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Design and development of a robotic predator as a stimulus in conditioned place aversion for the study of the effect of ethanol and citalopram in zebrafish

Romain J. G. Clément^{1,§}, Simone Macrì^{1,2,§}, Maurizio Porfiri^{1,3,*}

¹Department of Mechanical and Aerospace Engineering, New York University, Tandon School of Engineering, 6 MetroTech Center, Brooklyn, NY 11201, USA

²Centre for Behavioural Sciences and Mental Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy

³Department of Biomedical Engineering, New York University, Tandon School of Engineering, 6 MetroTech Center, Brooklyn, NY 11201, USA

Abstract

Zebrafish are becoming a species of choice in psychopharmacology, laying a promising path to refined pharmacological manipulations and high-throughput behavioral phenotyping. The field of robotics has the potential to accelerate progress along this path, by offering unprecedented means for the design and development of accurate and reliable experimental stimuli. In this work, we demonstrate, for the first time, the integration of robotic predators in place conditioning experiments. We hypothesized zebrafish to be capable of forming a spatial association under a simulated predation risk. We repeatedly exposed experimental subjects to a robotic heron impacting the water surface and then evaluated their spatial avoidance within the experimental tank in a subsequent predator-free test session. To pharmacologically validate the paradigm, we tested zebrafish in drug-free conditions (control groups) or in response to three different concentrations of citalopram (30, 50, and 100 mg/L) and ethanol (0.25, 0.50, and 1.00%). Experimental data indicate that, when tested in the absence of the conditioning stimulus, zebrafish displayed a marked preference for the bottom of the test tank, that is, the farthest location from the simulated attacks by the robotic heron. This conditioned geotaxis was reduced by the

* Author to whom correspondence should be addressed: mporfiri@nyu.edu.

§ These authors contributed equally to the work

Authors' contributions

SM and MP designed and supervised the research. RJC performed the experiments. RJC provided a first draft of the manuscript. All the authors performed statistical analysis. SM and MP wrote the final version of the paper. All the authors reviewed and approved the manuscript.

Ethics

All animal procedures were approved by the University Animal Welfare Committee of New York University under protocol number 13-1424.

Data availability

Datasets and codes used in the analyses are stored at the authors' home institution and will be provided upon request.

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administration of citalopram in a linear dose-response curve and ethanol at the low concentration. Ultimately, our data demonstrate that robotic stimuli may represent valid conditioning tools and, thereby, aid the field of zebrafish psychopharmacology.

Keywords

citalopram; conditioned placed preference; *Danio rerio*; ethanol; ethorobotics; predation risk

Introduction

Zebrafish (*Danio rerio*, Hamilton) are gaining momentum as an animal model in preclinical research, from genetics to neurosciences, and on to toxicology and psychopharmacology. Their high reproduction rate, small size, and similarity to human physiological pathways are some of the factors that have fueled their extensive use as laboratory animals [1, 2]. Particularly enticing is the possibility to perform high-throughput studies on behavioral phenotyping to elucidate the neurobiological determinants of psychiatric disorders [3, 4] and to inform the development of new drugs [5].

Hypothesis-driven research in psychopharmacology using zebrafish calls for the design and validation of targeted experimental paradigms, which are often derived from the rodent literature. For example, an approach-avoidance behavioral test, analogous to the light-dark test in mice, has been used to assess the efficacy of anxiolytic compounds in zebrafish [6-8]. Just as the light-dark test is based on fish tendency to prefer a brighter portion of the tank, the novel tank diving test rests upon their propensity to swim in the lower portion of an experimental tank [9, 10]. While these tests are constructed upon positional anxiety, other tests are based on the fear for natural predators [11]. For example, zebrafish exhibit fear-related behavior in response to the presence of predators, like the Indian leaf fish (*Nandus nandus*, Hamilton) [12] or the red tiger oscar (*Astronotus ocellatus*, Agassiz) [13]. All these tests have been shown to be sensitive to the administration of anxiety-modulating compounds [14].

Another viable paradigm in preclinical psychopharmacology using zebrafish is the conditioned place preference (CPP) test, which has been used extensively to examine the rewarding effects of drugs [15-17]. In this test, experimental subjects are first trained to form an association between the substance and a location (conditioning phase) and then tested in a drug-free state, to evaluate whether they exhibit a spatial preference for the drug-paired environment. During conditioning, subjects are repeatedly exposed either to a psychoactive drug in a compartment of a bi-partitioned conditioning arena or to the vehicle in the other compartment. Subjects are not allowed to move from one compartment to the other during conditioning. In the test phase, performed in a drug-free state, they are allowed to move between the drug- and the vehicle-paired compartments to ascertain the association between the drug and its respective compartment. The capability to associate a given environment with a specific underlying emotional state has also been leveraged to investigate the aversive nature of specific pharmacological compounds in a variant of the CPP, the conditioned place aversion (CPA) test. In this test, subjects are conditioned to an aversive substance, such as an

anesthetic drug [18], and then tested to evaluate whether they avoid the drug-paired compartment in a drug-free state.

