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Title

Low concentrations of the benzodiazepine drug oxazepam induce anxiolytic effects in wild-caught but not in laboratory zebrafish

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Keywords

Oxazepam; benzodiazepines; GABA; pharmaceutical pollution; schreckstoff; domestication

Abbreviations

¹ NTDT: Novel Tank Diving Test

² HPI axis: Hypothalamic-Pituitary-Interrenal axis

³ sNTDT: Novel Tank Diving Test with addition of conspecific skin extract

⁴ LMM: Linear Mixed-effects Model

Graphical abstract

Abstract

Pollution by psychoactive pharmaceuticals has been found to disrupt anti-predator behaviors of wild fish. The challenge is now to identify which of the many psychoactive drugs pose the greatest threat. One strategy is to screen for behavioral effects of selected pharmaceuticals using a single, widely available fish species such as zebrafish. Here, we show that although such high-throughput behavioral screening might facilitate comparisons between pharmaceuticals, the choice of strain is essential. While wild-caught zebrafish exposed to concentrations of the anxiolytic drug oxazepam as low as $0.57 \mu\text{g L}^{-1}$ showed a reduction in the response to conspecific alarm pheromone, laboratory strain AB did not respond to the alarm cue, and consequently, the anxiolytic effect of oxazepam could not be measured. Adaptation to the laboratory environment may have rendered laboratory strains unfit for use in some ecotoxicological and pharmacological studies, since the results might not translate to wild fish populations.

1. Introduction

Pharmaceuticals and pharmaceutical residues are frequently detected in freshwaters and coastal waters in concentrations in the ng L^{-1} range (Björlenius et al., 2018; Hughes et al., 2012). Especially psychoactive pharmaceuticals are of concern, since these have both high prescription rates and strong potency, resulting in environmental concentrations that come closest to human therapeutic plasma concentrations (Batt et al., 2015; Fick et al., 2010). Ecotoxicological studies initially focused on antidepressants (Brooks et al., 2003; Valenti et al., 2012), while recently the anxiolytic drug oxazepam was shown to reduce anti-predator behaviors of fish at low concentrations (Brodin et al., 2013; Klaminder et al., 2014). Subsequent investigation into this compound revealed that oxazepam accumulates in lake sediment (Klaminder et al., 2015) and bioconcentrates in the tissues of perch (Heynen et al., 2016).

Oxazepam belongs to a family of pharmaceuticals called the benzodiazepines, which were serendipitously discovered in the 1950s and 60s to have anxiolytic, sedative, anticonvulsant and muscle-relaxing properties (Bateson, 2002; Sternbach, 1979). Benzodiazepines are GABA_A receptor positive allosteric modulators, meaning that they enhance the effect of the major inhibitory neurotransmitter in brain, γ -aminobutyric acid (GABA), by binding to the GABA_A receptor at a binding site that is separate from the GABA binding site. Benzodiazepines facilitate the GABA-induced conformational change of the receptor resulting in the opening of the central ion pore (Masiulis et al., 2018), which in turn leads to the inflow of negatively charged chloride ions into the neuron, hyperpolarizing it and driving it away from the threshold for excitation (Olsen and Sieghart, 2008). Like many human drug targets (Gunnarsson et al., 2008), the GABA_A receptor is highly evolutionary conserved across the vertebrates (Darlison et al., 2005), with only few amino acid changes at positions crucial for binding benzodiazepines within the ray-finned fishes (H. Haines and D. Larhammar, personal communication, March 2019). This suggests that the potential for GABA_A receptor binding and behavioral changes could be common in teleost fish.

In European river water, oxazepam is the benzodiazepine that is detected with the highest frequency (85%), with a maximum concentration of 61 ng L^{-1} (Fick et al., 2017). Close to the outlet of waste water treatment plants (WWTPs), oxazepam concentrations can reach several hundreds of ng L^{-1} to low $\mu\text{g L}^{-1}$ concentrations, depending on the size of the receiving river and the season (Aminot et al., 2015; Brodin et al., 2013; Camilleri et al., 2015). A handful of studies have exposed wild fish in the laboratory to oxazepam; most have used a low dose ($\sim 1 \mu\text{g L}^{-1}$) to mimic WWTP effluent concentrations and a high dose ($\sim 100 \mu\text{g L}^{-1}$ or $\sim 1 \text{ mg L}^{-1}$) as a positive control, with exposures typically lasting a few days to a week (Brodin et al., 2013; 2017a; Sundin et al., 2019). A recurrent finding is that exposed fish show more swimming activity when

