured drug plasma levels. The probabilities are expressed as odds ratios: odds ratio = 2 means that a treated fish has a 2 times higher chance of being in the upper group than a control fish, and odds ratio < 1 indicates the opposite behavior, that the corresponding treatment leads the fish to stay more at the bottom.

Among all fish, those with oxazepam plasma levels above the quantification limit were more likely to be at the bottom than control fish, especially fish with low or median plasma levels (indicated by statistical significance). However, the median behavioral response for the grouping was 99.7%, and consequently many fish were allocated to the "upper location." A second grouping with fish having spent at least 90% of their time at the bottom revealed similar odds ratios below 1 but not statistically significant. The hypothesis is that the observed fish behavior in control tank 1 was mainly responsible for these findings as data analysis without these data revealed nonsignificant odds ratios at approximately 1 (data not shown). In none of the analyses was any indication for gender differences found (p values >0.3). Whereas the effects observed in the 4.7 $\mu g\,L^{-1}$ group were confirmed by the different statistical approaches used, additional experiments will be necessary to clarify the current uncertainty around potential effects in the 0.8 $\mu g\,L^{-1}$ exposure group (Table 4). It is also interesting that on the basis of a previous experiment with oxazepam, an increase in the exploratory behavior was expected [8]. However, in the present study a decrease of the exploratory behavior was observed at 4.7 $\mu g\,L^{-1}$.

Shelter-seeking test. No significant differences in the time spent under the shelter or the distance traveled in the tank were observed between the control and treatment groups (see Figure 4 and Table 3; Supplemental Data, Table S4). Male fish spent on average 20% more time under the shelter than female fish, which agrees with previous studies on fathead minnow behavior; but these differences were not supported by statistics ($p > 0.3$) [33]. Differences were observed between the 4 replicate tanks of the control group. In particular, fish in tank 1 (control treatment, males) spent more time under the shelter compared to the fish in the other tanks (Supplemental Data, Figure S3).

Logistic regression revealed a positive association between the time a fish spent under the shelter and the treatment (Table 4). Among all fish, those exposed to oxazepam were twice as likely to leave the shelter as the control fish (16.8% of their time under the shelter), which was used as the criterion for the balanced grouping. Refining the analysis by using instead the tertiles of the measured plasma drug levels, only drug plasma levels above 40.1 $\mu g\,L^{-1}$ showed a significant odds ratio, with a 3-fold higher chance of a fish leaving the shelter. However, when the grouping criterion was changed to "at least one-third time spent under the shelter," the outcomes from the

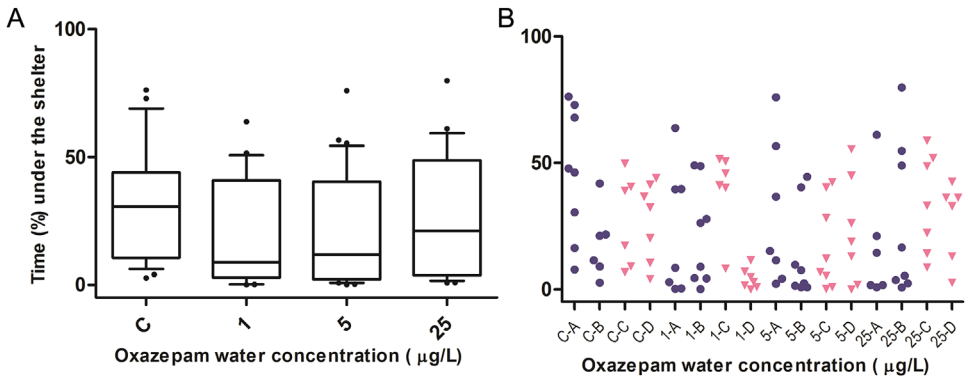

Figure 4. Effect of oxazepam on fish exploratory behavior quantified during a shelter-seeking test performed after 28 d of exposure. (**A**) Time spent under the shelter for all fish in treatment ($n = 32$). Boxes represent medians, with 10th and 90th percentiles. (**B**) Time spent under the shelter in each individual tank ($n = 8$), with males represented as blue circles and females as pink inverted triangles.

previous analysis were not confirmed, with odds ratios being always lower and nonsignificant. In none of the analyses did we observe significant gender-related differences ($p > 0.2$).

Ecological relevance of the observed behavioral effects

Despite the potential ecological consequences of behavioral alteration, the main challenges are 1) to be confident of the reproducibility of the results, and 2) to know how to translate any subtle effects observed in laboratory studies to the field. Previous studies have shown that fathead minnows exposed to environmentally relevant mixtures of 4 different psychiatric drugs impacted predator-avoidance behavior (an endpoint of high ecological relevance) [34]. However, results from other studies have been contradictory: while exposure to sertraline induced fathead minnow males to spend less time sheltering as a result of altered nest guarding behavior [17], exposure to fluoxetine, a drug with the same mode of action, resulted in an increase in the same endpoint [34]. Remarkably, fathead minnows have been shown to react in different ways when exposed to stress: sometimes they become motionless ("freezing"), for periods ranging from approximately 0.5 min to greater than 8 min; in other cases, they swim more slowly or they spend less time outside the shelter or the opposite: sudden unpredictable dashing may happen as an initial response to danger. In other cases, there is no apparent response [35]. This serves to highlight the inherent variability of behavioral data measured in fathead minnow as a model species.

The way to translate this contrasting information into ecological predictions is currently uncertain; however, steps forward have been achieved by Hellström et al. [36], who successfully applied Global Positioning System technology to track the exploratory behavior of individual fish in a Swedish artificial lake. In the future these technologies may promote an understanding of the ecological relevance of chemical-induced behavioral effects by monitoring such effects with the animals in their natural environment rather than one in which normal behavior is likely to be altered prior even to the introduction to test substances of interest.

CONCLUSIONS

In the present study, we successfully characterized the uptake and distribution of oxazepam in several tissues of fathead minnow, following 28-d exposure. Drug-related behavioral effects in exploratory behavior were observed in fish exposed at the medium concentration ($4.7\ \mu g\ L^{-1}$) for the novel tank diving test and at the highest concentration in the shelter-seeking test. Although the highest exposure concentration produced plasma concentrations of the drug that were close to, or within, the human therapeutic range, statistically significant effects on the exploratory behavior of the fathead minnow were only detected with very robust statistical analysis and only under certain conditions. According to the read-across approach [5,16], the closer the drug concentration in fish plasma is to the H_TPC, the higher is the probability of observing the effect (i.e., behavioral alterations) [16]. The present results provide no clear indications for a dose response–related trend and, therefore, leave a significant degree of uncertainty around the internal concentrations of oxazepam that do or do not cause behavioral effects in fathead minnow and what aspects of behavior are changed. Future behavioral studies involving fathead minnow should therefore be designed to have higher statistical power than that of the present study to minimize the risk of data misinterpretation. Moreover, previous studies with other fish species (*Perca fluvialis*) [8] observed increased activity and boldness after treatment with oxazepam. These apparent interspecies differences, together with the uncertainties in regard to the observed dose-response data in the present study and the difficulties developing an optimal experimental design, highlight the challenge to generate predictions on potential behavioral changes induced by psychoactive drugs in adult fish that can be systematically extrapolated to all species.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3448.

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Data Availability—Some of the chemistry data have been provided in the Supplemental Data. The raw data from the behavioral analysis can be provided on contact with the corresponding author (bhuerta@icra.cat).

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