The studies described above indicate that zebrafish have the capability to associate both rewarding and aversive psychoactive substances with a given location. Several efforts showed that zebrafish and other fish species are able to extend spatial associative learning capabilities to non-drug stimuli. For example, Arthur and Levin [19] conditioned zebrafish to avoid a compartment associated with a fear-eliciting stimulus (a moving net). Similarly, Vilhunen [20] showed that the Arctic charr (*Salvelinus alpinus*, Linnaeus) can be trained to avoid a compartment associated with the odor clues of Arctic charr-fed pikeperch (*Sander lucioperca*, Linnaeus).

Although Arthur and Levin [19] and Vilhunen [20] observed robust conditioning, since the publication of these efforts almost two decades ago, we have witnessed a range of new technologies that could help refining these methods and favor high-throughput screening. For example, several authors demonstrated that zebrafish exhibit antipredatory responses to moving images of a predator projected on a screen [21-24]. However, computer-animated images may bring their own drawbacks, which could restrain their value in conditioning experiments. In a binary choice test, we compared the fear responses of zebrafish elicited by a live red tiger oscar and a computer-animated image to discover that zebrafish exhibited a weaker avoidance for a computer-animated image than a live predator [13]. At the same time, it is difficult to advocate for the use live predators in conditioning experiments, due to methodological (that is, potential idiosyncrasies with focal subjects and fatigue between trials that might increase inter-individual repeatability) and ethical reasons (that is, the risk of predator-induced wounds or death of experimental subjects in case of contact with the predator).

Over the years, we designed and developed robotic stimuli inspired – in shape, size, and locomotory patterns – by their live counterparts [13, 25-31]. In these ethorobotics studies at the interface of animal behavior and robotics, we observed that zebrafish exhibit consistent avoidance responses when confronted with predator-like robots [13, 26, 28, 29, 31] and approach responses when faced with conspecific-like robots [25, 27, 30]. Beside eliciting predictable responses without harm, robotics allows standardization and fine-tuning of visual stimuli, which may be difficult to achieve through live predators or computer-animated images. We experimentally demonstrated that robotic stimuli could match the avoidance response evoked by live predators and lead to reduced inter-individual variability, compared to live stimuli or computer-animated images [13]. While the potential of robotics to aid in the study of animal behavior is vastly documented [32-34] and robotic predators are being increasingly integrated in a number of behavioral assays on multiple species [35-39], the feasibility of robotics-based solutions in place of conditioning experiments has remained elusive.

In the present study, we combined zebrafish natural tendency to form associations between a location and an aversive stimulus, and the advantages offered by robotics, to design a novel conditioning experiment. In this paradigm, we hypothesized zebrafish to be capable of forming an association between a given portion of a test tank and a robot resembling a

natural predator – the Indian pond heron (*Ardeola grayii*, Sykes) impacting the water surface. To that end, we repeatedly exposed experimental subjects to the robotic predator and then assessed the spatial avoidance in a stimulus-free test session. To pharmacologically validate the paradigm, after conditioning, we tested independent groups of adult zebrafish both in drug-free conditions and in response to three different concentrations of citalopram and ethanol.

Ethanol and citalopram have been selected based on their known efficacy in modulating fear- and anxiety-related behaviors across a number of taxa ranging from humans [40, 41] to rodents [42, 43] and fish [9, 23, 44]. In zebrafish, we have already demonstrated that ethanol administration modulates individual behavior in response to the same predatorial stimulus adopted in this study [26]. Specifically, we observed that the latency of zebrafish to retreat to a shelter following a simulated attack by a robotic heron tended to decrease with low doses of ethanol (0.25% and 0.50% w/v) while it significantly increased with a high dose (1.00% w/v) [26]. Sackerman and collaborators [9] demonstrated that a selective serotonin reuptake inhibitor citalopram (100 mg/L) increased the time in the upper part of the water column in zebrafish.