the animal is placed alone in a novel arena. In perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*), exposure to $\sim 1 \mu\text{g L}^{-1}$ increased the number of swimming bouts performed (Brodin et al., 2013; 2017b; 2014; Klaminder et al., 2014), while burbot (*Lota lota*) showed an increase in burst swimming only at a higher dose ($100 \mu\text{g L}^{-1}$) (Sundin et al., 2019). Other behavioral changes recorded for $\sim 1 \mu\text{g L}^{-1}$ include a shorter latency to feed and reduced spatial use close to a group of conspecifics in perch (Brodin et al., 2013), as well as increased shelter seeking in burbot (Sundin et al., 2019), but generally these effects are more pronounced at higher doses. Also Atlantic salmon (*Salmo salar*) seem sensitive; animals exposed to $1.9 \mu\text{g L}^{-1}$ migrated faster, both down a river tributary and in a laboratory setting (Hellström et al., 2016). Laboratory fathead minnows (*Pimephales promelas*) do not show anxiolytic responses; animals exposed to $4.7 \mu\text{g L}^{-1}$ (but not $30.6 \mu\text{g L}^{-1}$) spent more time in the bottom zone and traveled shorter distances in the novel tank diving test, while no changes were observed in shelter seeking behavior (Huerta et al., 2016). In zebrafish (*Danio rerio*) such novel tank diving test results would be considered indicative of an increased anxiety-like state and/or a sedative effect (Cachat et al., 2010), but as the authors point out, fathead minnows may not respond in the same way (Huerta et al., 2016). Reproductive behavior of fathead minnows appears unaffected by a 21-day exposure to diazepam (0.1 , 1.04 or $13.4 \mu\text{g L}^{-1}$) (Lorenzi et al., 2014). Taken together, these studies provide evidence that oxazepam can induce anxiolytic effects in wild fish populations close to WWTP outlets, although species differ in sensitivity. Effects at river concentrations (ng L^{-1}) have not yet been investigated but can currently not be excluded.

The effects of oxazepam need to be weighed with effects of other pharmaceuticals (and other chemicals) present in the aquatic environment (Saaristo et al., 2018; Tanoue et al., 2019). One possible strategy could be to screen for behavioral effects of a select number of pharmaceuticals (Fick et al., 2010) using a single fish species, to facilitate comparisons between pharmaceuticals. Zebrafish (*Danio rerio*) would be an obvious candidate for high-throughput behavioral screening, not in the least because many psychoactive substances have already been tested at higher concentrations (Kalueff et al., 2014). A problem with laboratory strains is however that inbreeding has led to reduced genetic variation compared to wild populations and divergence among strains (Whiteley et al., 2011). In addition, adaptation to laboratory conditions has caused laboratory strains to differ from wild zebrafish in a number of ways (Hutter et al., 2010; Robison and Rowland, 2005; Wright et al., 2006). Studies on salmonid species reared in hatcheries (which are released into streams to mitigate declines of wild populations) suggest that the absence of predation in the captive environment selects for reduced fright responses (Johnsson et al., 2001; 1996). In zebrafish, startle responses to a falling disk were significantly stronger in the Nadia strain (4 generations removed from wild-caught descendants) compared to a pet-store derived strain (24 generations in the lab) (Robison and Rowland, 2005). Differences between

zebrafish strains have also been reported in the behavioral responses to alcohol (Gerlai et al., 2008). Hence it is unclear how well the responses of laboratory strains predict the behavior of wild fish.

In the current study, we asked whether defensive behaviors of zebrafish are affected by short-term exposure to oxazepam (in the $\mu\text{g L}^{-1}$ range), and if laboratory and wild-caught zebrafish differ in this respect. To this end, we exposed AB laboratory strain and wild zebrafish from India to 0.6, 5.6 or 60.8 $\mu\text{g L}^{-1}$ oxazepam for 9 days. To evaluate the effect of our treatment on behavior we used the novel tank diving test (NTDT¹), with addition of alarm pheromone. We also measured a physiological endpoint, namely the primary stress hormone cortisol, to evaluate effects of oxazepam on the hypothalamic-pituitary-interrenal (HPI²) axis.

1 Material and Methods

1.1 Animals and housing

Zebrafish of the AB strain were bred by SciLifeLab (Evolutionary Biology Center, Uppsala University) and transferred to the Department of Neuroscience, Biomedical Center at Uppsala University 6 months prior to the start of the experiment, where they were kept in a stand-alone rack system (Aquaneering, San Diego, USA). Wild-caught zebrafish were caught as juveniles in the Northern Bengal region in India, kept at the Norwegian University of Science and Technology (NTNU) in Trondheim for 5 months, and arrived at the Biomedical Center (BMC) in Uppsala 1 month before the start of the first experiment. Wild-caught zebrafish were housed in mixed-sex groups of 150 individuals in aerated 200L tanks kept at 26° C. Fish were fed once a day in the morning with granular food (ZM systems, UK) and live *Artemia* nauplii (Argent aquaculture, USA) and the lab maintained a photoperiod of 14:10 light:dark. Rearing, handling and experimental procedures were approved by the ethical committee on animal experiments of Uppsala (ethical permit: C55/13 to SW).