Ultimately, based on the hypothesis that a robotic heron constitutes a strong conditioning stimulus, we anticipated zebrafish to be able to form an association between the stimulus and the portion of the tank associated to it, thereby avoiding such portion in drug-free state. Additionally, based on the known efficacy of citalopram and ethanol in regulating fear- and anxiety-related responses, we predicted that zebrafish exposed to these substances would display a differential spatial avoidance compared to vehicle treated counterparts. Beside positional (avoidance-related) parameters, in the present study we aimed at evaluating zebrafish behavior through the adoption of an in-house developed three-dimensional (3D) scoring system that, compared to traditional 2D approaches has been shown to reduce the rate of false positive and false negative findings [45]. This 3D analysis allowed a thorough phenotyping encompassing both horizontal and vertical movements performed in the entire test-tank.

Material & methods

Animal care and maintenance

A total of 64 adult zebrafish were used, with a 1:1 ratio of males to females. The fish were purchased from a commercial supplier (Carolina Biological Supply Co., Burlington, NC, USA) and were kept in 10 L holding tanks (Pentair Aquatic Eco-Systems Locations, Cary, NC, USA) with no more than 10 individuals per tank, under a photoperiod of 12 hours light/12 hours dark. Fish were fed commercial flake food (Hagen Corp. Nutrafin max, Mansfield, MA, USA) once per day after experiments ended. Temperature and pH of the water in the holding tanks were maintained at 26 °C and 7.2 pH, respectively. Stress coat was added to regular tap water to remove chlorine and chloramines. Fish were acclimatized in the holding tanks for a minimum of two weeks before experiments started.

One day before the first conditioning session, each fish was transferred from the initial housing tank to its individual tank (1.5 L) tank. Following each conditioning session, the fish

was returned to its individual tank. To prevent social isolation, fish were always kept in visual contact with other conspecifics. After the test was completed, the fish was placed in another housing tank.

Experimental setup

The experimental tank was 42 cm (length) $\times$ 30 cm (width) $\times$ 30 cm (height), filled up to a level of 15 cm with conditioned water maintained at 26°C. The tank was covered with white contact paper on three later sides and on the bottom, in order to create a background for enhanced contrast and minimal light reflection. The front side was left uncovered to allow filming from the front view. Two lights from the front and top were used to provide uniform lighting, facilitating automated tracking. The experimental arena was surrounded by a black curtain to minimize disturbance due to movement of the experimenter and light interference. Two cameras (Logitech C920 Pro HD webcams, Newark, CA, USA) were placed at the top and the front of the tank, respectively. The top camera was placed 98 cm from the bottom of the tank, directly above the front wall of the tank to minimize potential tracking error associated with the reflection of the fish from the front wall. The front camera was placed 43 cm from the front wall, at the same height of the water level (15 cm) to minimize potential tracking error associated with the reflection of the fish on water surface.

Two partitions (30 cm wide, made of transparent acrylic) with sliding doors (20 cm wide, made of transparent acrylic) separated the tank into three compartments (see figure 1a). The length of the central compartment was approximately 12 cm and that of each lateral, conditioning, compartments was approximately 15 cm. The doors were slightly tilted to avoid obstructing the view of the arena due to the perspective of each camera (see figure 1a). A robotic stimulus mimicking a sympatric predator of zebrafish, the Indian pond heron (figure 1b) was programmed via a microcontroller (Arduino Uno, Somerville, MA, USA) to strike the water surface approximately every 30 seconds in one of the lateral compartments, by rotating about the Z-axis (figure 1c). The duration of the attack was 2 seconds at a nominal angular speed of 90°/s, so that the stimulus struck the water surface in one second and then resumed its original configuration in another second. The efficiency of this stimulus to induce fear in zebrafish was demonstrated in previous work by our group, see [26] for a detailed description.

Experimental procedure

The experiments were performed from March to May 2019. The subjects were trained and tested in batches of eight individuals. Every batch contained four males and four females. In each batch, all the experimental conditions were tested with one male and one female. Trials were alternated between males and females, and the order of the conditions was alternated between batches. The side of the predator was shifted between the first four subjects and the last four subjects. The order of the door opening was also balanced so that in half of the trials the subject had access first to the predator compartment, while in the other half, the subject had access first to the empty compartment.

Each subject was trained in six sessions over three days (one in the morning from 8am to 12pm, one in the afternoon from 2pm to 6pm). In each training session, the subject was

hand-netted from its individual housing tank and released into the middle compartment of the test tank with both doors closed. After one minute of habituation, one of the doors was manually lifted, allowing the fish to exit the middle compartment. The door was kept open for up to one minute to let the fish swim into the conditioning compartment. The door was closed as soon as the fish left the middle compartment. In the case of a fish not moving within one minute, it was gently accompanied into the other compartment using a hand net. The subject was left in the first training compartment for two minutes, in which it was either subjected to attacks by the robotic heron or exposed to no stimulus. At the end of the two-minute period, the door was lifted again to allow the fish to swim back into the middle compartment, and the door was closed upon returning there. The fish was gently pushed using a hand net when it did not return to the middle compartment within 10 seconds. Upon returning to the middle compartment, the subject was kept therein for one minute before the other door was lifted. The door was left open for up to one minute to let the fish exit by itself, and the door was closed upon exit. A hand net was used to gently accompany the fish when it did not leave within one minute. The subject was then left in the second conditioning compartment for two minutes, where it experienced either attacks by the robotic heron or the empty compartment. At the end of these two minutes, the subject was placed back into its individual holding tank using a hand net.

On the fourth day, the subject was transferred from its individual housing tank into a 500 mL beaker containing the desired psychoactive compound using a hand net. In the citalopram condition, the subjects were kept for 5 minutes in the beaker with the following solutions: vehicle (0), 30, 50, 100 mg/L. In the ethanol condition, the subjects were kept for longer exposure time of 60 minutes, with the following solutions: vehicle (0), 0.25, 0.50, 1.00 % (volume/volume %). After the appropriate time, the subject was transferred from the beaker using a hand net and briefly dipped in an intermediary beaker containing the same water as in the experimental tank, to wash out traces of the aforementioned compounds. The subject was then released into the middle compartment of the test tank with both doors closed. After one minute of habituation, the doors were simultaneously lifted, allowing the fish to freely swim in the whole tank for 10 minutes. At the end of the test session, the subject was returned to its individual tank.

The differential exposure time between ethanol and citalopram was dictated by previous literature. Specifically, with respect to ethanol, as detailed elsewhere [26], this exposure time has been already reported to allow the attainment of maximal blood and brain concentrations of ethanol (90% of the concentration present in the beaker, [46]), and neither to increase mortality nor to affect visual acuity [47]. With respect to citalopram, administration schedule has been selected based on previous literature [9]. Sackerman and colleagues evaluated citalopram uptake in tissues (muscles and brain) and saturation binding in response to 3–4 min immersion time and observed that this administration regime allows a direct comparison between citalopram concentration in the administration tank and brain citalopram availability.

Tracking, 3D reconstruction, and behavioral outcome measures

The top and front view videos were recorded at the same time on a single computer. The fish positions were tracked using an in-house developed software, whose details can be found in [13, 48, 49]. The tracks from both the views of each trial were merged to reconstruct the 3D trajectories. From the trajectories, we computed the following behavioral parameters:

- *aversion* (measured as the ratio of the time spent in the empty compartment over the time spent in both the empty and conditioning compartments). Values close to one indicate avoidance of the robotic predator and values close to zero suggest preference;
- *geotaxis* (measured as the proportion of time spent in the bottom half of the water column). Large values are associated with anxiety-related response [50];
- *freezing* (measured as the proportion of time that the fish moved less than two centimeters anywhere in the tank over a rolling window of two seconds). Large values are associated with an anxiety-related response [50];
- *spatial entropy* (measured as Shannon entropy [51], computed on the subject's 3D positions binned across the tank using a uniform grid size corresponding to approximately two body lengths, resulting into $7 \times 5 \times 3$ bins). Borrowing the definition introduced by [52] to examine behavioral phenotyping in rodents, this measures the level of exploration, with higher values indicating a higher tendency of the fish to explore the entire experimental tank,
- *average speed* (computed as time-average of the first-order numerical differentiation of the position time series);
- *average acceleration* (computed as time-average of the magnitude of the first order numerical differentiation of the velocity time series). Measuring the acceleration in addition to the speed offers a more complete representation of the locomotory activity of the subject, which is useful to ascertain erratic movements associated with anxiety-related response [50, 53];
- *average angular speed* (computed on the basis of a finite difference approximation of the curvature of fish trajectories [54]). Together with the acceleration, the angular speed offers an indication of erratic movements [53];

Statistical analysis

Prior to the analysis, we removed from the data outliers defined as subjects with freezing values that fall outside of the 1.5 interquartile range. Four subjects with the highest freezing time proportion for each condition were thus removed as outliers, in both citalopram and ethanol treatments.

For positional parameters (avoidance and geotaxis), we tested whether experimental subjects showed a clear horizontal and/or vertical avoidance of the robot by comparing the avoidance and geotaxis indices against chance. To that end, we estimated a 95% confidence interval of the value for each concentration and examined whether it overlapped with 0.50. For ethological parameters (freezing, average speed, average acceleration, average angular

speed, and spatial entropy), the value for each individual fish was used as a response variable in a linear mixed model, with concentration and time (5 bins with a 1-minute interval) as fixed factors, and subject as a random effect to account for repeated measures.