1.2 Exposure to oxazepam

AB and wild-caught zebrafish were exposed for 9 days to one of 3 different oxazepam concentrations, low (0.57 $\mu\text{g L}^{-1}$), medium (5.65 $\mu\text{g L}^{-1}$), high (60.83 $\mu\text{g L}^{-1}$) or a tapwater control. In control treatments, oxazepam concentration above the LOQ was measured in 5 of 12 water samples with a mean concentration of 2.3 ng L⁻¹ (positive samples). Oxazepam treatments were prepared from a stock concentration of 22 mg L⁻¹, which was placed in an ultrasound bath for 4 hours. Fish were exposed in mixed-sex groups of 19-23 individuals in 12 aerated glass tanks of 22L (21 × 33 × 20 cm L×W×H), resulting in 3 tanks per concentration. For each concentration, 2 tanks contained AB and 1 tank contained wild-caught zebrafish. A full water

exchange was performed once. Temperature was maintained at 25.4° C. Water samples were taken on 2 occasions for analysis of pH, conductivity, NH₄-H, NO₃+NO₃-N and on 4 occasions for oxazepam water concentrations; also mortality was recorded per tank (**Table A1**). Novel tank diving tests were performed prior to exposure and on day 7. On exposure day 9, fish were subjected to confinement stress after which they were humanely euthanized.

1.3 Quantification of oxazepam concentration in experimental tanks

Water samples were analyzed for the occurrence of oxazepam and not for other pharmaceuticals. Concentrations of pharmaceuticals in Swedish tapwater are very low, usually below ng L⁻¹ (Wahlberg et al., 2011). A triple-stage quadrupole mass spectrometer Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA) equipped with an Accela LC pump (Thermo Fisher Scientific, San Jose, CA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for instrumental analyses after separation of target analytes in water samples by C18 phase Hypersil gold column (50 mm x 2.1 mm ID x 3 µm particles, Thermo Fisher Scientific, San Jose, CA, USA). Water analysis was done according to the protocol of Fick et al. (2017), based on an online solid phase extraction system connected to liquid chromatography-tandem mass spectrometry (SPE LC-MS/MS), which has been also previously described (Khan et al., 2012). Briefly, water samples were defrosted at room temperature and 5 mL of each was filtered through 0.45 µm Filtropur S (Sarstedt, Nümbrecht, Germany) syringe filter into a glass autosampler vial. Then 5 ng of ²H₅-oxazepam (CAS 65854-78-6) purchased from Sigma Aldrich was added to the sample as an internal standard. Acetonitrile (LiChrosolv—hypergrade) purchased from Merck (Darmstadt, Germany) and ultrapure water prepared by Milli-Q Gradient water system (Millipore, Billerica, USA) both acidified with formic acid (Sigma-Aldrich, Steinheim, Germany) were used as 0.1 % mobile phases during the analysis.

A six-point calibration curve over the range of 1–500 ng L⁻¹ was used for evaluation of linearity, limit of quantification (LOQ) determination, and quantification of oxazepam in samples. Calibration curve was prepared by spiking both surrogate standard (CAS 604-75-1) and internal standard of oxazepam in ultrapure water. The analytical method was linear ($r^2 > 0.999$) over the range measured in calibration curve and its lowest point had the signal/noise ratio less than 10, so 1 ng L⁻¹ was considered to be equal to the LOQ. No concentration of oxazepam above LOQ was measured in blank samples prepared from ultrapure water and acetonitrile as a part of quality assurance/quality control.

1.4 Novel tank diving test with alarm cue injection (sNTDT)

To check for any pre-existing differences in diving response between experimental groups, all animals were tested in a novel tank diving test (NTDT) (Cachat et al., 2010) for 12 minutes prior to exposure. Fish were fed at least 1 hour before behavioral testing. Animals were netted out of their housing tank and were transferred using a 500 mL beaker (20×9×7 cm L×W×H), and their sex was determined by visual inspection of the belly and fin color. Subsequently, each fish was released into an individual narrow and deep 1.8 L rack system tank (length top 26 length bottom 23, width 5 and depth 12 cm; Aquaneering, USA). 8 individuals (4 from each of 2 different exposure tanks) were tested simultaneously per trial/ video in the order that they were caught. Zebrafish typically show a “diving response” when they enter a novel tank alone, where the animal dives to the bottom of a tank where it remains still for some minutes, after which it gradually moves higher up in the water column arguably to explore the novel tank (Cachat et al., 2010). An infra-red camera (JVC SuperLoLux, Japan) filmed the diving arenas from the side and an infrared light board (Noldus, the Netherlands) was placed behind the tanks. Two photographic lights (Walimex daylight 1000, the Netherlands) provided moderate lighting. The experimenter was not present in the room and not visible during video recordings. We used video tracking software (Ethovision XT14, Noldus, The Netherlands) to record the duration of time spent in the bottom, middle and top third of the tank, velocity, and duration immobile (i.e. velocity of 1 cm s⁻¹ or less). Tracks were manually edited to correct any tracking errors caused by reflections of visible light.