We note that, although we tested male and female subjects, sex has not been taken into account in the statistical design. This decision rested upon the following grounding: given the novel nature of this study, in order to improve the generalizability of findings, we decided to test a heterogeneous experimental group. Toward this aim, we opted to test a population composed of male and female subjects. While this choice allowed testing a sex-counterbalanced population, it was underpowered to assess potential sex differences. Consequently, we considered sex as an intervening variable, and controlled for it. However, since in the present study we were not explicitly interested in sex differences, the number of males and females did not allow testing main effects of sex.

Statistical analyses were performed with R v3.5.0, using the package lme4 v1.1-17. When significant effects were detected, post-hoc tests were performed using Dunnett's test from the package emmeans v1.2.4, comparing control to other concentrations, and first minute to other minutes.

Results

Citalopram

Aversion: In addressing whether previous exposure to the robotic heron resulted in the avoidance of the compartment paired with the aversive stimulus, we observed that control subjects did not exhibit a clear preference for any of the compartments (95% CI: 13.17–65.20%). Similarly, experimental groups failed to show a preference for one or the other compartment (30 mg/L, 28.11–57.65%; 50 mg/L, 28.51–72.82%; and 100 mg/L, 26.92–84.34%) (figure 2a).

Geotaxis: Control subjects showed a clear positional avoidance of the robot by exhibiting a robust preference for the lower portion of the apparatus (95% CI: 74.11–100.08%). In accordance with our predictions, citalopram administration exerted a linear dose-response curve on anxiety, whereby subjects treated with 30 and 50 mg/L failed to show any preference for the lower or the upper portion of the apparatus (30 mg/L, 95% CI: 23.51–79.75%; and 50 mg/L, 11.62–66.38%;) and subjects treated with 100 mg/L exhibited a significant preference for the upper part of the test tank (–8.20–38.42%) (figure 2b).

Freezing: Although experimental subjects exhibited mild levels of freezing during the early stages of the test, freezing steadily declined throughout the test session to attain minimal levels during the fourth and fifth minutes of testing ($\chi^2_4 = 16.22$, $P = 0.003$ for time; Dunnett's test: $t_{108} < -2.81$, $P < 0.022$). The time dependence of the freezing response was indistinguishable among the four experimental groups ($\chi^2_{12} = 9.68$, $P = 0.644$ for interaction between time and concentration). Experimental groups were indistinguishable in terms of absolute values of freezing ($\chi^2_3 = 0.19$, $P = 0.980$ for concentration) (figure 2c).

Spatial entropy: Mirroring the freezing behavior, experimental subjects displayed an increasing tendency of exploration during the later stages of the test ($\chi^2_4 = 31.41$, $P < 0.001$ for time; Dunnett's test: $t_{108} > 4.67$, $P < 0.001$). Such an exploration tendency was not modulated by citalopram concentration, whereby spatial entropy did not significantly with the experimental group ($\chi^2_3 = 2.87$, $P = 0.412$ for concentration). Also, we did not register an interaction between time and concentration ($\chi^2_{12} = 9.79$, $P = 0.635$) (figure 2d).

Average speed: Average speed did not vary in time ($\chi^2_4 = 8.48$, $P = 0.076$) and was neither influenced by citalopram concentration, neither in absolute values ($\chi^2_3 = 0.51$, $P = 0.916$), nor as a function of time ($\chi^2_{12} = 5.97$, $P = 0.918$) (figure 2e).

Average acceleration: Experimental subjects displayed lower values of average acceleration during the second and third minutes of testing ($\chi^2_4 = 10.66$, $P = 0.031$; Dunnett's test: $t_{108} < -2.58$, $P < 0.040$). However, this was not significantly affected by the citalopram concentration ($\chi^2_3 = 0.36$, $P = 0.948$), and we failed to identify an interaction between time and concentration ($\chi^2_{12} = 10.40$, $P = 0.581$) (figure 2f).

Average angular speed.—Average angular speed decreased over time ($\chi^2_4 = 19.32$, $P < 0.001$; Dunnett's test: $t_{108} < -3.57$, $P < 0.020$), across all experimental groups in a similar temporal pattern ($\chi^2_3 = 1.06$, $P = 0.786$ for concentration; $\chi^2_{12} = 7.88$, $P = 0.795$ for interaction between time and concentration) (figure 2g).

Ethanol

Aversion: In addressing whether previous exposure to the robotic heron resulted in the avoidance of the compartment paired with the aversive stimulus, in contrast to our predictions, control subjects appeared to prefer the compartment paired with the robotic heron, but this preference failed to reach statistical significance (95% CI: 5.57–54.58%). Note that control subjects for ethanol treatment were kept in vehicle solution for one hour, while those in the citalopram condition only for five minutes. A preference for the bottom of the tank was not exhibited by the other experimental groups (0.25% concentration, 5.91–62.67%; 0.50%, 31.60–61.15%; and 1.00%, 31.70–80.30%) (figure 3a).

Geotaxis: Control subjects tended to avoid the robot by exhibiting a preference for the lower portion of the apparatus, although it was not statistically significant (95% CI: 48.61–101.44%). Such a preference was modulated by ethanol administration, whereby 0.25% subjects failed to show any preference for the lower or the upper portion of the apparatus (0.25% concentration, 28.33–91.80%). Ethanol administration followed a U-shaped dose-response curve whereby subjects treated with 0.50% and 1.00% exhibited a preference for the lower part of the test tank (0.50%, 52.46–96.08%; and 1.00%, 54.92–102.88%) (figure 3b).

Freezing: While experimental subjects exhibited mild levels of freezing during the early stages of the test, such a behavior was nearly absent towards the fourth and fifth minute of testing ($\chi^2_4 = 20.54$, $P < 0.001$ for time; Dunnett's test: $t_{108} < -3.38$, $P < 0.004$). Such a temporal patterning was indistinguishable among the four experimental groups ($\chi^2_{12} = 6.60$,

$P = 0.883$ for interaction between time and concentration). Overall, freezing was not different among concentrations ($\chi^2_3 = 2.45$, $P = 0.484$) (figure 3c).

Spatial entropy: Complementary to the freezing behavior, experimental subjects showed an increasing tendency to explore the entire experimental tank during the later stages of the test ($\chi^2_4 = 26.47$, $P < 0.001$ for time; Dunnett's test: $t_{108} > 2.66$, $P < 0.033$). Low values in spatial entropy during the early stages of the test corroborate the observation of increased freezing described above. Additionally, spatial entropy did not significantly vary as a function of ethanol concentration ($\chi^2_3 = 4.15$, $P = 0.246$) nor an interaction between time and concentration ($\chi^2_{12} = 15.77$, $P = 0.202$) (figure 3d).

Average speed: Average speed was different over time in all experimental subjects ($\chi^2_4 = 10.78$, $P = 0.029$). Specifically, 0.50% ethanol fish swam significantly faster than controls (Dunnett's test: $t_{24} = 2.59$, $P = 0.043$). Average speed was also influenced by ethanol administration ($\chi^2_3 = 8.64$, $P = 0.034$). There was no significant interaction between time and concentration ($\chi^2_{12} = 7.84$, $P = 0.798$) (figure 3e).

Average acceleration: Average acceleration did not significantly differ among ethanol concentrations ($\chi^2_3 = 6.75$, $P = 0.080$), time ($\chi^2_4 = 3.60$, $P = 0.462$), or an interaction between time and concentration ($\chi^2_{12} = 3.60$, $P = 0.462$) (figure 3f).

Average angular speed: Experimental subjects exhibited a steady decline in average angular speed over time ($\chi^2_4 = 22.77$, $P < 0.001$; Dunnett's test: $t_{108} < -2.53$, $P < 0.045$). There was no significant effects of concentrations ($\chi^2_3 = 2.34$, $P = 0.505$) or an interaction between time and concentration ($\chi^2_{12} = 3.54$, $P = 0.990$) (figure 3g).

Discussion

In the present study, we observed that repeated exposures to a predator-like robotic stimulus may represent a valid tool to condition zebrafish to associate a given portion of a test tank with a negative emotional state. After six conditioning sessions in which experimental subjects were exposed to periodic attacks by a robotic heron striking the water surface, we observed a robust avoidance response during a test session conducted in the absence of the conditioning stimulus. Importantly, we noted that such an association could be modulated via the administration of psychoactive compounds – citalopram (a selective serotonin reuptake inhibitor) and ethanol (a depressant of the central nervous system acting on GABAergic neurotransmission). We offer that such an association is specific to spatial avoidance, whereby zebrafish ethogram was marginally influenced by the pharmacological manipulation.

Through 3D tracking of animal behavior, we examined avoidance along the horizontal (aversion) and vertical (geotaxis) planes of the experimental tank. Specifically, we quantified the spatial conditioning effect of the robotic predator in terms of avoidance of the location where the attacks were performed, from front and top views. In this vein, aversion measures the tendency of the animal to prefer the compartment of the tank opposite to the robotic heron, while geotaxis quantifies fish tendency to occupy the farthest portion of the water

column with respect to the robotic heron. We found that zebrafish avoided the water surface in the absence of predatory attacks after the conditioning session, albeit with minor variations associated with the exposure protocol (control subjects were exposed to the beaker for five minutes in the citalopram experiment and one hour in the ethanol experiment). Zebrafish have been shown to swim towards the bottom of the test tank in a state of anxiety and fear, which can be modulated through pharmacological manipulations [8, 50]. As a result, it is tenable that the robotic heron was able to elicit a geotactic response that was captured from the front view of the tank, where we recorded the motion of the fish along the water column. In contrast with our expectations, however, we failed to observe a consistent aversion: subjects did not prefer either of the lateral compartments of the experimental tank during testing in a drug-free state.