On exposure day 7 the diving response was assessed a second time. This time we injected conspecific skin extract to the diving tank water at minute 12:00 of the recording, and filmed the fish for another 12 minutes. Fish of the superorder *Ostariophysi* possess specialized epidermal cells that contain a chemical alarm cue (Frisch, 1942; Pfeiffer, 1977), suggested to be hypoxanthine-3(N)-oxide (Brown et al., 2000; Parra et al., 2009) or another molecule with a NO group (Enjin and Suh, 2013). Upon injury to the skin such as during a predator attack, these potent alarm cues induce anti-predator behaviors, including reduction in activity and movement to the bottom (Korpi and Wisenden, 2001). Conspecific alarm cue was extracted from the skin of a euthanized donor AB zebrafish by making 6 superficial cuts to each flank. The fish was then submerged in 50 mL water for 5 seconds. Four mL of this skin extract was injected into the diving tank water within 10 min after preparation. We termed this 24-minute long test the sNTDT³, the ‘s’ denoting ‘schreckstoff’, the term von Frisch used in his original article (Frisch, 1942). Alarm cue has been used before to invoke anxiety in zebrafish (Egan et al., 2009).

1.5 Confinement stress and sampling

On day 9, all fish were subjected to confinement stress prior to sampling, which consisted of placing an individual fish in a 50 mL centrifuge tube for 30 minutes, to capture the peak in cortisol (Pavlidis et al., 2015; Ramsay et al., 2009). Confinement stress is considered a standardized stressor (Pottinger and Carrick, 1999) and post-confinement cortisol levels have been used successfully to select rainbow trout for low and high stress responsiveness (Øverli et al., 2005). Immediately after the confinement stress, the animals were euthanized with an overdose of MS-222 (Sigma-Aldrich, Sweden) and a cut to the spinal chord at the neck. After the brain of the fish was dissected out, sex was determined by dissecting the gonads and then whole-body samples were snap frozen on dry ice and stored at -80°C.

1.6 Whole-body cortisol concentrations

We used a cortisol-specific radioimmunoassay (RIA) to analyze the cortisol concentrations present in homogenized zebrafish bodies, using a protocol based on (Ramsay et al., 2006). Zebrafish bodies (brain removed) were thawed, individually weighed and homogenized in PBS in a 15 mL centrifuge tube with 1 mL PBS buffer, using a VDI12 dispenser with S12N-7S rotor (VWR international, Sweden). Ethyl acetate was added and the sample was thoroughly vortexed and centrifuged, after which the cortisol containing ethyl acetate phase was transferred into a borosilicate glass vial. Ethyl acetate was evaporated with a nitrogen sparge and a block heater set at 40° C (Bibby Scientific Stuart concentrator, Cole Parmer, USA). The sample was reconstituted in ethyl acetate, and 3 replicates of the sample were transferred into three 1.5 mL microcentrifuge tubes.

Samples were compared to a standard curve containing known concentrations of cortisol (Sigma-Aldrich, Sweden) ranging from 0.125 to 80 ng mL⁻¹. [1, 2, 6, 7-3H(N)]-Hydrocortisone (Perkin-Elmer, USA) was added to each sample and the remaining ethyl acetate was evaporated using a SpeedVac (Savant, Thermo Fisher Scientific, Sweden). The samples were incubated overnight at 4° C with rabbit polyclonal antibodies to cortisol (Cambio, UK) in a bovine serum albumin buffer (Sigma-Aldrich, Sweden). The next day, unbound antibodies were washed away using dextran coated charcoal (Sigma, Sweden) incubated at 4°C for 5 minutes and centrifuged. The supernatant was added to a scintillation tube with EcoLite(+) scintillation cocktail (VWR, Sweden) and analyzed with the Tri-Carb 2910TR liquid scintillation analyzer (Perkin-Elmer, USA). Three controls were added containing i) no antibodies and no DCC; ii) no antibodies and DCC; iii) antibodies and DCC. Each assay contained an internal standard consisting of a mixture with equal volume of each extracted sample. The detection limit was 0.25 ng mL⁻¹. The proportion of samples lying below the limit of detection or above the highest standard curve was

analyzed per group, after which these samples were disregarded from subsequent analyses.
Cortisol concentrations were corrected for dilution factor and weight of the fish.

1.7 Statistical analyses

Statistical analyses were performed in R version 3.5.0 (R Development Core Team, 2013) with addition of packages ‘lme4’ (Bates et al., 2015), ‘lmerTest’ (Kuznetsova et al., 2016), ‘car’ (Fox and Weisberg, 2002) and ggplot2 (Wickham, 2009). To analyze the four behavioral variables (duration in bottom, duration in top, velocity and duration immobile) prior to oxazepam exposure, we fitted ANOVAs with explanatory variables strain (AB or wild-caught), sex (male or female), oxazepam concentration (control, 0.6, 5.6 or 60.8 $\mu\text{g L}^{-1}$) and all their interactions. Behavioral variables in the sNTDT after exposure were analyzed by fitting a LMM⁴ with fixed effects of alarm cue (before or after injection), strain, sex, oxazepam concentration, and all interaction, and random effects of individual and trial. Duration in bottom and top as well as duration immobile were recalculated to proportions by dividing the duration spent in the bottom by the track duration, and a logit transformation was applied to conform with the assumption of normality of residuals. To analyze the cortisol data, we first fitted a binomial GLM to the proportion of samples lying inside or outside the standard curve, adding strain, sex and oxazepam concentration as explanatory variables. We subsequently analyzed all samples with values inside the standard curve using linear mixed-effects models (LMMs), adding fixed effects of strain (AB or wild-caught), sex, oxazepam concentration (control, 0.6, 5.6 or 60.8 $\mu\text{g L}^{-1}$) and all their interactions, and random effects of sample and assay number. Cortisol concentrations were log transformed.