These results parallel our recent observations collected in response to a robotic predator inspired by the oscar fish [31]. Different from the current work, experiments in [31] consisted of a binary choice test between an empty compartment and a compartment in which a robotic replica was maneuvered by an external platform to swim along three dimensions while oscillating its body. Therein, we observed robust geotaxis in response to the robotic replica, whereby the time spent at the bottom of the tank increased threefold upon the presentation of the robotic stimulus. Similar to the present study, geotaxis was not mirrored by aversion along the horizontal plane. By comparing the time traces of the motions of the fish and replica, we discovered that the observed avoidance responses were due to vertical movements of the fish away from the replica. This evidence may suggest that geotaxis could be a preferable escape response compared to aversion when zebrafish are presented with a robotic predator.

In the present study, the lack of horizontal spatial association with risk may also be due to the size of the test tank, which was not sufficient to allow fish to appraise one of the lateral compartments as the predator-free one. Although the robotic heron only struck the water surface in a localized region of the conditioning compartment, its length was about a half the size of the test tank. In nature, we might expect that fish escaping strategy from an aerial attack will trigger aversion for a region larger than the size of the tank. One might argue that animals may not be able to associate a predator threat with narrow location, as predators are likely to move. Interestingly, recent work exploring the use of robotic stimuli in conditioning experiments for chicken found that a robotic snake was less effective than air puff and water spray in inducing conditioned place aversion [55]. The ratio between the robotic stimulus and the experimental arena was comparable to ours, whereby the robotic snake was 60 cm long and the width of each compartment was 95 cm.

Just as zebrafish were a few tail beats away from the location of the attack of the robotic heron, so also the chicken in [55] were only a couple of steps away from the robotic snake. It is tenable that the presence of a robotic predator on one side does not exclude the perception of its presence on the other side. While the locomotory repertoire of chicken develops only in the plane of the arena, zebrafish may exploit also the third dimension along the water column of the test tank, which is what we observe in terms of geotaxis. Potential refinements of this test may contemplate a wider test tank and the presence of shelters. These modifications could be combined with systematic randomization in the localization of the

heron attacks to facilitate the association between the conditioning compartment and a potential threat.

An alternative explanation for the lack of conditioned placed aversion is that the fearful nature of the robotic stimulus was not sufficient to condition the response of the subjects. This proposition would conflict with our previous findings [26], wherein we reported a strong fear response in zebrafish exposed to simulated attacks by a robotic heron. Importantly, such a response was mitigated by acute ethanol administration and validated against two additional anxiety-related tests. In addition, the proposition that the robotic heron was not sufficiently fearful would also contrast with the observed geotaxis and its modulation in response to the administration of classical anxiety-regulating compounds. Perhaps, the lack of aversion, along the horizontal plane, could be related to the difference between “defensive approach” and “defensive avoidance,” underlying different aspects of anxiety in zebrafish, as defined by [56].

In accordance with studies conducted across numerous animal taxa, the administration of the selective serotonin reuptake inhibitor citalopram monotonically decreased the observed anxiety-related response [9, 41, 42]. The possibility of modulating anxiety-related behavior through serotonergic agonists is widely documented in zebrafish [57-60]. For example, acute administration of the selective serotonin reuptake inhibitor fluoxetine or the 5-HT_{1a} receptor agonist buspirone was found to reduce geotaxis in a novel tank diving test [58]. Beside citalopram, we observed that ethanol also reduced predator-induced anxiety, albeit at a lesser degree than we had initially predicted. Specifically, of the three concentrations tested, only 0.25% abolished preference for the bottom of the test tank. Although we anticipated 0.50% and 1.00% ethanol concentrations to reduce geotaxis, we note that a U-shaped behavioral response is widely documented in both clinical and preclinical research. For example, a study on humans reports that while low ethanol concentration had a disinhibitory effect, higher concentrations increased anxiety [61]. Likewise, in our previous study [62], we determined that zebrafish treated at low ethanol concentration have a higher tendency to acquire a leader role in a group, while subjects treated at high concentrations tend to be followers.

The robust effect of citalopram on geotaxis was not mirrored by variations in zebrafish ethogram, scored through a wide array of behavioral measures. While we registered a steady decrease of anxiety-related measures over time due to progressive habituation to the environment, we failed to identify an effect of citalopram. During the five minutes of the test, subjects spent less time freezing, increased the tendency to explore the environment (measured through spatial entropy), and reduced erratic movements (measured through the average angular speed and acceleration). However, none of these ethological parameters was modulated by citalopram concentration. Similar to citalopram administration, we did not register a strong effect of ethanol exposure on ethological parameters. Although, these ethological parameters displayed a predictable variation in time, equivalent to the analysis of citalopram data, only the average speed was influenced by ethanol concentration. Specifically, we determined that acute 0.50% ethanol concentration caused an increase in the average speed that could, again, be related to disinhibitory effects of alcohol. Similar U-shaped dose-response curves in response to acute ethanol administration have already been

observed in zebrafish locomotion [63, 64]. While we originally selected citalopram and ethanol based on their known capability to affect anxiety-related behaviors in zebrafish, we acknowledge that their mechanisms of action are not exclusively targeting anxiety. While citalopram acts as a selective serotonin reuptake inhibitor, ethanol potentiates GABAergic signaling. Based on these considerations, we believe that future studies shall test whether zebrafish behavior in this test is also sensitive to compounds selectively acting on anxiety like benzodiazepines.

From a methodological point of view, this study contributes to the integration of robotic stimuli in behavioral neuroscience, by demonstrating, for the first time, the use of robots in conditioned place aversion. There is a wide body of ethorobotics research offering a compelling case in favor of the use of robotic stimuli to replace live predators, toward improved consistency, repeatability, and tailorability, while reducing potential harm to live animals. In our previous work [13, 26, 29, 31], we have shown that robotic stimuli can elicit avoidance response similar to live predators across a number of experimental tests involving binary choice, shelter-seeking, and spatial preference. Analogous observations have been conducted in other freshwater species, clarifying the possibility of triggering complex escape responses in groups [37, 38] and helping explain the process of identification of potential threat [39]. Compared to these studies, this is the first effort in which the fear-evoking nature of the robotic predator is utilized as a conditioning stimulus.

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Figure 1.

(A) Experimental setup, showing the central compartment and the lateral, conditioning compartments, along with the location of the two cameras and the sliding doors. For the sake of illustration, the right door is closed and the left is open. Note that the tilting of the partitions is exaggerated for clarity of presentation. (B) Head of the robotic heron used as an aversive stimulus during conditioning. (C) Overview of the robotic arm used to control the attacks of the heron.

Figure 2.

(A) Aversion (inverted triangle) with 95% CI (whiskers) measured as the time spent in the compartment opposite to the one paired with the predatory stimulus divided by the total time spent in both of the conditioning compartments. The dashed line represents a chance level. A CI intersecting the dashed line indicates that avoidance was not statistically different from chance; \$ significantly different from chance. (B) Geotaxis (inverted triangle) with 95% CI (whiskers) measured as the time spent in the bottom half of the test tank divided by the duration of the test session (300 s). The dashed line represents a chance level. A CI above the dashed line indicates that fish showed a significant preference for the bottom half of the water column; a CI intersecting the dashed line indicates that fish did not show a preference for either half of the water column; a CI below the dashed line indicates that fish showed a significant preference for the top half of the water column. Mean $\pm$ standard error for (C) freezing, (D) spatial entropy, (E) average speed, (F) average acceleration, and (G) average angular speed across the five 1-min bins for subjects treated with the following citalopram concentrations: 0; 30; 50; and 100 mg/L. Horizontal bar with an asterisk denotes a significant difference.

Figure 3.

(A) Aversion (inverted triangle) with 95% confidence intervals (CI, whiskers) measured as the time spent in the compartment opposite to the one paired with the predatory stimulus divided by the total time spent in either of the conditioning compartments. The dashed line represents a chance level; a CI intersecting the dashed line indicates that avoidance was not statistically different from chance; \$ significantly different from chance. (B) Geotaxis (inverted triangle) with 95% CI (whiskers) measured as the time spent in the bottom half of the test tank divided by the duration of the test session (300 s). The dashed line represents a chance level; a CI intersecting the dashed line indicates that fish did not show a preference for either half of the water column. Mean $\pm$ standard error for (C) freezing, (D) spatial entropy, (E) average speed, (F) average acceleration, and (G) average angular speed across the five 1-min bins for subjects treated with the following ethanol concentrations: 0.00; 0.25; 0.50; and 1.00 % volume. Horizontal bar with an asterisk denotes a significant overall difference over time. The filled symbol on the right of panel E indicates a significant overall difference ($P < 0.05$) between ethanol 0.50 % and vehicle.