2 Results

2.1 Effect of alarm cue on diving response

The diving tests performed prior to exposure confirmed that there were no differences in diving responses between assigned exposure groups and there were no sex differences or interactions, but there was a main effect of strain, with AB bottom dwelling more than wild-caught zebrafish (76% resp. 56%; ANOVA, $F_{1,204}=34.704$, $p<0.001$), spending less time in the top zone (11% vs. 25%; ANOVA, $F_{1,204}=59.412$, $p<0.001$), swimming at a lower speed (2.95 vs. 3.15 cm s⁻¹; ANOVA, $F_{1,204}=11.047$, $p=0.001$) and were longer immobile (37% vs. 33%; ANOVA, $F_{1,204}=6.097$, $p=0.014$). This strain difference persisted after exposure, but in spite of AB bottom dwelling more, AB did not respond to the alarm cue (**Fig 1**). Significant alarm cue by strain interactions indicated that wild-caught zebrafish upon injection of conspecific skin extract increased their time spent in the bottom (LMM contrast, $t_{1,189}=-5.163$, $p<0.001$; **Fig 1**), reduced their time in the top (LMM contrast, $t_{1,25}=5.228$, $p<0.001$; **Fig B1**), reduced their velocity (LMM contrast, $t_{1,27}=6.335$, $p<0.001$; **Fig 2**) and were longer immobile (LMM contrast, $t_{1,30}=-5.918$, $p<0.001$; **Fig B2**), while no such effects were observed for AB zebrafish (LMM contrasts, $t_{1,191}=-0.327$, $p=0.744$; $t_{1,22}=1.080$, $p=0.292$; $t_{1,24}=0.583$, $p=0.565$; $t_{1,26}=-0.983$, $p=0.335$, resp.).

2.2 Effect of oxazepam on diving response

Oxazepam reduced the response to the alarm cue in wild-caught but not AB zebrafish. Wild-caught control females increased their time in the bottom after the alarm cue was added (LMM contrast, $t_{1,189}=-3.946$, $p<0.001$; **Table 1**), while females exposed to all three oxazepam concentrations did not (LMM contrasts, 0.6 µg L⁻¹: $t_{1,318}=-0.721$, $p=0.471$; 5.6 µg L⁻¹: $t_{1,318}=-0.669$, $p=0.504$; 60.8 µg L⁻¹: $t_{1,189}=-0.139$, $p=0.889$). Male wild-caught zebrafish showed an increase in bottom dwelling in the low and medium concentrations while the high concentration did not respond (LMM contrasts; 0.6 µg L⁻¹: $t_{1,189}=-2.194$, $p=0.029$; 5.6 µg L⁻¹: $t_{1,189}=-2.577$, $p=0.011$; 60.8 µg L⁻¹: $t_{1,189}=-1.348$, $p=0.179$). AB control females decreased rather than increased their time in the bottom (LMM contrast, $t_{1,189}=2.972$, $p=0.003$) while exposed AB females showed no response to the alarm cue (0.6 µg L⁻¹: $t_{1,196}=0.742$, $p=0.459$; 5.6 µg L⁻¹: $t_{1,189}=-0.342$, $p=0.733$; 60.8 µg L⁻¹: $t_{1,189}=-0.417$, $p=0.677$). AB males showed no response to the alarm cue, except for the lowest dose showing increased bottom dwelling (LMM contrasts; control: $t_{1,199}=-0.629$, $p=0.530$; 0.6 µg L⁻¹: $t_{1,189}=-2.766$, $p=0.006$; 5.6 µg L⁻¹: $t_{1,189}=-0.489$, $p=0.626$; 60.8 µg L⁻¹: $t_{1,189}=-0.457$, $p=0.684$). Both male and female wild-caught zebrafish showed strong reductions in movement in response to the alarm cue, and movement variables were less affected by oxazepam treatment (**Table 1**). In all but the middle oxazepam concentration, female wild-caught zebrafish reduced their velocity (LMM contrasts; control:

$t_{1,120}=3.916$, $p<0.001$; $0.6 \mu\text{g L}^{-1}$: $t_{1,160}=2.506$, $p=0.013$; $5.6 \mu\text{g L}^{-1}$: $t_{1,137}=1.326$, $p=0.187$; $60.8 \mu\text{g L}^{-1}$: $t_{1,100}=3.445$, $p<0.001$). Male wild-caught zebrafish reduced their velocity in all oxazepam concentrations (**Table 1**). AB males and females on the other hand, showed no reduction in movement in response to the alarm cue (**Table 1**). An exception were AB control females, which again responded in an opposite way to wild-caught animals, i.e. this group showed an increase in velocity and decrease in duration immobile (LMM contrast; $t_{1,98}=-2.342$, $p=0.021$ resp. $t_{1,128}=2.463$, $p=0.015$).

We also compared the behavior during the first half of the post-exposure diving test (i.e. prior to the addition of skin extract) between oxazepam exposed and respective control groups (**Table 2**). We detected overall few differences between oxazepam treatments. AB females exposed to $5.6 \mu\text{g L}^{-1}$ spent less time in the bottom (LMM contrast, $t_{1,318}=2.010$, $p=0.045$). AB males exposed to the $5.6 \mu\text{g L}^{-1}$ and $60.8 \mu\text{g L}^{-1}$ reduced velocity (LMM contrasts, $t_{1,306}=2.954$, $p=0.003$; $t_{1,307}=2.089$, $p=0.038$, resp.); the $5.6 \mu\text{g L}^{-1}$ group also were longer immobile (LMM contrast, $t_{1,315}=-2.481$, $p=0.014$) and the $60.8 \mu\text{g L}^{-1}$ group spent less time in the top (LMM contrast, $t_{1,329}=2.093$, $p=0.037$). Wild-caught males exposed to the highest concentration spent less time in the bottom (LMM contrast, $t_{1,334}=-2.511$, $p=0.013$).

2.3 Effect of oxazepam on cortisol

Some of our collected whole-body samples could not be measured for cortisol concentration due to a failed batch of antibodies; therefore we pooled the data for the sexes. There was no three-way interaction of strain, sex and oxazepam concentration on the proportion of samples inside the standard curve (GLM, $\chi^2=5.550$, $p=0.136$), indicating that the number of samples below LOQ did not differ between oxazepam concentrations within strain/ sex groups. Whole-body cortisol concentrations after 30 min confinement stress were $153 \pm 0.4 \text{ ng g}^{-1}$ tissue (grand mean $\pm$ SEM). We did not detect differences between oxazepam concentrations, or between the sexes, nor where there any interaction effects, but there was a main effect of strain (LMM, $F_{1,109}=5.139$, $p=0.025$, **Fig 3**), with AB zebrafish having higher cortisol concentrations than wild-caught zebrafish (AB: 171 ± 0.7 ; wild-caught: $116 \pm 0.7 \text{ ng g}^{-1}$ tissue; mean $\pm$ SEM).

3 Discussion

3.1 Anxiolytic effect of oxazepam

Oxazepam had an anxiolytic effect on the behavior of female wild-caught zebrafish for all investigated concentrations. While control females showed a strong response to addition of conspecific skin extract, this reaction was weakened in the oxazepam exposed groups. Oxazepam reduced the cue-induced movement to the bottom, while reduction in activity was still prominent in both oxazepam and control exposed groups, suggesting that some anti-predator behaviors were unaffected by oxazepam. The lowest concentration used here ($0.57 \mu\text{g L}^{-1}$) is the lowest water concentration reported to produce an effect on fish behavior, although this might not have been the lowest brain concentration since fish species differ substantially in bioconcentration rates (Brodin et al., 2013; Heynen et al., 2016; Klaminder et al., 2015; Sundin et al., 2019). Nevertheless, it presents an environmentally relevant dose since an oxazepam concentration of $0.58 \mu\text{g L}^{-1}$ was measured in a river receiving WWTP effluent from the city of Uppsala, Sweden (Brodin et al., 2013), which is a small to medium-sized urban area (OECD, 2019).

3.2 Sex differences

Male wild-caught zebrafish showed a reduced diving response only for the highest dose ($60.8 \mu\text{g L}^{-1}$). This sex difference was not present before exposure to oxazepam, indicating that it represents a sex difference in sensitivity to oxazepam. Previous studies on wild-caught zebrafish did detect sex differences in anxiety-related behaviors (Dahlbom et al., 2011). Males spent more time in the center of the open field and females spent more time under the roof in the shelter test (Dahlbom et al., 2011; Wong et al., 2012), suggesting that females are more ‘risk-averse’ or ‘shy’. Since benzodiazepines are GABA_A receptor modulators and not full agonists, GABA needs to be present in order for benzodiazepines to exert an effect (Millan, 2003). This could explain why males, if they are less anxious, are less sensitive to the effects of benzodiazepines.

3.3 Strain differences

Our results suggest that AB strain zebrafish have not retained the diving response to conspecific alarm pheromone, despite high levels of bottom dwelling in this strain, which have been noted before (Gerlai et al., 2008). As a consequence, the anxiolytic effect of these low concentrations of oxazepam could not be detected for AB zebrafish. It is possible that oxazepam had an anxiolytic effect on other behaviors that our behavioral test failed to capture. Alternatively, AB might exhibit altered reactions to a variety of stressors. Some of the patterns in our data provide evidence for the latter. Contrary to our expectation, AB control females spent less time in the

bottom and moved more after the alarm pheromone was added. Such an opposite reaction to a normally anxiogenic stimulus has also been described in a study employing an inhibitory avoidance learning paradigm; while TL strain zebrafish increased their latency to enter a dark compartment where they had previously received a shock, AB showed decreased latencies over time (Gorissen et al., 2015). Conversely, the AB strain has also shown anxiogenic responses to anxiolytic substances; intermediate doses of alcohol, which normally have an anxiolytic effect, were shown to increase bottom dwelling in AB (Gerlai et al., 2008). We found a similar anxiogenic effect of oxazepam in one of our exposure groups; AB males exposed to $60.8 \mu\text{g L}^{-1}$ oxazepam showed an increased diving response compared to the control. In summary, these results question the use of AB zebrafish for behavioral screens in ecotoxicology.

We emphasize that we would not have arrived at the same conclusions would we have performed a standard diving test, i.e. without the addition of conspecific skin extract. We observed very few differences between wild-caught exposure groups before the alarm pheromone was added. It is possible that the variation between individuals in anxiety-related behaviors overshadowed drug effects, making retesting of the same fish with increasingly stressful stimuli a more reliable testing method.

3.4 Cortisol concentrations

We found no differences between oxazepam concentrations in whole-body cortisol concentrations, but AB showed higher cortisol concentrations than wild-caught zebrafish. It is possible that wild-caught zebrafish were chronically stressed, after having to acclimatize to laboratory conditions. Chronic elevation of cortisol can inhibit secretion of cortisol by the interrenal tissue (Bonga, 2002). Alternatively, AB might exhibit higher cortisol concentrations than other strains. A study on AB and TL zebrafish found that AB fish had higher basal cortisol levels than TL, while TL but not AB showed an increase in cortisol in response to an inhibitory avoidance task (Gorissen et al., 2015).

3.5 Consequences for the use of laboratory strains

The laboratory zebrafish used in our study did not respond to the dose of conspecific skin extract applied under the test conditions. Since dose-dependent changes in diving responses have been documented after acute treatment with a variety of anxiolytic and anxiogenic drugs in laboratory strains (Bencan et al., 2009; Maximino and Herculano, 2010), the failure to display a diving response is likely specific to the stressor used here (alarm pheromone). It is possible that AB zebrafish have not completely lost the ‘schreck reaction’ (Frisch, 1942) but are only less sensitive than wild-caught zebrafish. Perhaps AB would have displayed a diving response to a

higher dose of alarm pheromone or if tested under more threatful conditions (although conditions should not be too threatful to avoid contextual fear conditioning (LeDoux, 2000)). This lower sensitivity could result from a loss of (part of) the specialized olfactory and neural mechanisms needed to detect and respond to alarm pheromone (Enjin and Suh, 2013). This points to a larger issue that domesticated animals may not be the most convenient organisms to study questions on defensive behaviors/ brain circuits within the fields of neuroscience, pharmacology and ecotoxicology (Krogh, 2004). Finally, it can currently not be excluded that the aversive nature of conspecific skin extract to some extent has to be learned from conspecifics.

4 Conclusions

Our results show that oxazepam concentrations as low as $0.57 \mu\text{g L}^{-1}$ reduce the response to conspecific alarm pheromone in wild-caught zebrafish. Zebrafish from the laboratory AB strain did not respond to the alarm cue, and consequently, the anxiolytic effect of oxazepam could not be measured. The lack of this key anti-predator response in AB zebrafish under the conditions tested can be an exciting avenue to study the genetic architecture underlying this behavior (Enjin and Suh, 2013). Finally, our results indicate that laboratory strains, such as AB, can differ from wild-caught animals in defensive behaviors and therefore their response to behaviorally modifying drugs. As such, laboratory strains may be unfit to use in ecotoxicological studies since the results might not translate to wild fish populations.

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7 Competing interests

The authors declare no competing interests.

8 Data availability

Data will be made available online.

9 References

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Tables and figures

Table 1 Response to the alarm cue. Post-hoc pair-wise comparisons (least square means) of behavior in the sNTDT before vs. after injection of alarm cue (conspecific skin extract) to the tank water. Comparisons were made within each experimental group, i.e. group of a specific strain (AB or wild-caught), sex (F: female or M: male) and oxazepam exposure concentration (control, 0.6, 5.6 or 60.8 $\mu\text{g L}^{-1}$). Numbers indicate p-values and arrows indicate an increase ($\uparrow$) or decrease ($\downarrow$) after addition of alarm cue.

Variable	Strain	Sex	control	0.6	5.6	60.8
% in bottom	AB	F	0.003 $\downarrow$	0.459	0.733	0.677
	AB	M	0.530	0.006 $\uparrow$	0.626	0.648
	wild	F	<0.001 $\uparrow$	0.119	0.601	0.078
	wild	M	0.184	0.029 $\uparrow$	0.011 $\uparrow$	0.179
% in top	AB	F	0.054	0.923	0.901	0.615
	AB	M	0.329	0.011 $\downarrow$	0.611	0.377
	wild	F	<0.001 $\downarrow$	0.311	0.312	0.139
	wild	M	0.060	0.009 $\downarrow$	0.011 $\downarrow$	0.133
Velocity (cm s^{-1})	AB	F	0.021 $\uparrow$	0.953	0.834	0.600
	AB	M	0.136	0.252	0.893	0.270
	wild	F	<0.001 $\downarrow$	0.013 $\downarrow$	0.187	0.001 $\downarrow$
	wild	M	0.030 $\downarrow$	0.046 $\downarrow$	<0.001 $\downarrow$	0.003 $\downarrow$
% immobile	AB	F	0.015 $\downarrow$	0.585	0.490	0.434
	AB	M	0.124	0.264	0.679	0.109
	wild	F	0.001 $\uparrow$	0.012 $\uparrow$	0.586	0.001 $\uparrow$
	wild	M	0.101	0.085	0.005 $\uparrow$	0.046 $\uparrow$

Table 2 Comparison of oxazepam exposed to control groups. Post-hoc pair-wise comparisons (least square means) between groups exposed to oxazepam (0.6, 5.6 or 60.8 $\mu\text{g L}^{-1}$) and the control group. ‘Before alarm cue’ resp. ‘After alarm cue’ columns present comparisons of the sNTDT variables before injection of conspecific skin extract in the tank water (first 12 minutes) resp. after addition (second 12 minutes). sNTDT variables measured using video-tracking software were ‘% in bottom’: percentage of time spent in the bottom third of the tank; ‘% in top’: percentage of time spent in the top third; ‘Velocity (cm s^{-1})’: swimming speed in cm per second; ‘% immobile: percentage of time moving with a velocity of 1 cm s^{-1} or less. Numbers indicate p-values and arrows indicate an increase ($\uparrow$) or decrease ($\downarrow$) in the experimental group compared to the control.

Variable	Strain	Sex	Before alarm cue			After alarm cue		
			0.6	5.6	60.8	0.6	5.6	60.8
% in bottom	AB	F	0.230	0.045 $\downarrow$	0.104	0.014 $\downarrow$	0.617	0.363
	AB	M	0.082	0.156	0.013 $\uparrow$	0.003 $\uparrow$	0.208	0.023 $\uparrow$
	wild	F	0.471	0.504	0.889	0.524	0.003 $\downarrow$	0.089
	wild	M	0.708	0.997	0.308	0.998	0.524	0.387
% in top	AB	F	0.184	0.154	0.149	0.012 $\downarrow$	0.903	0.713
	AB	M	0.150	0.200	0.035 $\downarrow$	0.022 $\downarrow$	0.387	0.058
	wild	F	0.304	0.603	0.819	0.307	0.007 $\uparrow$	0.042 $\uparrow$
	wild	M	0.793	0.961	0.453	0.996	0.809	0.387
Velocity (cm s^{-1})	AB	F	0.608	0.624	0.291	0.049 $\downarrow$	0.334	0.380
	AB	M	0.823	0.003 $\downarrow$	0.030 $\downarrow$	0.808	0.047 $\downarrow$	0.084
	wild	F	0.920	0.053	0.298	0.495	0.672	0.604
	wild	M	0.828	0.995	0.490	0.882	0.601	0.978
% immobile	AB	F	0.549	0.258	0.173	0.102	0.290	0.383
	AB	M	0.229	0.013 $\uparrow$	0.131	0.516	0.106	0.185
	wild	F	0.980	0.422	0.476	0.901	0.011 $\downarrow$	0.428
	wild	M	0.791	0.894	0.603	0.921	0.505	0.919

Fig 1:

Bottom dwelling in response to alarm pheromone and oxazepam. Percentage of time spent in bottom third of the tank before (open bars) and after (hashed bars) the injection of conspecific skin extract into the testing arena, for AB females (upper left panel), AB males (lower left), wild-caught females (upper right) and wild-caught males (lower right) exposed for 7 days to control tapwater (pale yellow bars), or oxazepam concentrations of 0.6 (mint green), 5.6 (aqua blue), 60.8 $\mu\text{g L}^{-1}$ (dark blue), or tap-water control (pale yellow; for exact concentrations see table A1). Bars indicate means $\pm$ SEM. Asterisks indicate significant differences ($p < 0.05$).

Fig 2: Swimming speed in response to alarm pheromone and oxazepam. Velocity (in cm per second) before (open bars) and after (hashed bars) the injection of conspecific skin extract into the testing arena, for AB females (upper left panel), AB males (lower left), wild-caught females (upper right) and wild-caught males (lower right) exposed for 7 days to control tapwater (pale yellow bars), or oxazepam concentrations of 0.6 (mint green), 5.6 (aqua blue), 60.8 $\mu\text{g L}^{-1}$ (dark blue), or tap-water control (pale yellow; for exact concentrations see table A1). Bars indicate means $\pm$ SEM. Asterisks indicate significant differences ($p < 0.05$).

Fig 3: Post-confinement cortisol concentrations after oxazepam treatment.

Whole-body cortisol concentrations (in ng per mg tissue) following a 30-minute confinement stress in AB and wild-caught zebrafish after a 9-day exposure to oxazepam concentrations of 0.6 (mint green), 5.6 (aqua blue), 60.8 $\mu\text{g L}^{-1}$ (dark blue), or tap-water control (pale yellow; for exact concentrations see table A1). In order to produce errorbars that reflect variation between individual fish, for this figure replicate samples were averaged per individual; however in the LMM random effects of sample and assay number were included (see section 1.7). Bars indicate means $\pm$ SEM. Asterisks indicate significant differences ($p < 0.05$).